FREQUENCY AND DISTRIBUTION OF TLR GENES IN SOME HUMAN POPULATIONS OF NORTH BENGAL AND THEIR ASSOCIATION WITH RHEUMATOID ARTHRITIS

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BY

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JULY 2018

DEDICATED TO

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DECLARATION

I declare that the thesis entitled, **—FREQUENCY AND DISTRIBUTION OF TLR GENES IN SOME HUMAN POPULATIONS OF NORTH BENGAL AND THEIR ASSOCIATION WITH RHEUMATOID ARTHRITIS‖** has been prepared by me under the guidance of Prof. Tapas Kumar Chaudhuri, Professor, Department of Zoology, University of North Bengal.

No part of this thesis has formed the basis for the award or any fellowship previously.

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I certify that Mr. Avishek Das has prepared the thesis entitled "**FREQUENCY AND DISTRIBUTION OF TLR GENES IN SOME HUMAN POPULATIONS OF NORTH BENGAL AND THEIR ASSOCIATION WITH RHEUMATOID ARTHRITIS**" for the award of Ph.D degree of the University of North Bengal, under my guidance. He has carried out the work at the Department of Zoology, University of North Bengal.

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Avishek Das

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PREFACE

I started my research work in 2013 which has been documented in this dissertation entitled **"FREQUENCY AND DISTRIBUTION OF TLR GENES IN SOME HUMAN POPULATIONS OF NORTH BENGAL AND THEIR ASSOCIATION WITH RHEUMATOID ARTHRITIS"** under the supervision of Prof Tapas Kumar Chaudhuri, Department of Zoology, University of North Bengal, Darjeeling.

Toll like receptors are very interesting field of study in recent times. It is one of the major receptor in innate immunity. This receptor plays a vital role against the pathogen present in the environment. TLR genes reside in diverse chromosome in the human genome and recognize conserve molecular pattern molecules known as PAMPs. The pathogens present in the environment plays a direct role to interact with these receptors. TLRs expression has been observed against various types of diseases. After interaction with the pathogens via TLR signaling with the help of various molecules like MyD88, IRF3, TRAF etc. and produce various cytokines like IFN- gamma, TNF-α etc.

In various population expressions of TLRs vary according to the presence of the pathogens in the environment. Polymorphic variation has been seen in TLR genes and these polymorphic variations sometimes susceptible or resistant for various diseases in different populations. It has also very important for us to know the frequency of all ten human TLR genes in different ethnic and tribal populations in the world.

The Northern part of West Bengal or the North Bengal region is inhabited by different tribes and caste populations. They have their own cultural heritage and linguistic variations. Different populations of this region arose due to blending of the gene pool of different population groups in this region. On the other hand, they also share the same environment in which convergent evolution also came into play for the TLR genes which we have to explore by studying the population genetics of this region which is untouched for many times in respect of their TLR genes.

The study also aimed to observe the frequency distribution and association of TLR genes with rheumatoid arthritis in the Siliguri and adjacent areas. This disease is characterized by

progressive deterioration of articular region leading to joint destruction inflammation and pain. Blood samples were collected from the patients with the proper consent and unrelated individuals were selected for the study. RA was diagnosed by expert physician and recruited from the Department of pathology, North Bengal medical college and Hospital, Siliguri and 3gen diagnostic Pvt. Ltd, Siliguri. All the experiments were done in the Cellular Immunology Laboratory, Dept. of Zoology, University of North Bengal.

In addition the study aimed to observe the frequency pattern and association of TLR genes with typhoid fever patients. Typhoid fever is an enteric disease mainly caused due to *Salmonella typhi*. It is caused mainly in the rural areas due to sanitation problem and unhygienic conditions in the tea garden and related areas in the Siliguri region. The samples gas been collected from different pathology lab of Siliguri and adjacent areas and all experiments were performed in the Cellular Immunology Laboratory, Dept. of Zoology, University of North Bengal.

In the North Bengal region HIV is also a major disease which attacks our immune system and destroyed it completely and leads to increase of viral load in the body. The study aimed to observe the association of TLR genes with the HIV+ patients in the Siliguri and adjacent areas. Blood samples of HIV positive patients were collected from Malda District Hospital, Malda with proper consent. CD4+ count results of all the positive samples were taken for the confirmation of the positive samples. The published articles of our findings in the reputed journals and are discussed in details in the result and discussion part of the dissertation.

ABSTRACT

Host defense against the invading microbial antigens is recognized by the immune system. The immune system consists of two major components, innate immunity an acquired immunity. Two different types of immunity that is interrelated recognize invading microorganisms as non-self molecules which triggers the immune responses to eliminate them from the body. At the beginning of the 21th century, Toll protein was shown to be an essential receptor for host defense against the fungal infection in Drosophila, which only helps in innate immunity. Thereafter, a mammalian homolog of the Toll receptor (TLR4) was shown to induce expression of genes involved in inflammatory responses. After that one point mutation in the region of TLR4 gene has been observed in a mouse strain that is unresponsive to Lipolysaccharide. These studies have made TLR genes a very promising subject of research.

Human Toll like receptor genes comprise a large family consisting of at least 11 members. TLR1–9 is very much conserved between the human and mouse. However, TLR10 is functional in the human, whereas C-terminal half of the mouse TLR10 gene is substituted to an unrelated and non-productive sequence and indicating that mouse TLR10 is non-functional. Similarly, mouse TLR11 is functional, but the stop codon present in the human TLR11 gene, which results in lack of production of human TLR11. All the TLR genes are located in diverse chromosomes in mammalian genome. TLR receptors of innate immune system known as pattern recognition receptors (PRRs) recognizes a conserved molecular pattern, also known as Pathogen associated molecular patterns (PAMPs). TLRs signaling pathway occur via different signaling molecules like MyD88, IRAK, and IRF3 etc. which helps in the production of various kinds of cytokines for the inflammatory and other types of diseases. TLRs are mainly dependent on environmental pathogen for their recognition of diverse pathogens. Extensive variation among the TLR genes helps to understand the gene- disease- environment associations against various kinds of diseases and also their frequency distribution in different populations.

The present study was aimed to estimate the frequency distribution and phylogenetic relationship of ten human TLR genes among the studied four populations namely, Rajbanshi, Rabha, Gurkha and Muslim in the North Bengal region of India where different types of tribal populations reside. Present study has also aimed to study the frequency and distribution patterns of TLR

genes in the patients of Rheumatoid arthritis, Typhoid fever and in HIV and their association with the TLR genes if any.

Blood samples were collected from the volunteers with prior informed consent. In both population- based and disease related study, three generation pedigree of the volunteers were taken into account and only unrelated individuals were included in the study. DNA samples were extracted using standard protocol. Ten TLR genes were analyzed using PCR-SSP typing. Different available software was used for the analyzing of the data.

It has been documented from population based section that TLR8 and TLR9 are having very high frequency among Rajbanshi in respect to the other three populations. In Gurkha population TLR4 and TLR5 are showing highest frequencies. On the other hand, in Rabha population, frequency of TLR4 is highest, whereas in Muslim population TLR3, TLR5 and TLR7 are showing the highest frequencies.

The present study has also documented the phylogenetic relationship of the above mentioned four populations and found that Gurkha and Muslim are very close to each other, whereas Rabha is distantly related. Rajbanshi is close to Gurkha population as evident from the Nei‘s genetic distance analysis. Principal component analysis demonstrated the relationship and genetic structure of Gurkha and Muslim population. Rabha is more distantly related from the other three populations. It may occur due to the environmental pathogens present in their surroundings and that may have the direct relationship with the change of the frequency pattern of the TLR genes among the four studied populations. It is inferred from the results that Rajbanshi population is susceptible for various kinds of viral chronic diseases. On the other hand in Gurkha and Rabha population the frequency of TLR4 is high. TLR5 is also very high in Gurkha. They may probably susceptible for bacterial diseases and various gastro-insetinal diseases. High frequencies of some of the endosomal TLRs in Muslim population suggested that they are probably susceptible to get infected with viral and entero- bacterial diseases. The study also gives a light on the convergent evolution/selection pressure of the TLR genes among the population. Convergent evolution has occurred in TLR genes among the above-mentioned populations due to the sharing of similar environmental conditions. It is quite interesting to observe that although the Rajbanshi, Gurkha and Rabha populations have shared ancestry due to their emergence from a common East-Asian stock, but there is no similarity in the distribution of TLR genes as has been recorded in the

present study. However, there exist considerable similarities in the distribution of TLR genes between the Muslim and the Gurkha population who share the same environment but differ considerably in their ethnicity. This striking observation may depict the impact of environmental selection on the distribution of TLR genes. Such influences of the environment on TLR distribution may depend on the constant presence of specific pathogens in respective environment. Thus, it may be assumed that TLR genes play a significant role in shaping the genetic ancestry of the above mentioned populations from North Bengal region of India as well as in determining the exposure of the diseases in these populations.

Rheumatoid arthritis is a chronic inflammatory disease that affects the joints. It is an autoimmune disease by producing different types of autoantibodies in the serum. 110 numbers of patients were selected for association study and the numbers of healthy control samples were 100. Patients were selected by evaluating the diagnostic result of anti-CCP and RF titre assay and compared with the control group. The patients were also selected on the basis of American college of Rheumatology (ACR) and European League against Rheumatism (EULAR) criteria 1987. It has been found that the median range of anti-CCP is 182.7 (16.5 to 504.93) compared to the control group which is 10.8(8.2 to 13.1), whereas the RF titre concentration is 142.3 (41.20 to 198.0) in case of rheumatic patients and 16.8 (12 to 20.5) for control group.

Molecular typing of ten human TLR genes was performed in the rheumatoid arthritis patients and the control group. Significant associations are found in case of TLR2, TLR4, TLR5, TLR8 and TLR9. The frequency of TLR1, TLR6 and TLR8 are highest among patients. Relative risks are also high for TLR4, TLR6, TLR7, TLR8 and TLR9. Door line association has been found in case of TLR1 and TLR3.

Odd ratio is very high for TLR1, TLR4, TLR6, TLR8 and TLR9. Low odd has been found in case of TLR2, TLR5 and TLR10. The sensitivity for the positive association with the disease was also calculated. High sensitivity has been observed for TLR1, TLR3, TLR6 and TLR8, whereas low sensitivity has been observed in case of TLR2, TLR5 and TLR10.

In conclusion, the results may help to find out the association of TLR genes with the rheumatoid arthritis which was not previously studied in this region. The genetic profile study of the TLR genes in human and their association with the RA has got immense importance. It has been

found that some of the TLRs those are highly associated with the disease, need further investigation to establish their role in case of RA as the toll like receptors are also regulated by the environmental factors.

Another study with typhoid fever caused due to *Salmonella typhi*, a gram- negative bacterium, was carried out. It is restricted in human and causes a wide range of food- and water-borne diseases ranging from self-limiting gastroenteritis to systemic typhoid fever. Typhoid patients were screened by Widal test positive result carried out by serum agglutination test. The serum antibody titre of 1: 80 or above was considered positive for the typhoid fever.

Molecular documentation of ten human TLR genes in typhoid patients and the control group were performed. Significant associations are found for TLR8. TLR10. TLR1, TLR5 and TLR6 are highly up-regulated among typhoid patients. It proves that the flagellin protein and other antigens from *S. typhi* up- regulate the TLR genes. Positive association are found for TLR1 (5.54) and TLR6 (4.77) in respect to their odd ratio. Door line association of TLRs with the disease has been observed when the relative risk was calculated for TLR2 (1.72), TLR3 (1.21) and TLR10 (1.98). The result indicates the higher association in case of some of the TLRs with typhoid fever in the region of Siliguri. It also signifies the risk factor of typhoid fever with TLR genes.

Human immunodeficiency virus infection is characterized by a gradual dysfunction, mainly in cell-mediated immunity but also in humoral immunity. It is also characterized by CD4+ cell depletion and leading to high levels of HIV RNA and development of opportunistic disease. Positive HIV patients were selected based on the viral infection and CD4+ cell count results (CD4+ count range- 156- 756 x 10^6 cells/L).

Molecular typing was performed for all ten TLR genes for patients and control group. It has been observed that the gene frequency of TLR8 (0.809) and TLR9 (0.865) are very high. Chi-square analyses ($\chi 2$) were performed to compare the differences in carrier frequencies (OF) of TLR genes among the patients and control samples. Significant differences are found in case of TLR2, TLR4, TLR8 and TLR9. Odd ratio is observed very high in case of TLR4 (9.56), TLR8 (6.04) and TLR9 (10.06), increased multiple times between patients and control group. Risk ratio was also high for TLR8 and TLR9 which manly recognize viral RNA and dsDNA. Furthermore, this

study suggested the significant association between the clinical parameters and the role of TLR genes in occurrence of HIV. It also deciphers that the viral RNA/DNA, which is present in the endosomal compartment of the cell, activates the TLRs in course of the disease.

It can be concluded that the results will not only help to understand the genetic background of the studied population in respect to their TLR genes, but also the gene- environment interaction in the northern part of West Bengal. This study also helps in illustrating the convergent evolution of TLR genes in respect to their ethnic background. Furthermore, this study will help to understand the degree of association of TLR genes with the disease.

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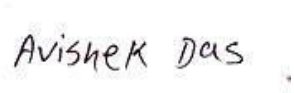

Dated: 23.06.2018 **Avishek Das**

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ACCP2A	Anti cyclic citrullinated 2 peptide antibody
ACR	American College of rheumatology
AGT	Angiotensin gene
AICD	Activation induced cell death
AICD	Arthritis impact measurement scale
APC	Antigen presenting cell
CC	Cysteine containing
CD	Crohn‘s disease
CDAI	Clinical disease activity index
CRP	C- reactive protein
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent Assay
ESR	Erythrocyte sedimentation rate
HAQ	Health assessment questionnaire
HLA	Human leukocyte antigen
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma

Ig	Immunoglobulin super family
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factors
KIR	Killer cell immunoglobulin like receptors
MHC	Major Histocompatibility complex
mt-DNA	Mitochondrial DNA
M ϕ	Macrophage
MyD88	Myeloid differentiated protein 88
PAD	Peptidyl arginine deaminase
PCR	Polymerase chain reaction
PS	Plant specific
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RI-like	Ribonuclease inhibitor-like
SCID	Severe combined immunodeficiency
SDAI	Simplified disease activity index
SNP	Single nucleotide polymorphism
STR	Short tandem repeats
TCR	T cell receptor
TLR	Toll like receptor

TNF-α	Tumor necrosis factor A
TpLRR	Treponema pallidum LRR
TRAF	Tumor necrosis factor receptor-associated factors

CHAPTER 1

INTRODUCTION

1. Introduction

Human genome project and genetic research has generated an enormous amount of data about the genetic differences among the world and Indian populations. Investigations of these differences have transformed the understanding of the origin and nature of the human diseases and their connection with the genetic background (**Bamshad *et. al*., 2004., cavalla- Sforza, 1994**). Geneticists have studied the genetic variation among the individuals forming a species (**Cavalla- Sforza and Feldman, 2003., Jorde and Wooding, 2004**). Different human traits like hair, eye color and others vary among individuals in many populations due to the genetic variations present in the individuals. These traits contribute to the understanding of the extent of variations in humans. The first genetic variation study is of ABO blood groups which were described long time ago (**Landsteiner, 1927**).

These studies were then extended to other blood group systems and to other markers to show that the different human populations have different proportion of blood groups. The first site of the staggering magnitude of genetic variation came later in the beginning of 50s and coming to full development in 60s when individual differences for proteins could be systematically studied. Different protein chains vary considerably in their amino acid compositions and serve different functions. The same protein structure may show small strictly inherited differences between individuals. In case of sickle cell anemia it was first observed that replacement of one amino acid by another in hemoglobin molecule was the determinant of the hereditary disease. The amino acid change in sickle cell amino acid involved change in the electric charge of the hemoglobin molecule. Electrophoretic analysis has been developed and helped to detect the variation in the proteins (**Glazko, 2005**).

Recent trends mainly focused on the DNA variations. Changes in the DNA sequences in different human populations helped to find out the variations among the populations. It is a very unique tool to study variations at the DNA level.

1.1 Evolutionary process affecting population diversity

Anthropologist tried to construct evolutionary relationships and history on the basis of genetic background and for many years by the analysis of cephalic index (percentage of skull breadth to

length) introduced in the middle of last century. However, with a single trait two populations of different origin could well turn out to be more or less identical. The anthropometric traits are not well established because the characters are under the control of inheritance and variations are short term and under control of environmental changes. Single gene frequency study is not very authentic as that the frequency for one gene in different human populations may not change during many years (**Itoh, 2002**). By studying more than one gene and many alleles of different genes. It will definitely reconstruct the human evolutionary history (**Jorde and Wooding, 2004)**.

Different statistical analysis such as Univariate for single trait and multivariate analysis for multiple genes helped to combine information from the population data. Multivariate analysis is useful for understanding the evolutionary process such as migration, random genetic drift etc. The construction of human evolution including migration, mutation, genetic drift and natural selection is difficult and challenging. Results from the genetic data should be compared with the existing data to infer some conclusion.

Different genetic types of different proteins and genes exist and one needs to count individuals carrying it. These proportions vary from population to population because they change over time (**Nei, 2005**). The change is very slow but incessant over generations. Therefore, the primary interest is to understand the evolutionary process.

Population is a local group of organism of the same species that interbreed. A group of individuals within whom marriages are constructed is called a Mendelian population. The genetic information carried by a population is called its gene pool. Gene pool transfers from one generation to the next. The gene pools of a new generation have different alleles or gene frequencies than their parental pool. The changes in allele frequency can cause changes in phenotypic frequency which causes evolutionary change. Members of the same local group are very close than the group separated from geographic barriers or other. These results in the change of behavior occur at the micro or macro level. The evolution also changed the genotypic and phenotypic constitution of the population (**Joaquim, 2004**).

To understand the genetic structure of human populations one needs to calculate the gene frequencies or the allele frequencies at different loci. Gene frequencies are the estimates of the relative frequencies of alleles and are of widest application in human evolution. The gene

frequency estimation is observed by the phenomenon of sampling fluctuation and misclassification of genotypes which can be solved by using right markers and more sophisticated software or statistical analysis tools. In any population, the genotype frequencies among zygotes are determined in large part by the patterns in which genotypes of previous generation come together to form mating pairs. In random mating, genotypes form mating pairs in the proportions expected from random collisions. It is solved by the theory of Hardy-Weinberg equilibrium (HWE). For a gene with two alleles A and a in a random mating, the expected genotype of AA, Aa and aa are given by P^2, 2pq and q^2 respectively (where p and q are the allele frequencies of A and a, with p+q= 1). Statistical tests of HWE are often based on the χ^2 test, but this test is relatively weak in detecting departures from expected frequencies especially those caused by admixture of subpopulations differencing in allele frequency. The gene frequencies can be changed by several factors like mutation, genetic drift, gene flow, natural selection etc (**Cavalla- Sforza, 2003**).

Gene is being extensively studied now-a-days and the alternative form of this is called allele. This allele is produced due to mutations in the populations (**Roychoudhury, 1988**). The geographic distribution of a particular allele may give information on the place of origin of the genetic changes that generated it. Mutation occurs in both coding and non coding region and causes polymorphism in the DNA. The geographic distribution of a particular allele may give information on the place of origin of genetic changes that generated it. Correlation of distributions of gene frequencies with environmental parameters at the geographic level has been instrumental in the discovery of specific genetic adaptations (**Nei, 2005**). The proportion of allele varies from place to place but greatest variation observed in case of large distances.

Random genetic drift is also one of the major factors that change the gene frequencies among populations. Frequencies change over time because of the accumulation of random sampling errors while passing from one generation to other. When a small population migrates to a new place, the sampling error is large and allele frequencies in new population may be different from the parent population. In course of time the errors are decreased and constitute a very different frequency. This is called ―founder effect‖ (**Strachan *et. al*., 1996**).

Gene flow is a process by which interbreeding among certain groups of individuals results in those populations which become similar to each other. Mating can be either positive assortative

or negative assortative. Positive assortative mating increases the homozygosity. While mating is carried out between individuals with different genotypes then it is called negative assortative (C**avalla- Sforza, 1998**) which results in high heterozygosity in populations.

Natural selection is a shifting process. Organisms that can adapt in particular changing environment are at an advantage than the others. It is influenced by the organism‘s biology but also with the interaction of biology with environment (**Sternberg, 2004**).

Adaptation and migration are also two factors that change the genetic diversity of human populations. It is possible that much of the variation seen among the groups of human populations indirectly resulted from the pattern of expansion and migrations accompanied by genetic drift. Over time frequencies of DNA variants changed only in terms of the total DNA composition but changed enough to produce differences (**Harding *et. al*., 2000**).

1.2 Genetic tools for studying human genetic diversity

Advancement of various techniques in molecular biology enabled us to study the human genetic diversity and the association with different diseases. Previously, DNA markers were that of functional genes. However, these were very less informative. In recent times development of DNA markers has been discovered along with more robust DNA typing technology. These markers are neutral and not affected by selection pressure. These markers are also polymorphic, making them more informative in studying genetic diversity among human populations. The different genetic tools are as follows-

1.2.1 Short tandem repeats (STR)

STR DNA markers are short fragments of DNA that are commonly used in forensic identification. These markers are useful in studying human genetic diversity because of their polymorphism. STR marker analysis estimates the exact number of repeating units in DNA. Single nucleotide polymorphisms are characterized by single base changes in DNA sequence. SNPs are highly stable and preserved across populations and help for genetic diversity study.

1.2.2 Mitochondrial DNA (mtDNA) and Y chromosome markers

Mitochondrial DNA markers are essentially variations in DNA sequences. mtDNA is highly stable allowing lineage analysis among the populations across time and different genetic geographic areas. mtDNA, SNPs are very effective markers for analyzing genetic diversity and disease association study among populations.

Y chromosome markers are exclusively paternally inherited, allowing genetic analysis of the male lineage. As mtDNA is maternally inherited it allows genetic analysis of maternal line.

Along with the MHC and KIR, another marker came into focus that is TLR or Toll like receptors. This marker is mainly of innate immunity genes. Gene- environmental interaction has been described with the help of this marker.

1.3 Population of Indian Sub- continent

Indian subcontinent is located between 8 degree N to 37-degree N latitude and 68 degree to 97 degree longitude. This country assembled over 100 million people in the country with all the different populations and their different cultural background (**Cann, 2001**). The populations reside in this country are mixed between the western Caucasians and the Oriental in the East (**Chakraborty, 1992., Balakrishnan, 1996., Agarwal & Arundhati, 1999., Jaini, 2002., Chhaya, 2005**).

Investigation of the genetic diversity among the present human populations can be useful in reconstructing concepts regarding population diversity and migration routes and also in identifying the ancestral populations. The Indian subcontinent not only exhibits enormous morphological, cultural, and linguistic diversity but also stands only second to Africa in its genetic richness (**Cann, 2001**). Therefore, people of the Indian continent have been and continue to be of interest for investigation in different areas, all aimed at exploring their vast genetic wealth. Moreover, the continent has served as a major corridor for the dispersal of modern humans that started from Africa about 10,000 years ago (**Majumdar, 1998**). Thus, India occupies a center stage in human evolution.

Various socio-cultural practices have led to a unique gene pool of the human population of India and thus there is a need to study these unique populations at genetic level (**Srivastava, 2007)**. It

has been reported by the population geneticists that unique marital patterns, such as endogamous caste groups, could be one such contributory factors for the creation of unique gene pool. India, on the other hand also has undergone several historical invasions, as a result of which a significant admixture might have taken place. The most salient feature of Indian population is that in India socio-cultural barriers also plays an important role in determining the gene flow between populations (**Balakrishnan, 1978**), thereby establishing the diversity at the genetic level. This could have led to the formation of numerous close gene pools, which has remained virtually undisturbed for many generations that may have disturbed the original gene pool of the Indian population.

North Bengal region has got full of cultural and linguistic variations. The presence of various ethnic populations in this region signifies the variation in the gene pool of the populations. In the present study such four ethnic populations namely Rajbanshi, Gurkha, Muslim, Rabha were chosen to reveal the gene-environment interaction of ten human TLR genes among the populations.

Therefore, in the present study the objectives were formulated as mentioned below.

OBJECTIVES OF THE STUDY

1. To study the distribution and frequency of TLR genes of some human populations of North Bengal Region of West Bengal.

2. To study the heterogeneity among the local population(s) and trace their phylogenetic relationships.

3. To correlate the association of Rheumatoid arthritis with TLR genes if any.

4. To study the association of typhoid fever with TLR genes if any.

5. To study the association of HIV positive (+ve) patients with TLR genes if any.

CHAPTER 2

Review of literature

2. Review of literature

Infectious pathogens/antigens are the major forces for the selection procedure in human history **(Admetlla, 2008)**. Human migration in different parts of the world results in the exposure of the immune response genes to different local infections and thereby gets modified as per the demand of the environment. Thus, the pressure that has been exerted by the local pathogens causes the positive selection of some genetic markers in the population for developing protection against the pathogens **(Admetlla, 2008)**. The immune system comprised of two parts, innate and adaptive immunity. Innate Immunity plays a vital role in the recognition of the diverse set of foreign pathogens via some receptors and generates immune responses **(Schroder and Schumann**, **2005)**. They recognize it through some molecular receptors and send some signals through which different cell types release different cytokines by which they counteract the pathogens. Various families of molecular markers are there for the recognition of the pathogens of which TLRs or Toll like Receptors are the most important. TLRs are group-1 membrane glycoproteins that are conserved from ***C. elegans*** to human (**Kwai and Akira, 2010**). They are also known as the pattern recognition receptors (PRRs) or Danger associated molecular pattern (DAMPs). These receptors mainly act in innate immunity. There are mainly ten types of TLRs present in the human and mouse of which some are represented as pseudogenes due to the evolutionary constraints (**Barrio, 2009**).

Table 1: Toll like receptors and their ligands

Receptor	Ligands	Adapter(s)	Location
TLR 1	multiple triacyl lipopeptides	MyD88/MAL	cell surface
TLR 2	Multiple glycolipids, multiple lipoproteins, lipoteichoic acid	MyD88/MAL	cell surface
TLR 3	double-stranded RNA, poly I:C	TRIF	cell compartment
TLR 4	Polysaccharide, fibrinogen, Various opioid drugs	MyD88/MAL/TRIF/TRAM	cell surface
TLR 5	Bacterial flagellin, Profilin	MyD88	cell surface
TLR 6	multiple diacyl lipopeptides	MyD88/MAL	cell surface
TLR 7	single-stranded RNA	MyD88	cell compartment
TLR 8	small synthetic compounds; single-stranded Viral RNA, phagocytized bacterial RNA	MyD88	cell compartment
TLR 9	unmethylated CpG Oligodeoxynucleotide DNA	MyD88	cell compartment
TLR 10	Unknown	unknown	Unknown

(Sources: Waltenbaugh, 2008)

2.1 Toll Like Receptors

There are ten different TLR genes present in human and there is strong correlation among the different TLRs. These ten TLRs are located in different chromosomes. TLR 1, 2, 3, 6 and 10 are present in the chromosome number 4, TLR4 in chromosome 9, TLR7 and 8 in X chromosome and TLR9 on chromosome 3. These receptor genes are not like that of HLA and KIR because they are locus specific, presents in the single chromosome but TLRs are present in the different chromosome set. So, linkage study is much more complicated for the TLRs.

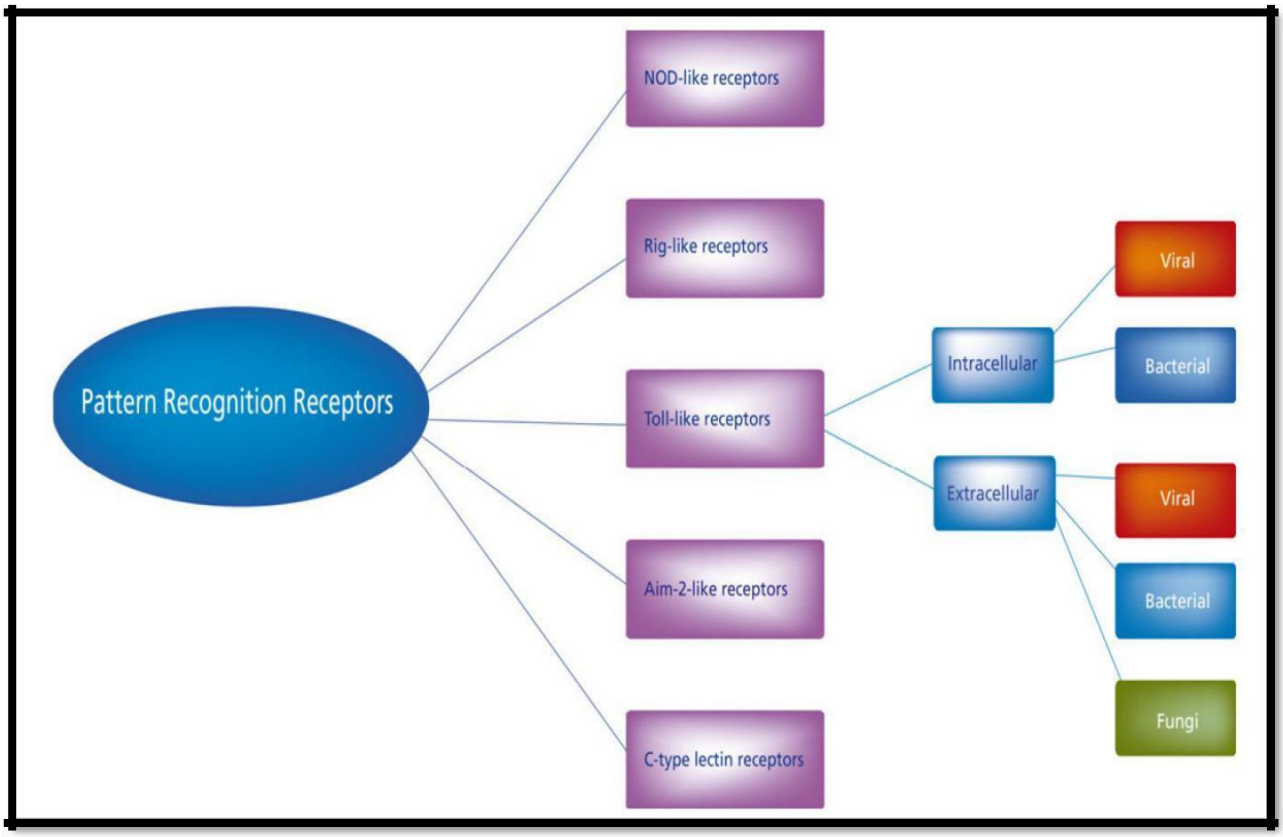

Fig. 1: Pattern recognition receptors and their function against antigens (Rietdijk *et. al.*, 2016)

2.2 Structure of Toll like receptors

Structural studies of TLR-ligand complexes have become an attractive area of research as the structural information is critical in understanding the innate immunity as well as designing novel drugs **(Mi Sun Jin, 2008)**. TLRs are type I transmembrane glycoproteins composed of extracellular, transmembrane and intracellular signaling domains **(Gay and Gangloff, 2007)**. The extracellular domain contain leucine-rich repeat (LRR) and are responsible for binding so-called pathogen associated molecular patterns (PAMPs) **(Janeway, 1989., Medzhitov, 2001)**. The extracellular domains of all TLR family proteins contain 16–28 LRRs **(Matsushima *et. al.*, 2007)**. On the basis of their sequences and structural patterns, LRR family proteins can be classified into seven subfamilies such as RI-like (ribonuclease inhibitor-like),CC (cysteine containing), PS (plant specific), SDS22-like, bacterial, and TpLRR (Treponema pallidum LRR) **(Kobe and Kajava, 2001., Matsushima *et. al.*., 2007)**. TLRs, typical subfamily proteins, have LRR modules of 24 amino acids with the conserved motif of xLxxLxxLxLxxNxLxxLPxxxFx.

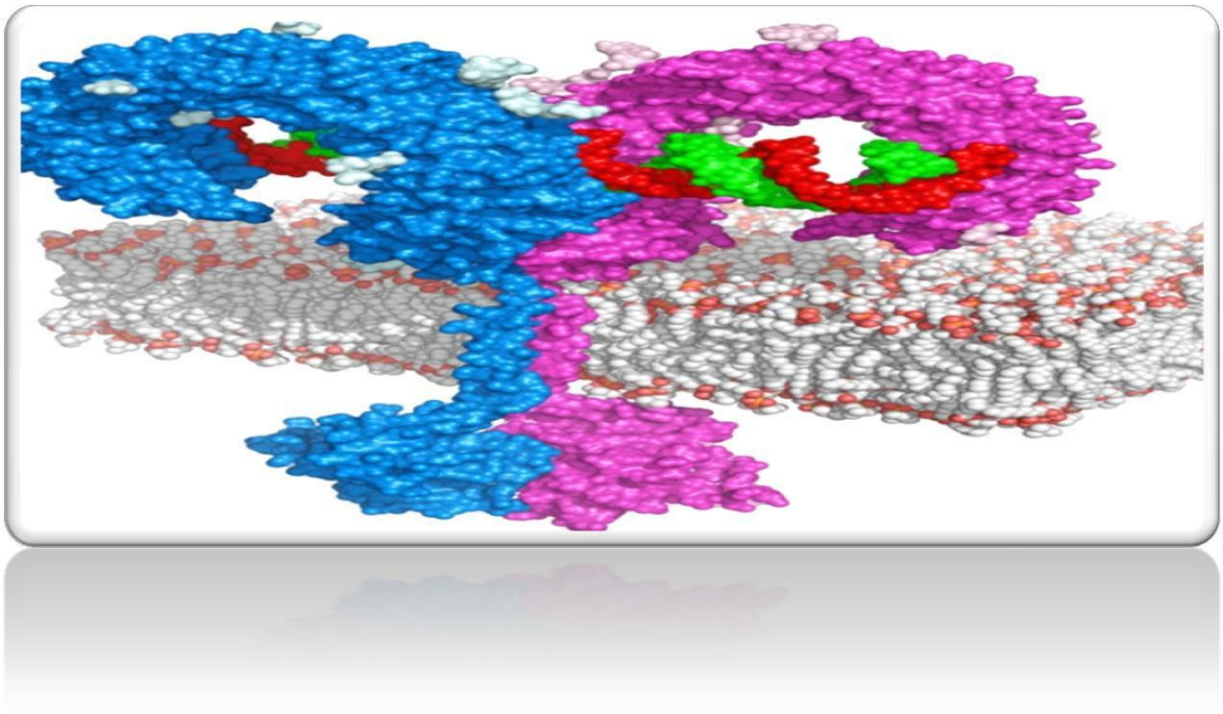

Fig 2: Structural model of the full-length TLR3/dsRNA (Sources: Botos, 2011)

2.3 Signalling pathways of TLR

The activation pathway of TLR signalling originates from the cytoplasmic TIR domains. The downstream signaling pathway via TIR domain, a TIR domain-containing adaptor, and MyD88 was first characterized to play a crucial role. In addition, recent accumulating evidence indicates that TLR signaling pathway consists of a MyD88-dependent pathway that is common to all TLRs, and a MyD88-independent pathway that is restricted to the TLR3- and TLR4 **(Akira *et. al.*, 2001., Takeda and Akira, 2004).** There are other similar pathways present that help in TLR signaling.

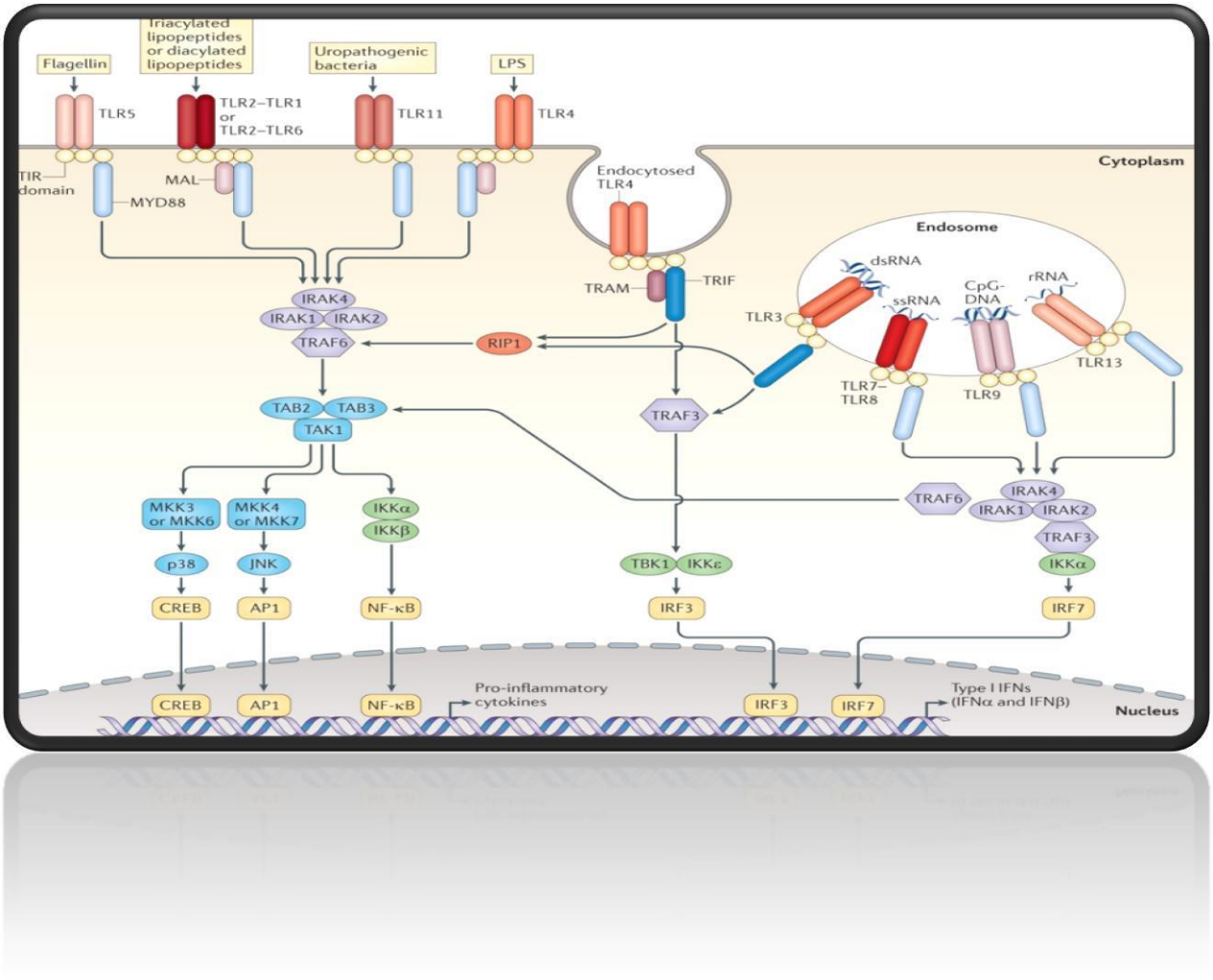

Fig 3: Mammalian TLR signalling pathways (Sources: O'Neill, 2013)

2.4 MyD88-dependent pathway

MyD88 pathway contains the TIR domain in the C-terminal position, and a death domain in the N-terminal portion. After TLR engagement with the pathogen, MyD88 forms a complex with IRAK kinase family members, known as the Myddosome **(Kawasaki, 2014)**. During the complex formation IRAK4 activates IRAK1, which is then autophosphorylated. IRAK1 associates with the RING-domain E3 ubiquitin ligase TRAF6. TRAF6 is a member of the tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family that initiates cytokine signaling pathways **(Arch, 1998)**. TRAF6, along with ubiquitin-conjugating enzyme UBC13 and UEV1A, promotes K63-linked polyubiquitination of both TRAF6 and TAK1 protein kinase complex **(Kawasaki, 2014)**. Then TAK1 then activates two different pathways that lead to the activation of IKK complex-NF-κB pathway and -MAPK pathway. TAK1 then binds to the IKK complex through ubiquitin molecule after that allows it to phosphorylate and activate IKKβ. The IKK complex phosphorylates the NF-κB inhibitory protein IκBα, which undergoes proteasome degradation, allowing NF-κB to translocate into the nucleus to induce proinflammatory gene expression **(Akira, 2006 ., Kawai, 2010)**.

2.5 Other molecules

Tollip (Toll-interacting protein) is another protein that complex with IRAK- 1. Tollip-IRAK-1 complex is then recruited to the IL-1 receptor complex. IRAK-1 then phosphorylated and leads to the rapid dissociation of IRAK-1 from Tollip, thereby inducing activation of TRAF6. Tollip also negatively regulates the TLR- mediated signaling pathways (**Zhang, 2002., Bulut, 2006)**.

2.5.1 MyD88-independent pathway

Another pathway for TLR signalling is MyD88-independent pathway. This pathway activates through two different molecules, one is TIRAP/Mal and another is TRIF. TIRAP/Mal specifically interacts with TLR4, and then involved in the TLR4-mediated MyD88-independent signaling pathway **(Horng, 2002)**.

TRIF interacts with TRAF6 and TRAF3. TRAF6 recruits another molecule that is RIP- 1 kinase which again interacts and activates the TAK-1 complex leading to the activation of NF-κB and MAPKs and production of inflammatory cytokines **(Akira, 2006., Kawai, 2010)**. On the other

hand, TRAF3 activates IKK related kinase along with NEMO for IRF3 phosphorylation. Subsequently, IRF3 forms a dimer and translocate into the nucleus from the cytoplasm and induces the expression of type- I IFN genes for the production of cytokines.

2.6 BALANCED ACTIVATION BETWEEN MyD88- AND TRIF-DEPENDENT PATHWAYS

TLR4 activates both the MyD88-dependent and TRIF-dependent pathways. Activation of these pathways is controlled by several other molecules to induce appropriate signaling. Balanced production of inflammatory cytokines and type- I IFN is very important for controlling the tumor cell growth and autoimmune diseases. In case of TLR4 signalling TRAF3 has been shown to be incorporated into the MyD88 complex as well as with the TRIF complex. TRAF3 within the MyD88 complex has been degraded and causes activation of TAK1. Thus it plays a vital role for inhibiting the MyD88- dependent pathway along with its role in promoting TRIF-dependent pathway activation. NRDP-1, an E3 ubiquitin ligase is consisting of two molecules that bind and ubiquitinates MyD88 and TBK1. It is inducing the degradation of MyD88 and augmenting the activation of TBK1. This attenuates inflammatory cytokine production and induces preferential type I IFN production **(Wang, 2009)**.

2.7 Negative regulation of TLRs

The excessive activation of TLR signaling which associated with autoimmunity and inflammatory diseases is negatively regulated by a number of other molecules through various mechanisms to prevent or terminate the excessive immune responses that lead to the detrimental results. Negative regulatory proteins target each of the key molecules present in TLR signaling pathway. Excessive activation of MyD88-dependent pathway is suppressed by ST2825, SOCS1, and Cbl-b, and activation of TRIF-dependent pathway is suppressed by SARM and TAG molecules **(Palsson, 2009., Han, 2010)**. These molecules associate with MyD88 or TRIF to prevent them from binding to TLRs or downstream molecules. TRAF3 activation is negatively regulated by SOCS3 and DUBA **(Kayagaki, 2007)**. A20, USP4, CYLD molecules target the TRAF6 and inhibit the molecules for cytokine production **(Skaug, 2011., Yuk, 2011., Kondo, 2012)**. Now TAK1 activation is inhibited by other molecules TRIM30α and A20 **(Shi, 2008)**. Not only these signaling molecules but the transcription factor NF- κB is also suppressed by Bcl-

3, IκBNS, Nurr1, ATF3, and PDLIM2. While IRF3 activation is inhibited by Pin1 and RAUL **(Saitoh, 2006)**. The stability of mRNAs encoding signaling molecules is regulated by miRNAs such as miR-146a, miR-199a, miR-155, miR-126, miR-21, miR-29, miR-148/152, and miR-466l **(Kondo, 2012)**. In addition to the mRNAs stability for signaling molecules, cytokine production is regulated by Regnase-1 and TTP **(Kondo, 2012)**.

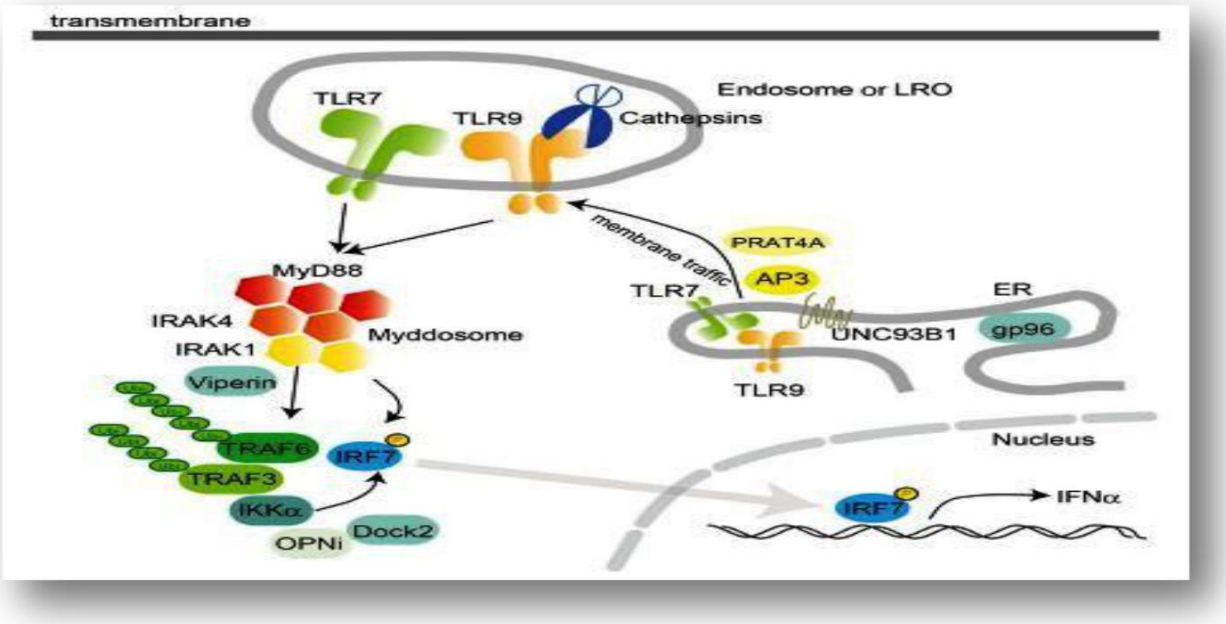

Fig 4: TLR signaling and membrane processing in pDCs Polymorphic variation of the TLRs in Human populations (sources: Kawasaki T, 2014)

Toll like receptors recognize very conserve molecular patterns. Ten different TLR genes are associated with different diseases in human populations. Different polymorphic varieties of the ten different TLR genes have been identified. Some polymorphic TLR genes may undergo mutation or single nucleotide polymorphisms in the TLR genes and causes diverse susceptibility for particular diseases. Synonymous substitution in the TLR genes produces no change in the production of amino acid whereas nonsynonymous substitution produces substitution in the amino acid **(Cheung, 2002)**. Different SNPs have been identified in human for the different

TLR genes and correlation study done on the disease association among different populations. Different diseases are caused by particular pathogens and the TLRs identified through the receptors and send the signals via IRAK or MyD88 pathway. So, the mutation in the coding or some conserve sequence region in the TLR genes causes the modification of the amino acid of particular region of the receptors and modified it. Thus the substitute pattern may induce the susceptibility for a particular disease and make it epidemic in the population **(Yi-Tzu Lin, 2012).**

Work on different SNPs study revealed that different polymorphic variety of TLR genes associate with the different diseases. Out of ten TLR polymorphic varieties, TLR4 has been extensively studied in different populations. The present investigation has elaborately studied the TLR polymorphic regions and how these relate to different diseases and correlate the association among the gene and disease.

2.8 TLR1 and disease association (cluster of differentiation 281-CD281)

TLR1 receptor mainly recognizes peptidoglycans and lipoproteins in association with the TLR2. It is mainly found on the surface of macrophage and neutrophil **(Farhat, 2008**) and designated as CD281. Structure of TLR1 consists of 786 amino acid residue of which extracellular domain contains 581-amino acid (leucine-rich), a 23-amino acid transmembrane domain and a 181-amino acid cytoplasmic domain (**Hawn, 2005**). It is situated on the chromosome number 4p14. There are different types of nonsynonymous polymorphic mutations found in gene sequence of TLR1, susceptible to different diseases like sepsis, leprosy, and candidemia. It has been found that TLR1 variants associated with leprosy. *Mycobacterium tuberculosis* is also inducing TLR1 for the susceptibility of the tuberculosis (**Hawn, 2003**). In case of TB lack of surface expression was found for TLR1 negative cell (**Uciechowski, 2011**). Some of the SNPs found in case of TLR1 gene causes susceptibility for that disease. Three common SNPs are found in the TLR1 of which -7202G/A, Asn248Ser, Ile602Ser are susceptible to sepsis and leprosy.

2.9 TLR2 and disease association (cluster of differentiation 282-CD282)

TLR2 is expressed on the cell surface of different cell types except T cells. This receptor recognizes lipoproteins from different bacteria like *Borrelia, Treponema* and *Mycobacterium.* Common SNPs found on the TLR2 are Arg677Trp, Arg753Gln. Mutation on the Arg753Gln make less responsive to the bacterial infection. Arg677Trp mutation on TLR2 suppresses the Nf-

kB signalling and makes susceptible to *Mycobacterium leprae* (**Kang, 2002**) and Tuberculosis (**Bochud, 2003**) and also increase the susceptibility for leprosy (**Kang, 2002**) and tuberculosis (**Ben-Ali, 2004**). TLR2 is also associated with other diseases like Lyme disease, urinary tract infection and staphylococcal infection.

2.10 TLR3 and disease association (cluster of differentiation 283-CD283)

There are 100 of SNPs are found in the TLR3 but none of the mutations are associated or susceptible with any diseases. Out of which four mutational regions are detected for the disease susceptibility for TLR3 gene. Asn284Ile, Tyr307Asp, Leu412Phe, and Ser737Thr are the four SNPs commonly found for TLR3 gene. But neither of these is related with each other and nor common with any diseases. Association has been found with Leu412Phe that correlates with the colorectal cancer and it has been found that 50% of the patients have the mutation for that disease (**Gorbea, 2010**). Pro554Ser, a rare mutation in the TLR3 gene was found to be associated with the herpes simplex virus that is not found in the healthy individuals by impairing the function of the signalling molecules (**Zhang, 2007**).

2.11 TLR4 and disease association (cluster of differentiation 284-CD284)

TLR4 have been extensively studied for association of various diseases in the different population. TLR4 is the cell surface receptor and recognize bacterial polysaccharide, LPS, taxol (**Akashi, 2001**) and other pathogens. The first identified TLR gene polymorphism encodes an Asp299Gly mutation in TLR4. This polymorphism is associated with a decreased signalling response to LPS *in vitro* and decreased airway response to inhaled bacterial LPS (**Arbour, 2000**). Thr399Ile Asp299Gly are two most common identified TLR4 gene SNPs found in the populations .This two are the co- segregating missense mutation (**Arbour, 2000**). Asp299Gly are related with different diseases like atherosclerosis, crohn‘s disease and asthma. Studied revealed that the patients with atherosclerosis has the higher level frequency of TLR4 (**Kiechl, 2002**). It has been found that for sepsis TLR4 is present in higher frequency when compared to the control. These common SNPs are found in higher frequency in some of the populations where they have increased the disease probability for that population. Indeed, it is not only related to atherosclerosis or sepsis but also with the other diseases common in different regions.

2.12 TLR5 and disease association (cluster of differentiation 285-CD285)

TLR5 recognizes the bacterial flagellin as a ligand for the receptor (**Akira, 2006**). TLR5 is expressed on the basolateral, but not the apical side of intestinal epithelial cells (**Takeda and Akira, 2003**). Only one common SNP found in the TLR5 gene which is a stop codon mutation Arg392Stop. This mutation in the TLR5 gene causes less responsive to bacteria like *Legionella pneumophila* which is responsible for pneumonia. This allelic variation also transmitted in the case of SLE where it protects the population from infection.

2.13 TLR6 and disease association (cluster of differentiation 286-CD286)

No extensive data till date have been found regarding TLR6 gene polymorphism or any disease relationship. TLR6 has expressed on the cell surface and recognize the lipopeptides and lipotechoic acid (**Irvine, 2013**). One common SNP found on TLR6 is Ser249Pro where serine changed to proline at 249 positions. It is also related with decreased risk of asthma (**Tantisira, 2004**). TLR6 Ser249Pro SNP has been associated with susceptibility to infection with aspergillosis (**Kesh, 2005**). The allelic variation in the TLR6 also caused the ventricular wall thinning (**Sales, 2010**).

2.14 TLR7 polymorphism and disease association (cluster of differentiation 287-CD287)

TLR7 was found on the endosomal part in the human. They mainly recognize single stranded RNA (ssRNA) as their ligands. There are three common variants found in TLR7. The common SNPs are Gln11Leu, 1-120T / G found in intron 1 and a synonymous SNP in TLR7 (**Schott, 2007**). The common polymorphism Gln11Leu is associated with the Hepatitis C infection and enhances the viral infection. It reduces the expression of the IFN- γ (**Askar, 2010**). It has also been provided evidences that TLR7 also related with the HIV infection (**Oh, D, 2009**).

2.15 TLR8 and disease association (cluster of differentiation 288-CD288)

TLR8 gene mainly encoded by the X chromosome and also known as CD288, it senses the bacterial nucleic acid as their ligands. TLR8 is highly expressed on monocytes, macrophages and dendritic cells and in the lung tissue (**Alexopoulou, 2012)**. TLR8 gene variation or the single

nucleotide polymorphism made susceptible for the disease. Met1Val and 129G/C are two common polymorphic variety found in the TLR8 gene. Study on the Met1Val SNP analysis on pulmonary tuberculosis patients provides evidence that this variation increase the disease susceptibility of the male patients (**Dalgic, 2011)** Association with TLR7 suggested that the frequency is highest in case of HCV (Hepatitis C virus patients) **(Chiou-Huey Wang, 2011).** As it senses the nucleic acid in the endosome compartments it senses the HIV-1 nucleic acid (**Akira, 2006**).

2.16 TLR9 and disease association (cluster of differentiation 289-CD289)

TLR9 gene encodes the receptor that resides in the endosome. It recognizes the CpG DNA as their ligands. As the bacterial DNA contain high amount of this DNA motifs so its automatically stimulate TLR9 and induce production of inflammatory cytokines such as IL-12 and TNF-a (**Akira, 2006**). The mutational regions which are associated with various diseases like cerebral malaria, lupus nephritis, cervical cancer. There are evidences that mutations like -1237T/C +1174G/A +2848G/A increased the frequency for the symptomatic malaria in the Ghanaian children (**Omar, 2012**).SNPs like -1237T/C +1174G/A increased the IFN-γ level in case of cerebral malaria in Ugandan children (**Sam-Agudu, 2010**) (**Nadia, 2010**). Common mutation likes 2848G/A in the TLR9 causes increased risk of cervical cancer and also induce the HPV infection (**Zeng-Zhen Lai, 2013**). A rare TLR9 SNP Pro99Leu prevents the receptor from being activated by ligands as it binds to the ligand normally (**Kubarenko, 2010**).

2.17 TLR10 and disease association (cluster of differentiation 290-CD290)

The lack of sufficient data for the TLR 10 reveals close examination of this receptor and lots of works have to be done on this. TLR 10 is one of the members of the TLR family that resides in the endosomal membrane and recognizes profilin as their ligand. The two common SNPs found in the TLR10 are Pro344Pro and Iso775Val which those are related with the asthma (**Lazarus, 2004**).

2.18 Correlation among the TLR polymorphic varieties and diseases

Till to date it was found that TLRs are the main components of innate immunity. But somehow it is also strongly correlated with the adaptive immune system. Vertebrate immune system is very specialized system, thus it comprised multiple defensive system to protect our body. So, in course of the vertebrate evolution various modifications occurred as the pathogens also modified itself in the evolutionary adaptive processes. As the TLRs are much conserved molecular pattern from teleost to human, there was very less change of the structural identity of this molecular marker. Some in course of the evolution has been deleted from the organisms and some are modified. Mutations are also played a very vital role for the adaptation of these receptors. Also, environmental factors are responsible for the modifications. Therefore, mainly ten TLRs still in humans are recognized (now TLR11 and TLR12) and various mutational regions or single nucleotide polymorphisms are found in the populations.

TLRs are very common in case of inflammation. As we know that mutations almost created a negative pressure on the receptors and if this negative selection (**Smirnova, 2001**) acts on it during infection it may be deleted from the population (**Smirnova, 2003**). But it does not occur in the population as well as in the case of immune system. Sometimes it has also been seen that the disease that related with the Single nucleotide polymorphism for certain TLRs may not be related in the other population.

2.19 TLRs and rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of synovial membrane of the joints caused by the infiltration of activated immune cells including CD4+ T cells, B cells, and antigen-presenting cells. It typically results in warm, swollen, and painful joints, thereby causing disability, deformities, premature deaths, and economic loss. Estimates of heritability suggested that genetic factors contribute more or less 50% to the risk of developing RA. Indeed, the development of autoimmune diseases like rheumatoid arthritis (RA) depends on the interaction between the genetic background and our surrounding environment.

2.19.1 Markers of RA

During the onset of the disease, various serological factors like CRP, anti-CCP, rheumatic factor (RF) are produced and identified as markers of the disease. C- Reactive proteins are produced in the hepatocytes of the RA patients due to the influences of certain cytokines like IL-6, TNF-α etc (**Kim, 2015**).The high risk concentration of this protein is above 3.0 mg/L. Rheumatoid factors are the autoantibodies, produce against IgG and present in lymphoid follicles of synovial area (**Dissick, 2010**). This marker is found to be positive in over 70% of RA patients. CD14-positive cells (monocytes) from the bone marrow stimulate RF-producing B cells (**Hirohata, 1996**). IgM-RF is also one of the major markers and can be detected in 60-80% of RA patients. It was measured up to 50IU/ml (**Nell-Duxneuner, 2010**). The physiological role of RFs is to enhance the clearance of immune complex by increasing its avidity and size, also to help the B cells to uptake the immune complex, and efficiently present to T cells.

Anti-CCP is another well known marker of RA pathogenesis. It is sensitive and specific than RFs. Citrullinated proteins, a non standard amino acid, are produced by post translation modification of arginine by peptidylarginine deaminase (PADI) enzymes. The apoptotic cells also activate the enzyme. So, when apoptotic cells are not cleared properly, the level of this protein and enzyme are raised in the inflamed area (**Vossenaar, 2004**). These proteins are mainly found in the form of filaggrin and cyclic citrullinated proteins. Autoantibodies are produced by the immune system against this altered peptide in case of RA in the synovial tissue and increase the severity of the disease. Connection to this, antibodies has been produced against this altered circular protein in the body and it has been the prime marker for the disease. Anti- CCP is locally produced in RA joints and very high sensitivity and specificity for the diagnosis of the disease (**Mimori, 2005**). The antibody titer has a prognostic value in destruction of the joints in this disease with 88% sensitivity and 98% specificity.

Now-a-days, it has been observed that in addition to the above mentioned serological markers, various different genetic markers are detected for the pathogenesis of the RA. Polymorphism of the markers in different populations around the world predicted the susceptibility or resistant to the disease. Certain allelic variation or mutation may influence the prognosis of the disease in

various populations. Recently two different markers have been identified which may influence susceptibility or prognosis of rheumatoid arthritis.

Genetics of TLR with RA

2.19.2 Epidemiology of RA

RA is the most common inflammatory joint disease with prevalence between 0.5% and 1% worldwide (**Dejaco *et. al*., 2006**).Epidemiological data showed that Native American populations such as Pima Indians have a high prevalence of RA and it is low in countries like China, Japan and Africa compared to Caucasians (**Wolfe, 1968**). RA can occur at any age, but its incidence increases with age and may vary depending upon the type of classification criteria used and demographics of the population studied (**Symmons, 1994**). The peak age of onset has risen to 50 years or more and is more common in women than men with a ratio of 3:1 (**Young, 2000., Symmons, 2002., James, 2004**). Several prevalence and incidence studies of RA have been reported during the last decades, suggesting a considerable variation of the disease occurrence among different populations.

2.19.3 Disease etiology

RA is an autoimmune disease of unknown cause (**Rindfleisch and Muller, 2005**) and interaction between genetic and environmental factors play an important role in the development of disease in susceptible individuals (**Jawaheer *et. al*., 2004**).

2.19.4 Genetic factors

Family and twin studies indicate that first degree relatives of patients with RA have an increased frequency of developing this disease, particularly if the patients had severe disease or were seropositive for rheumatoid factor (**Lawrence, 1970**). Identical twins have higher concordance rates of the disease compared to non-identical twins, supporting genetic susceptibility (**Lawrence, 1970; Silman, 1993**). However, RA is a polygenic and genetically heterogeneous disease and non-inherited factors are also of great importance. In RA, the causative role of different genes may vary between individual patients and various combinations of polymorphisms in a selection of different genes (genotype) may predispose to the clinical picture

(phenotype). Some genes are responsible for severity of the disease rather than occurrence. Only few genes have been consistently associated with RA.

The major Histocompatibility complex (MHC) is a large genetic region on the short arm of chromosome 6, which has been consistently linked to RA. A large part of the MHC comprises human leukocyte antigen (HLA) genes, which encode individual‘s tissue type and are divided into class I (HLA-A, HLA-B, HLA-C) and class II (HLA-DR, HLA-DQ, HLA-DP) genes. The encoded proteins are crucial in determining the individual‘s immune response to antigenic stimuli. HLA class II genes, in particular HLA-DR4 and HLA-DRβ1, have been strongly linked to RA. Particular HLA-DRβ1 molecules in RA share a sequence that influences the peptides that are bound and viewed by the immune system. This core amino acid sequence is named the ―shared epitope‖ and these epitopes have been linked with both predisposition to, and severity of RA **(Gregersen, 1987., Del Rincon, 2003.,Gorman, 2004)**.

2.19.5 Role of Environment in case of Rheumatoid Arthritis

The main environmental factors that affect the patients are the non-inherited factors like smoking and infections play a major role in the etiology of RA. Epstein-Barr virus (EBV), Parvovirus B19, *Mycobacterium tuberculosis*, *Escherichia coli* and *Proteus mirabilis* have all been implicated as possible trigger factors for RA, but the results have been inconsistent **(Holoshitz,1986., van Eden, 1988.,Venables, 1988., Ebringer, 1989., Albani,1995., Rashid, 2007)**. Environmental agents are considered as triggers rather than as being directly involved in the disease process and complex interplay between genetic and environmental factors are probably important for the initiation of the disease process in susceptible hosts. Certain viruses and bacterial agents contain identical peptide sequence to auto antigen and infection with these microbial agents can induce an immune response that cross-reacts with the auto antigen, termed ―antigen mimicry‖. Antigen mimicry is one hypothesis to explain induction of autoimmunity by environmental triggers. Another concept proposes that a local immune response to any environmental agents may release pro-inflammatory cytokines to up regulate antigen-presenting capacity resulting in an immune mediated inflammatory cascade (**Feldman, 1989)**. Hormonal factors may also play a possible role in the etiology of the disease as suggested by increased female preponderance, high incidence during the premenopausal or post-partum period and protective effect of oral contraceptive pills presumably due to its progesterone content (**Lahita,**

1990). Diet and stress have also been considered to play a possible role in the disease expression (**Buchanan, 1991)**. Vitamin D and its metabolites may have an inverse relationship with disease activity in inflammatory polyarthritis or RA, due to their immunomodulatory effects (**Patel, 2007)**. The higher consumption of olive oil, oil-rich fish, fruit, vegetables and beta-cryptoxanthin may have a protective effect on the development of RA, whereas lower consumption of foods rich in antioxidants, could be associated with an increased risk of RA, but the results were inconclusive. Also, high intake of red meat and low intake of vitamin C might play a role in the development of inflammatory polyarthritis (**Pattison, 2004)**.

2.19.6 Cellular destruction in case of Rheumatoid Arthritis

The most pronounced and fundamental pathology in RA is destruction of articular cartilage and subchondral bone by ectopic and hyperplastic synovium. T lymphocytes and macrophages are also seen in large numbers along with dendritic cells, plasma cells and B-lymphocytes in the synovial fluid and membrane. The lining layer of the synovial membrane, which is normally two cells thick, becomes much thickened with increased numbers of both macrophage like and fibroblast-like cells (**Isaacs, 2011)**. In case of RA, the synovium becomes highly vascular with increased number of new blood vessel formation termed ―angiogenesis‖. The junction between synovial tissue, cartilage, and the bare area of bone within the joint capsule is prone to develop erosions early in RA. The synoviocytes proliferate as the disease progresses and invade the adjoining articular cartilage, where the secretion of cytokines, and cartilage and bone-degrading enzymes, results in characteristic destructive changes of RA. The invading, hyperplasic synovium is called pannus and the zone of invasion is called cartilage-pannus junction. The proliferative ―pannus‖ behaves as a locally invasive malignancy, burrowing into and destroying articular cartilage and subchondral bone. Synovial membrane, lines the tendons and bursae, also develops similar proliferative changes leading to destruction and deformity **(Szekanecz, 2001., Yamanishi, 2001., Isaacs, 2011)**. Although the pathogenesis of RA is still unclear (**Hayer *et. al*., 2005**), genetic studies have pointed to the association of RA with the HLA–DRB1 alleles. The HLA–DRB1 alleles are encoding for the SE. The SE hypothesis postulates that the SE motif is directly involved in the pathogenesis of RA by allowing the presentation of an antigenic peptide to T cells. The Ag could be either an exogenous Ag, such as a viral protein, or an endogenous protein. Recently, a number of possible endogenous Ags including citrullinated

protein, human cartilage glycoprotein, and heavy chain binding protein, have been identified (**Blass *et. al*., 1999**). Ag-activated CD4+ T cells stimulate monocytes, macrophages, and synovial fibroblasts to produce the cytokines IL -1, IL-6, and TNF α through cell-surface signaling by means of CD69 and CD11, as well as through the release of soluble mediators such as interferon-γ and IL-17. IL -1, IL-6 and TNF α that drive inflammation in RA. TNF α and IL-1 stimulate synovial fibroblasts, osteoclasts and chondrocytes that release matrix metalloproteinases, in particular stromelysin and collagenases. These enzymes degrade connective-tissue matrix and are thought to be the main mediators of joint damage in RA. Furthermore, TNF α and IL-1 inhibit the production of tissue metalloproteinase inhibitors by synovial fibroblasts. These activated macrophages, lymphocytes, and fibroblasts, as well as their products can also stimulate angiogenesis, which may explain the increased vascularity found in the synovium of patients with RA. Endothelial cells in the synovium are activated and express adhesion molecules that promote the recruitment of inflammatory cells such as neutrophils into joints. Neutrophils release elastase and protease, which degrade proteoglycan in the superficial layer of cartilage.

Rheumatoid synovium contains a number of pro and anti-inflammatory cytokines, which are mainly of T-cell and macrophage origin. Prominent pro-inflammatory cytokines are TNF-α, IL-1, IL-6, IL-8, IL-12, IL-15, IL-18 and interferon-gamma (IFN-γ), whereas the main anti-inflammatory cytokines are IL-4, IL-10, IL-11, IL-13, TGF-β, and cytokine neutralizing factors such as soluble TNF-α receptors and IL-1 receptor antagonist (IL-1ra). An imbalance between pro and anti-inflammatory cytokines may be the main pathogenic mechanism in RA as pro-inflammatory mediators, in particular TNF-α and IL-1, appears to play a major role in the immune mediated inflammatory cascade leading to the various articular and systemic manifestations **(Feldmann,1996., Fox, 1997., Zhang, 2001., Isaacs, 2011)**. Nitric oxide, prostaglandins, leukotrienes, and free oxygen radicals are the other pro-inflammatory factors present within the RA synovium. Rheumatoid synovium is characteristically highly vascular with angiogenesis and this is stimulated by various factors including hypoxia, soluble factors such as vascular endothelial growth factor (VEGF) and soluble vascular cell adhesion molecule-1 (VCAM-1), which stimulate endothelial cell growth. There are other adhesion molecules that are abundantly present on the vascular endothelium such as E-selectin and intercellular adhesion

molecules (ICAMs). Their expressions are stimulated by proinflammatory cytokines, particularly TNF-α and IL-1, resulting in the recruitment of inflammatory cells via specific receptors. Chemokines such as monocyte chemotactic protein-1 (MCP-1), IL-8 and MCP-2 are highly expressed in RA synovium and they stimulate progression of inflammatory cells into the joint **(Liao, 1995., Panayi, 2001., Isaacs, 2011)**.

Tissue hyperplasia and lymphocyte proliferation as a result of immune response are normally counteracted by programmed cell death or apoptosis to prevent over accumulation of cells. In rheumatoid joints, apoptosis is actively inhibited despite the presence of pro-apoptotic stimulants such as hypoxia and TNF-α in rheumatoid synovium. Impaired synoviocytes apoptosis may contribute to the pathogenesis of RA (**Isaacs, 2011)**.

The exact mechanism of cartilage and bone destruction in RA are not understood, but may be related to a variety of destructive enzymes secreted by pannus. The important ones are MMPs, which include collagenases, stromelysin and gelatinases, and serine and cysteine proteases such as cathepsins. These enzymes destroy the articular cartilage by acting upon collagen and proteoglycan matrix but are normally controlled by physiological inhibitors such as TIMPs. An impaired regulatory mechanism between these destructive enzymes and their inhibitors may partly be responsible for the destructive nature of the disease (**Goldring, 2000., Gravallese, 2000.,Tak, 2000., Isaacs, 2011)**. Other destructive factors include the cytokines TNF-α and IL-1, which activate osteoclasts leading to bone resorption. Bone destruction may also be mediated by factors such as osteoclast differentiation factor (ODF) or TNF-related activation induced cytokine (TRANCE) and receptor activator of nuclear factor κB ligand (RANKL). ODF interacts with membrane RANK that is present on osteoclast precursors, resulting in their differentiation and activation and subsequent bone destruction. The combination of TNF-α, IL-1 and ODF probably contributes to periarticular as well as systemic osteoporosis in RA. Activated CD4+ T cells express osteoprotegerin ligands that stimulate osteoclastogenesis, which then leads to bone degradation.

Activated CD4+ T cells also stimulate B cells through cell-surface contact and through the binding of αLβ2 integrin, CD154 (CD40 ligand), and CD28, to produce immunoglobulins,

including ACCP2A and RF. The precise pathogenic role of RF is unknown, but it may involve the activation of complement through the formation of immune complexes (**Anderson, 2004., Choy and Panayi, 2001**).

2.19.7 Role of different cells in case of RA.

2.19.7.1 Monocytes/Macrophages

Macrophages (MΦ) have phagocytic capacity and are central effectors of synovitis (**Haringman *et. al.*, 2005**). They are found both in the synovial tissue and SF. There are two types of macrophages in the RA synovial tissue. The macrophage-like type A synoviocytes in the lining and the sublining macrophages migrated as monocytes from the circulation and are diffusely distributed in the synovium. Both types have multiple functions such as clearance of immune complexes, antigen presentation (MHC class II are over expressed on MΦ), mediation and regulation of local and systemic inflammation, tissue remodeling through release of different cytokines and growth factors (TNFα, IL-1, IL-6, IL-10, IL-13, IL-15, IL-18 and GM-CSF), mediation and regulation of monocyte migration, stimulation of angiogenesis by chemokines and chemo attractants, tissue degradation and post-injury tissue remodelling by matrix metalloproteinases (MMPs) (**Kinne *et. al.*, 2007**). They express several markers of the resident macrophage population including CD68, CD163 and CD14 (**Bartok and Firestein, 2010**). In addition to the monocytes/MΦs central role in inflammation, they are also involved in bone erosions due to their ability to differentiate into osteoclasts. Upon stimulation with TNF-α, IL-1, IL-6 and IL-17 synovial fibroblasts and activated T cells can up regulate RANKL expression on their surface which can engage its receptor RANK on the surface of monocytes and drive them into osteoclastogenesis (**Davignon *et. al.*, 2013**).

2.19.7.2 Fibroblast-like synoviocytes

FLS are non-phagocytic mesenchymal-derived cells. The FLSs found in the lining layer are highly activated and exhibit features with aggressive invasive properties. They are important in both initiation and perpetuation of RA and can contribute to the maintenance of chronic inflammation through cell–cell contact and through elaboration of soluble products. In response to environmental stimuli and interactions with various cell types in the inflamed synovium, FLS

can secrete several mediators like cytokines, chemokines, growth factors and several other proinflammatory molecules like prostaglandins and leukotrienes. It has recently been shown in a SCID mouse model of arthritis that FLSs can migrate to a distant unaffected joint and invade and degrade the cartilage and thereby promote articular involvement (**Lefevre *et. al*., 2009**). In a very recent study, citrullinated fibronectin (cFn) was shown to inhibit apoptosis and increase proinflammatory cytokine secretion of RA FLSs (**Fan *et. al*., 2012**). This could be one possible explanation for the increased number of FLSs that contribute to the hyperplasia in RA synovial membrane.

2.19.7.3 T and B cells

The T cells constitute around 30-50% of all cell types in the sublining and the majorities are CD4+ with T helper (Th) 1 phenotype (**Bartok and Firestein, 2010**). T cells are identified as CD3+ cells in the synovial tissue and are either CD4+ Th cells, CD8+ cytotoxic T cells or CD4+ regulatory T cells (**Wagner *et. al*., 1998**). The Th1 subset mediates cellular immunity and is defined by IFNγ secretion. The Th2 is involved in humoral immunity and forms mainly IL-13 and IL-4, while Th17, the newest member of the T cell family is identified through its signature cytokine, IL-17. Th17 cells are important promoters of autoimmunity in RA (**Gaffen, 2009**). Synovial-derived T cells have a phenotype that indicate chronic immune activation but express low levels of cytokines and show signs of anergy (**Cope, 2002**).

B cells and plasma cells are mainly found in the sublining layer of synovial membrane. Around 5% of sublining synovial cells is B cell. The pathogenic roles of B cells in autoimmune disorders have historically been attributed to autoantibody production that would drive the inflammation locally either in soluble form or as immune complexes (**Marston *et. al*., 2010**). B cells contribute to RA through both antibody-dependent and antibody independent mechanisms. Examples of antibody-independent functions are antigen presentation, T cell activation and polarization, organisation of other inflammatory cells and dendritic cell modulation. B cells display considerable phenotypic diversity (**Anolik *et. al*., 2009**).

2.19.7.4 Neutrophils

The phagocytic neutrophils are the most numerous and most important cells in innate immune responses. In the RA joint neutrophils are the first cells to be recruited at the sites of

inflammation and accumulate mainly in the inflamed SF and to a lesser extent in synovial membrane at the site of active destruction where they phagocyte immune complexes and release degrading proteases (**Cascao *et al*., 2010**). Resting peripheral blood neutrophils are relatively short lived while primed and activated neutrophils within tissues undergo molecular changes that extend their life span and alter their molecular properties, thereby allowing them to carry out many functions. Delayed apoptosis, together with synthesis of inflammatory mediators like IL-8, TNF-α, IL-1, IL-6, IL-12, TGF-β and BLyS, and ability to present antigen to T cells via MHC II, makes tissue neutrophils capable of driving inflammatory processes. Several recent reports have suggested a possible direct contribution of neutrophils in early RA pathophysiology and bone remodelling (**Poubelle *et. al*., 2007**) by mediating Th17-responses (**Cua and Tato, 2010**), expressing PRRs (**Hayashi *et. al*., 2003; Kerrigan *et. al*., 2009., Greenblatt *et. al*., 2010**) and mediating bone resorption via activating osteoclastogenesis (**Chakravarti *et. al*., 2009**). Neutrophil adheres to the endothelial wall using selectins, integrins and adhesion molecules to pass from the peripheral blood to the site of inflammation. Rolling arrest precedes transmigration through the endothelial lining of the blood vessel, and chemo taxis to sites of inflammation, for example the joint.

2.19.7.5 Dendritic cells

DCs play an essential role in the initiation and perpetuation of inflammatory arthritis by presentation of arthritogenic antigens to auto-reactive T cells. Through their potent antigen-presentation ability they stimulate naïve T cells, direct effecter cell‘s function and polarize the T cell repertoire towards the Th1, Th2, or Th17 phenotypes. Myeloid DCs (mDCs) are considered especially important in promoting synovial inflammation. Plasmacytoid DCs (pDCs) are recruited in RA ST and comprise an antigen presenting cell (APC) population. That might contribute to the local inflammatory environment, particularly as a result of their capacity to produce cytokines *in situ* such as IFN-α, IFN-β, Il-15, Il-18 and Il-23p19. The number of synovial pDCs is specially increased in RA patients that are ACPA positive (**Lebre and Tak, 2009**).

2.19.7.6 NK cells

Several reports have indicated that NK cells may have direct or indirect role in RA (**Ahern and Brennan, 2011**). Dalbeth and Callan reported that a subset of NK cell (CD56bright) is greatly expanded within inflamed (synovium) joints (**Dalbeth and Callan, 2002**), in which they produce more IFN-γ compared to the blood NK cells from the same patients (**Aramaki *et. al*., 2009**). Moreover, these NK cells could induce the differentiation of monocytes into DCs. The communication between NK cells and other cell types through cytokines and chemokines make a potential risk for autoimmune diseases. Other example of this phenomenon is the crosstalk between NK cells and myeloid DCs, referred to as ―DC editing,‖ which may lead to NK cell activation and DC maturation. In this way, activated NK cells may in turn kill immature DCs that fail to undergo proper maturation (**Moretta *et. al*., 2006**). Furthermore, it has been reported that NK cells can function as APCs in some instances, which complicate the involvement of these cells in the immune responses (**Hanna *et.al*., 2004**).

Yen *et al*. have found that patients with RA complications have an expansion of unique population of CD4+CD28− T cells which is uncommon in healthy individuals (**Yen *et. al*., 2001**). Interestingly, CD4+CD28− T cells are functionally distinct from classical CD4+ TH cells and share some features with NK cells. For instance, they do not express CD40 ligand, but express CD57 (an NK cell marker), and produce large amounts of IFN-γ, and produce granzyme B and perforin (**Yen *et. al*., 2001**). One of the most potent osteoclastogenic cytokines which is pivotal in the pathogenesis of RA is TNF-α (**Di Santo, 2006**). TNF-α induces receptor acquisition by NK cells and the combination of TNF-α and IL-15 can enhance this effect (**Lee *et. al*., 2010**). It has been demonstrated that NF-κB is an important factor in regulation of NK cell growth and differentiation. NF-κB is activated in presence of TNF-α plus IL-15(**Lee *et .al*., 2010**).

2.19.8 Diagnosis

2.19.8.1 Laboratory diagnosis

Bony erosions and deformities seen in case of RA are largely irreversible. Initiation of therapy within three months after the diagnosis of RA is crucial since a delay of as little as three months in the introduction of these medications result in substantially more radiographic damage at five

years. Therefore, early diagnosis, although challenging, is critical. Laboratory quantifications of antibodies and inflammatory markers provide a way for early diagnosis of RA.

2.19.8.2 RF antibodies

The serum of RA patients contain a variety of Antibodies (Abs) directed against self-antigens. The most widely known of these Abs are the RF and anti CCP antibodies. Rheumatoid factors (RF) are autoantibodies directed against the Fc portion of IgG and found in every immunoglobulin subclass (IgM, IgA and IgG). However, the IgM class is being the most common (**Halldorsdottir *et. al*., 2000**). Rheumatoid factor is a well-established diagnostic and prognostic test in Rheumatoid Arthritis. Normal human lymphoid tissue commonly possesses B lymphocytes with RF expression on the cell surface. However, RF is not routinely detectable in the circulation in absence of an antigenic stimulus. Modified IgG could be a stimulus to RF production and may become an important component of RA pathogenesis (**Newkrik *et. al*., 2003, Das *et. al*., 2004**). This concept is supported by studies that observed an association of RF and more severe RA with autoantibodies to advanced glycated end product-damaged IgG or agalactosyl IgG. Co-stimulation of B cells, perhaps mediated by toll-like receptors (TLRs), may allow B cells with low affinity receptors for IgG to become activated. TLRs are components of the innate immune system, and they provide signals after engaging various bacterial and viral products (**Shlomchik *et. al*., 1993, Rifkin *et. al*., 2005**). CD14-positive cells (monocytes) from the bone marrow stimulate RF-producing B cells (**Hirohata *et. al*., 1995)**. Synovial fluid RF may be produce by synovium-derived CD20-negative and CD38-positive plasma cells (**Van Esch *et. al*., 2003).** Circulating B cells require interleukin-10 (IL-10) for RF production (**Perez *et. al*., 1995**). Cigarette smoking, a risk factor for more severe RA, is associated with an increased prevalence of RF (**Padyukov *et. al*., 2004**). RFs possess significant heterogeneity related to mutations within heavy and light chain genes (**Youngblood *et. al*., 1994**). Thus, IgM RFs from patients with RA react with a variety of antigenic sites on autologous IgG (**Carson, 1993**). The potential physiological role of IgM RF includes the following:

1) Binding and processing of antigens embedded in immune complexes.
2) Presentation of antigens to T lymphocytes in presence of HLA molecules.
3) Immune tolerance.
4) Amplification of the humoral response to bacterial or parasitic infection.

5) Immune complex clearance.

2.19.8.3 Anti-cyclic citrullinated peptide (CCP) antibodies

Anti-citrullinated protein antibodies are highly specific for RA (**Masson-Bessiere *et. al*., 2001**). Citrulline, the antigenic determinant for this Abs, is a nonstandard amino acid. It does not incorporate into proteins during translation. It can, however, be generated post-translationally by enzymatic citrullination (deimination) of arginine residues. The citrullination is catalyzed by peptidyl arginine deiminase; arginine residues on fibrin and fibrinogen may be favoured sites for deimination within rheumatoid joints (**Van Boekel *et. al*., 2002; Vossenaar and van Venrooij, 2004, Vossenaar *et. al*., 2004, Vossenaar and Robinson, 2005**).

An enzyme linked immunosorbent assay (ELISA) was developed to detect antibodies directed against filaggrin derived from human skin and has high specificity and sensitivity for the diagnosis of RA (**Palosuo *et. al*., 1998**). The target amino acid in filaggrin is citrulline, a post-translationally modified arginine residue (**Schellekens *et. al*., 1998**). Subsequently, an ELISA assay for the detection of antibodies to a cyclic peptide containing citrulline was made commercially available, easier to standardize, and also has high sensitivity and specificity for the diagnosis of RA. This has become the assay for the detection of anti-cyclic citrullinated peptide (anti-CCP) antibodies.

2.19.9 Different Inflammatory markers in case of RA (ESR and C-reactive protein)

While multiple blood markers of inflammation have been identified and shown to be useful in the evaluation and treatment of RA, to date, ESR and CRP have been most commonly studied and used in clinical practice. Elevation of ESR and CRP are the strongest predictors of persistent, progressive disease in RA. If they are elevated in early disease and do not show any improvement with therapy, this may lead to joint damage and other worse outcomes (**James *et. al*., 2004; Lindqvist *et. al*., 2005**). ESR and CRP are very sensitive to change in disease activity. The ESR, an indirect assessment of inflammation, measures the distance that RBCs fall in a capillary tube over the course of an hour. The presence of inflammation causes the cells to fall more quickly due to the action of inflammatory proteins, such as fibrinogen or immunoglobulins, blocking the normal charge inhibition on RBCs. In many RA studies, an ESR level greater than

20 to 30 mm/h has been considered abnormal; however, considerable individual variability between normal and abnormal tests exists.

CRP is a pentameric protein released in response to inflammatory stimuli. CRP levels are a more accurate measure of inflammation than the ESR. Measuring CRP in inflammatory conditions is preferred over the ESR as CRP responds much more quickly to inflammatory stimuli and can, therefore, be used as a timely marker of active inflammation. The CRP level that has been determined to be abnormal in RA studies is generally greater than 1.0 mg/dL. But caution must be taken when interpreting this value as many clinical labs report CRP in mg/L, resulting in a 10-fold higher value that may still be a normal result.

2.19.9.1 Assessment of Disease Activity

In case of RA, measurement of disease activity at specific point of time or at a regular intervals helps to evaluate the disease progression and it is vital to assess treatment response, outcomes and prognostic factors. Various methods have been introduced and validated to measure disease activity in RA over the last few decades. These methods have been designed and modified to evaluate three different but interrelated aspects of the disease progression: clinical, radiological and functional.

2.19.10 Measurement of clinical disease activity

In the early 1990s, core sets of disease activity measures have been proposed by the American College of Rheumatology (ACR, formerly ARA), European League Against Rheumatism (EULAR) and World Health Organization (WHO) / International League of Associations for Rheumatology (ILR), to standardize the disease activity assessments in the clinical trials involving RA patients **(Tugwell, 1982., Felson, 1993., Boers, 1994)**. These measures included swollen joint count (SJC), tender joint count (TJC), and patient assessment of pain, global assessment of disease activity by the patients (PGA) and by the evaluators (EGA) and acute phase reactants such as erythrocyte sedimentation rate (ESR) and C - reactive protein (CRP). The core set also included structural damage on radiographs and functional status (**Aletaha and Smolen, 2006., Tugwell and Bombardier, 1982**). These measures are also very useful and crucial to assess disease activity and treatment response in day-to-day clinical practice.

2.19.10.1 Pain, Swollen and tender joint counts

Pain is the serious symptom for patients with RA and it is measured on a 100-mm visual analogue scale (VAS), evaluating symptom for one week before the study point. Horizontal VAS is more commonly used than vertical scales and there are also other reliable methods of pain assessment such as, arthritis impact measurement scale (AIMS) and McGill pain questionnaire (**Aletaha and Smolen, 2006**).

A number of different joint indices and counts have been developed over the years and they vary by the number of joints assessed or by the way several joints are aggregated to represent joint regions (**Aletaha and Smolen, 2006**). Ritchie *et al.,* introduced a graded tender joint count, assessing 26 joint areas with grades ranging between 0 to 3 depending upon the severity of joint tenderness (**Ritchie *et. al*., 1968**). Further modifications of the joint indices and simplifications of the extensive joint counts were carried out by other groups over the years, reducing the number of joints assessed (**Egger *et. al*., 1985; Fuchs *et. al*., 1989; van der Heijde *et. al*., 1992**). These simplified joint counts have been validated and are reliable and easy to use in clinical practice (**Prevoo *et al*., 1993; Smolen *et. al*., 1995**).

2.19.11 Disease activity scores and indices

Composite disease activity scores have been developed over the years to give reliable identification of disease activity and to overcome methodological problems. These scores use special formulas integrating SJC, TJC, ESR or CRP and GH to measure overall disease activity (**Aletaha and Smolen, 2006**). Van der Heijde *et al*, introduced disease activity score (DAS) in 1990 based on 44-swollen joint count (**van der Heijde *et. al*., 1990., van der Heijde *et. al*., 1993;**). This was later modified to include the reduced 28-joint count, DAS28, which shows similar validity and reliability compared to DAS and has been widely used (**Prevoo *et. al*., 1993; Prevoo *et. al*., 1995; Smolen *et. al*., 1995**). Both DAS and DAS28 have been modified in several ways to exclude the assessment of GH (DAS-3 and DAS28-3) and to include CRP instead of ESR (DAS-CRP and DAS28-CRP) (**Aletaha and Smolen, 2006**). Formulae to calculate DAS28 with 4 or 3 variables and with ESR or CRP

2.19.12 Criteria to assess disease activity

After the introduction of the composite disease activity indices, a number of criteria have been validated, based on DAS, DAS28, SDAI and CDAI, to assess different levels of disease activity including remission (**van Gaestel *et. al*., 1996., Smolen *et. al*., 2003., Balsa *et. al*., 2004., Fransen *et. al*., 2004., Paulus, 2004., Fransen and van Riel, 2005., Aletha *et. al*., 2005**).

EULAR has adapted disease activity criteria based on DAS and DAS28, which have been widely used in several studies.

EULAR criteria based on DAS

DAS < 1.60 - remission

DAS ≥1.60 and ≤ 2.40 - low disease activity

DAS >2.40 and ≤ 3.70 - moderate disease activity

DAS> 3.70 - high disease activity

The United States (US) Food and Drug Administration (FDA) has also proposed remission criteria, which is based on ACR remission criteria, but also takes into account the structural damage on x-rays and treatment status at the time of assessment. According to this, 5 out of 6 ACR remission criteria have to be fulfilled plus radiographic arrest for ≥ 6 months with no drug therapy (**Paulus, 2004**).

2.19.13 Radiological progression

Conventional radiography has been traditionally used to assess structural damage in RA. X-rays of hands and feet and/or large joints have been used to define radiological damage at a given point of time as well as progression of structural damage over a period of time. The advantage of radiographic assessment of disease progression over other methods is that the damage seen on x-rays largely irreversible and it represents the cumulative measure of disease activity and destructive process over time. Another major advantage is that apart from providing permanent records, radiographs can also be randomized and blinded for clinical investigations of new therapeutic agents in clinical trials (**Wollheim *et. al*., 1988., van der Heijde, 2000**). It has been widely recognized that radiological damage on x-rays has to be quantified to define the disease status of the patients and more importantly to assess disease progression, treatment response and outcome (**Weisman, 1987., van der Heijde, 2000., Rau and Wessenberg, 2005**). Semi-

quantitative methods have been developed to translate the amount of structural damage on x-rays into a score value as no truly quantitative methods are available(**Rau and Wessenberg, 2005**). There are lot of abnormalities that can be seen on radiographs in patients with RA among which erosions and, to a lesser extent, JSN are widely accepted to be included in the scoring methods as they give reliable and additive information on radiological progression (**Sharp *et. al.*, 1985., Fries *et .al.*, 1986**).

2.19.14 Included Joints

It is known that synovial joints can be affected in RA but not feasible to include all joints in scoring radiological damage. Hands (including wrists) and feet have been chosen to represent the overall radiological status of the disease as they are the most commonly involved joints in a majority of patients with RA, wherein erosions and JSN can be seen very early (**Scott *et. al.*, 1986., Drossaers- Bakker *et. al.*, 2000**).The joints that are usually evaluated in the scoring methods include PIP joints, MCP joints, IP joints of thumbs, wrist joint as a whole or as individual joints, MTP joints and IP joints of the 1st toe(**van der Heijde *et. al.*, 1999**). RA is typically a symmetrical polyarthritis, radiological changes can appear asymmetrically. So both hands and feet should be included in the radiographic evaluation (**van der Heijde *et. al.*, 1999**). Postero-anterior (PA) views of the hands and feet x-rays are the most commonly used technique for radiographic assessment (**Mewa *et. al.*, 1983**).

2.19.15 Different Scoring methods for RA

Several scoring methods have been developed and subsequently modified over the last few decades to quantify the radiographic damage in RA (**Steinbrocker *et. al.*, 1949., Kellgren, 1956**). The global method of scoring have been designed to score erosions and JSN together with one overall score, while the composite method scores erosions and JSN individually with a separate score for each that are added together at the end to give a overall score. Although there are several scoring methods available to measure radiographic damage, the modifications made by Larsen and Sharp mainly SvdH have been the most commonly used practice. Each of these scoring methods has their own advantages and disadvantages. The advantage of Larsen‘s score is that an experienced reader can perform it quickly, whereas SvdH method is more time consuming (**Sharp *et. al.*, 2004., Rau and Wessenberg, 2005**). However, inclusion of soft tissue

swelling in the Larsen's score may lead to a relatively high baseline score, decreasing with response to treatment. This may reduce the total possible increased score due to progressive damage, contributing to low sensitivity to change (**Rau and Wessenberg, 2005**). It has been shown that SvdH method is better than others in respect to its sensitivity to detect a real change in x-ray progression over time (sensitivity to change) and in detecting changes that are clinically meaningful, termed minimal clinically important difference (MCID) i.e. smallest radiographic change that necessitates the physicians to alter their treatment (**Pincus *et. al*., 1997., Paimela *et. al*., 1998., Lassere *et. al*., 1999., Drossaers- Bakker *et. al*., 2000., Bruynesteyn *et. al*., 2002., Bruynesteyn *et .al*., 2004., Sharp *et. al*., 2004., Guillemin *et. al*., 2005**).

2.19.16 Assessment of function

Functional assessment in patients with RA is a vital component in the evaluation of disease progression as it significantly correlates with disease activity, structural damage and long-term outcomes (**Wolfe and Hawley, 1998., Barrett *et. al*., 2000., Scott *et. al*., 2000., Young *et. al*., 2000**). The Health Assessment Questionnaire (HAQ) or HAQ-disability index (HAQ-DI) is a 20-question instrument, which assess the degree of difficulty a patient has in accomplishing his or her tasks in eight functional categories such as dressing, rising, eating, walking, hygiene, reaching, gripping and usual day to day activities. For each question there is a four-level difficulty scale ranging from 0 to 3. The final score is the mean of the highest scores across eight categories and it ranges from 0 to 3, with higher levels indicating more disability (**Fries *et al*., 1980., Ramey *et. al*., 1992**). The HAQ has been modified several times subsequently to simplify it and to make it user friendly and also to include other domains such as depression and anxiety (**Pincus *et. al*., 1983., Pincus *et. al*., 1999., Wolfe *et. al*.,2004**).It has been shown that during the early stages of the disease (<5 years duration), the HAQ score is mainly influenced by joint pain and swelling due to inflammation, which can improve with treatment (reversible); whereas in the late stages, the HAQ scores strongly correlate with structural damage (irreversible) and so the reversibility of HAQ in patients with established RA may not be as significant as in early RA (**Aletha *et. al*., 2006**).

The Arthritis Impact Measurement Scale (AIMS) is another form of patient self reported functional questionnaire, which include assessment of depression and anxiety (**Meenan *et. al*.,**

1980). There are longer and shorter versions of the AIMS, which have been used to evaluate function in patients with arthritis including RA (**Aletha and Smolen, 2006**).

Objective quantitative instruments have also been used to assess function and these include measures of grip strength and locomotion (Pincus and Callahan, 1992) using a vigorimeter or a dynamometer, indicating the pressure attained by squeezing a compressible rubber bulb (**Jones *et. al*., 1991; Pincus *et. al*., 1991**).

2.20 Relation of TLRs with RA

TLRs constitute one of the major markers of innate immunity which recognize various conserved antigens like LPS, bacterial flagellin, double stranded DNA, RNA. These markers are expressed on a variety of cell types such as NK cells, monocytes, epithelial cells etc. TLR1, 2, 4, 5 and 6 are expressed on the cell surface and they interact with ligands found on the surface of pathogens. In contrast, TLR3 and 7, 8 and 9 are located intracellularly on endosomal membranes and their ligands must be taken up into the endosome in order to activate the downstream signaling pathways (**Huang, 2009**). Binding of the TLRs with its corresponding ligands results in the activation of either the MyD88-dependent or the MyD88-independent pathways. The MyD88-dependent pathway leads to the activation of NF-κB and promotes in cytokine gene expression (**Huang, 2009)**.

TLR2 and TLR4 are primarily expressed in the blood monocytes of RA patients. Expressions of TLRs can also be found in the synovial tissue. (**Radstake, 2004; Huang, 2009)**. Endogenous TLR ligands in synovial tissue of RA patients include fibrinogen, heat shock protein 60 and 70, and fibronectin. These ligands activate certain TLRs in the synovial tissue and induce the production of various cytokines.

A Plethora of researches are being conducted throughout the World to explore the association of TLR with RA. Non-missense single nucleotide polymorphism in TLR4 was identified to be risk factors for the development of RA in Chinese Han population (**Yang, 2013**) yet several other studies have reported the lack of association with RA (**Xu, 2012**).

Cytokines such as TNF, IFN- y, IL-6 also regulates the expression of TLRs in the synovial fluid in case of RA pathogenesis (**Steiner, 1999., Radstake, 2004**). Chamberlain *et. al*., in 2012 reported strong correlation of TLR5 and TNF-α with RA progression. TLR5 has been postulated to be a TNF responsive gene and it is possibly linked to RA progression through induction of angiogenesis (**Chamberlain, 2012**). Study with transmission disequilibrium test on the French families revealed no association with the major RA related TLRs (TLR1, 2, 6 and 9) with RA (**Orozco, 2005; Jaen, 2009**).

It can be inferred from the different reports that TLR plays significant roles in RA pathogenesis. Genetic variations in the TLRs may influence the susceptibility or resistant to the disease in various populations. Auto reactivity of the synovial cells and production of the cytokines in RA pathogenesis significantly related with the markers activation. Autoimmune cells in the synovial tissue trigger TLR for the signalling and influence the cytokine production for the disease progression. So, signalling pathways can modulate the expression and cytokine production for the improvement of the disease. Initial screening of TLRs is needed in various populations, as that may reveal the association of RA with the populations and may help for controlling of the disease.

2.21 TLRs and typhoid fever

Free living organisms have the ability to cope up with the new environment by modifying their gene expression patterns (**Groisman and Mouslim, 2006**). Enteric fever is an important infection now-a-days among the populations in endemic countries like India (**Meltzer and Schwartz, 2010**). *Salmonella enterica* serotype *typhi* (*S. typhi*) is a gram negative bacteria that is restricted to human and causes a wide range of food and water-borne diseases ranging from self-limiting gastroenteritis to systemic typhoid fever (**Manon, 2012)** . The occurrence of typhoid fever is less in developing and industrialized countries, but it is high prevalent in countries like India and South- East Asia (**Meltzer and Schwartz, 2010)**. According to Crump *et. al*., typhoid fever caused 21,650,974 illnesses and 216,510 deaths during the year 2000 (**Crump, 2004)**. The Poor sanitation, lack of a safe drinking water supply, unhygienic condition and low socio economic conditions have amplified the disease progression in India which increased the morbidity and mortality among population (**Kanungo, 2008)**.

The virulence factors of different Salmonella serotypes can perform a powerful model for studying the host adaptation mechanisms, because these pathogens are physiologically well characterized for genetic analysis (**Baumler, 1998)**. The Salmonella genus is divided into two distinct species, *Salmonella bongori* and *Salmonella enterica*. The serotype typhi and paratyphoid A, B and C is present in human and in other higher primates (**De Jong, 2012**). Salmonella produces multiple Pathogen associated molecular patterns (PAMPs) like flagella, fimbrae, LPS (Vi antigen), and bacterial DNA and develop survival mechanism from the host cells by producing superoxide dismutase, salmonella containing vacuole, type I secretion system etc.(**Ibarra and Steele-Mortimer, 2009)**. These virulence factors have been recognized by the pattern recognition receptors like Toll like receptors (TLRs) (**De Jong, 2012)** which initiate an immune response and form a link between the innate and adaptive immunity (**Kawai and Akira, 2010)**. Primarily TLR4 and TLR5 play a major role in activation of the immune responses against LPS and flagellin. TLR4 polymorphisms among Asian Malay population express a higher risk for typhoid infection in case of *S. typhi* (**Bhuvanendran, 2011).** Genetic association study among the Vietnam population could not prove any association of TLR5392STOP stop codon with typhoid fever patients (**Dunstan, 2005)**. The modulation or variation of binding site of TLR gene receptors against the LPS, flagellin or other antigens of *Salmonella typhi* evokes host immune response during typhoid fever (**Sivaji, 2016)**. Several association studies have been conducted on TLR with typhoid fever on a different population of the worldwide, especially on TLR4 and TLR5. So, a complete screening of all ten TLR genes on typhoid patients is needed to interpret if there is any association present with other TLRs.

2.21.1 Toll-Like Receptor 4 (TLR4) and Typhoid Fever

The detection of genotype and mutation study within the TLR4 gene in typhoid fever patients and controls in the Vietnamese population postulate that genetic variations present within TLR4 may affect the recognition of *S. typhi* and altering activation of innate immunity and hence severely affecting the first line of defense against this pathogen. Out of the ten mutations identified seven are novel mutations found in the Vietnamese population. Besides the two common polymorphisms that has been reported (Asp299Gly and Thr399Ile), most polymorphisms within TLR4 occur at low frequencies in different populations in the world. Therefore it is difficult to establish their role in genetic susceptibility to infectious disease. The

TLR family has been described as type I transmembrane pattern recognition receptors (PRR) that contain varying numbers of extracellular N-terminal leucine-rich repeat (LRR) motifs, followed by a cysteine-rich region, a transmembrane domain, and an intracellular Toll/IL-1 R (TIR) motif. Several lines of evidence argue that TLRs play an important role in innate immunity, and changes in TLR structure could potentially lead to functional changes of those receptors. The extracellular TLR4 region which contains Glu24-Lys631 is the functional domain for LPS and MD-2 binding. It was identified five low frequency missense mutations (Ser73Arg, Ala97Val, Tyr98Cys, Thr175Ala, Thr399Ile) in the ectoplasmic LRR domain. The amino acid substitutions may alter protein structure and function as the structure and side chains of some of the substituted amino acids differ from wild-type TLR4. One of these, Ser73Arg, showed a slightly higher frequency in cases of typhoid fever than controls. These LRR region mutations may potentially alter phosphorylation of TLR4 altering downstream signaling of inflammatory mediator activation, ultimately contributing to disease susceptibility. The mutations Thr399Ile and Asp299Gly, which also lie in the ectoplasmic domain, are significantly associated with a blunted response to inhaled LPS and a variety of diseases. A mutation in the hydrophobic region adjacent to the transmembrane domain of TLR4 did not respond to LPS. A low frequency missense mutation (Val651Phe) in the transmembrane domain of TLR4 was identified and the possibility exists that it may alter the function of TLR4 in response to LPS produced by the *S typhi* (**Hue *et. al*., 2009**).

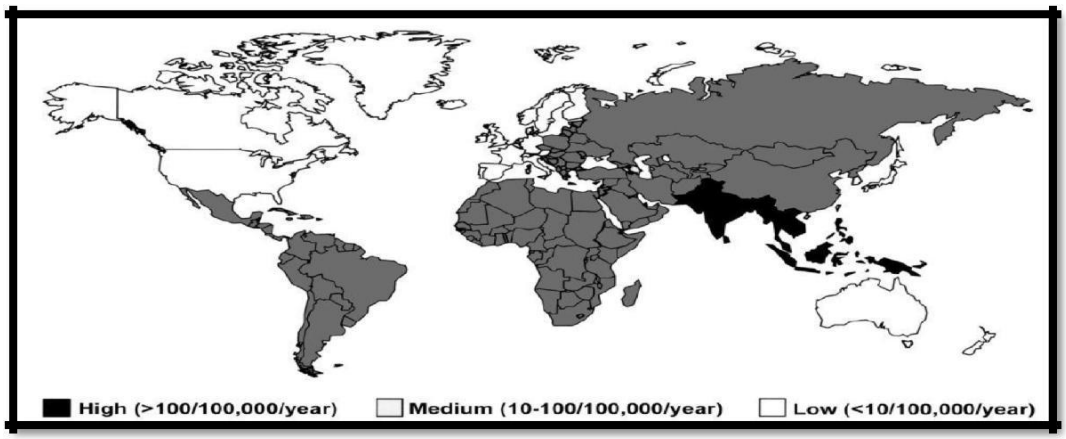

Fig 5: Geographical distribution of Typhoid fever (Source: Crump et al. 2004).

2.21.2 Toll-Like Receptor5 (TLR5) and Typhoid fever

TLR5 is a good candidate gene to use in the study of genetic association of typhoid fever. In one case of *in vitro study*, Flagellin from *S. typhimurium* binds to TLR5 and activates proinflammatory cytokines in the intestinal epithelia. In another *in vivo* murine studies, including experiments that involved Salmonella infections indicate that flagellin is an important stimulant of both innate and adaptive immune responses. In the third case, the murine TLR5 gene lies within a locus that is associated with the susceptibility to Salmonella infection.

In addition, TLR5 is associated also with legionnaire‘s disease caused by infection with *Legionella pneumophila*. Legionella and Salmonella are similar in that they both are gram-negative, flagellated pathogens, but, most importantly they occupy the same intramacrophage niche within the host (**Dunstan *et. al*., 2005**).

The presence of antiflagellin antibody responses in patients with typhoid fever clearly indicates that flagellin is expressed *in vivo* in humans and therefore is available for interaction with TLR5. TLRs also regulate innate immune responses and also play a crucial role in the initiation of adaptive immunity. TLRs influence the activation of adaptive immune responses by two mechanisms. Primarily, TLRs initiate signalling pathways by up-regulating co- stimulatory molecules, and this leads to the maturation of dendritic cells. In addition, TLR-induced cytokines, mostly interleukin 6 (IL-6) are essential if T helper cells want to overcome the suppressive effect of CD4+ and CD25+ regulatory T cells and to generate pathogen specific adaptive immune responses. Individuals who have one or two copies of the stop codons in TLR5, have significantly decreased IL-6 production after stimulation with flagellin, and this decreased production affect the mechanism of TLR-activated adaptive immunity. There may be sufficient redundancy in the TLR pathway to obviate the requirement of TLR5 for a protective immune response to *S. typhi*. The frequency of stop codon in TLR5, which functions as a dominant negative and severely impairs signalling, was not significantly associated with typhoid fever. Despite *in vitro* and murine studies describe the recognition of Salmonella flagellin by TLR5; this pattern recognition molecule may not play an important role in TLR- stimulated innate immune responses to human infection with *S. typhi*. Initiation of these responses may rely on other TLRs recognizing different bacterial ligands (**Dunstan *et. al*., 2005**).

2.21.3 Association of Toll like receptors with HIV

Toll like receptors regulates both the innate and adaptive immune response and polymorphism in the TLR genes has been investigated in case of various diseases (**Schwartz, 2005**). Susceptibility to the human immunodeficiency virus (HIV) infection and disease progression are variable among different populations and also it has been genetically determined (**Willie, 2014**). A small percentage (0.2%) of HIV-1 sero-positive patients is able to control the HIV-1 infection over several years. The adult HIV prevalence at national level has 0.26% in 2015 (**India HIV Estimations, 2015**). It defines that they can maintain a viral load which is fewer than 50 copies of HIV-1 RNA per ml **(Nunez, 2011)**. Different TLRs expressed on different cell types in the human immune system and up regulated by the effect of cytokines. IFN- γ has also induced the expression of TLR4 in peripheral blood monocytes (**Mita, 2001**).

Several association studies have been reported in case of TLRs with HIV disease progression. It has been reported from the previous study that depletion of CD4+ cells in HIV positive individuals release some viral proteins that directly activates the TLR4 (**Brenchley, 2006**). According to **Baenziger *et. al.***, in murine model the chronic activation of TLR7 leads to immune dysregulation that is almost similar found among humans (**Chang, 2009**). Several other TLRs are also associated with HIV disease progression. It was also reported that some polymorphism in TLR3 gene (Leu412Phe) plays a protective role against the disease (**Huik, 2013**). Asp299Gly, Thr399Ile the two variants which recognize lipopolysaccharide (LPS) is associated with increased infection risk in HIV patients (**Papadopoulos, 2010**). According to **Martinelli *et. al*.,** pDCs which normally secretes the IFN- γ and activates the natural killer cells also gets suppressed due to gp120 viral envelope produced by the HIV virus. It also inhibits the TLR9 mediated induction of proinflammatory cytokines in pDCs (**Martinelli, 2007**). Some of the polymorphic variation in the TLRs which related to disease progression or as a set point for the disease depends on the ethnicity among different populations of the world (**Mackelprang, 2014**).

CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

The technical details and analytical parameters used for collection, analysis, and interpretation of the data have discussed here. Also, described the criteria and procedures for collection of the human blood samples followed by experimental details of ten human TLR genes, and statistical analysis of the samples.

3.1 Sample Collection

The present study has been carried out on four endogamous caste groups of North Bengal region of West Bengal, India. Four populations selected in the present study belong to the northern region of West Bengal. They include Gurkha, Muslim, Rabha and Rajbanshi which is elaborated in the discussion part.

3.1.1 Selection criteria of samples

Randomly selected normal healthy individuals from each population have been included in the present study. The major criteria for selection of the samples includes-

A. Un- relatedness of individuals from each other at least for last two or three generation.

B. They were the resident of the area for at least two or three generations.

C. Individuals excluded which have inter- caste or inter religion marriages for two or three generations.

3.1.2 Sample collection strategy

Prior to the sample collection, regional addresses and detailed lists of the samples were prepared. Random numbers were generated with the help of computer and adult individuals living in different parts of North Bengal region were questioned about their ethnicity and tribal affiliations, surnames and birth places of their parents. Only un- related subjects were considered eligible to participate. Whole blood was then obtained by vein punctured and about 5mL blood after obtaining the informed consent from the subjects. The study was performed with the approval of Human ethics committee of Department of Zoology, University of North Bengal.

3.1.3 Demographic profile of the samples

A total of 400 Human blood samples belonging to four populations were collected from different parts of North Bengal like Muslims blood samples were collected from Malda district, Gurkha from Kurseong, Rabha and Rajbanshi from Jalpaiguri and Coochbehar districts. Demographic profile of the collected samples is shown in **Table 2**.

3.2 The four populations that were selected for our study include:

[1] Rajbanshi: an ethnic caste population from Terai and Dooars region of West Bengal,

[2] Rabha, a primitive tribal population group from Northern part of West Bengal,

[3] Gurkha, a Nepali speaking ethnic population from Siliguri and adjoining areas of West Bengal and

[4]. Muslim from northern part of the state

Detailed description about the possible origin, socio-cultural practices and the position of these population groups in the social hierarchy network of Indian society have been discussed in detail below

3.2.1 Rajbanshi

Rajbanshis are highly diversified ethnic community with rich cultural, linguistic and social background. The Rajbanshis account for 18.4% of the total Scheduled Caste population of West Bengal (26° 20'- 27° 03' N and 88° 18'- 89° 29' E) as per 2001 Census of India. Although distributed dispersedly throughout the state, the Rajbanshis are mainly inhabitant of Terai and Dooars region of West Bengal especially the districts of Jalpaiguri and Coochbehar. They also have adequate population in the neighboring state of Assam. They have an Indo-European linguistic background. Beside their own dialect they also speak Bengali, Assamese and some other minor languages. They practice Hinduism and they have their own rich cultural identity and heritage. According to Risley (1892) there are many sub-castes of the Koch Rajbanshis in North Bengal (**Risley, 1891**). Thus, it can be said that the ethnicity and genetic richness of the Rajbanshis may enhance their importance in genetic diversity studies and interpretation of the migratory routes in this part of the country.

3.2.2 Rabha

Rabha is a very little known small endogamous scheduled tribe community of India (**Chakraborty, 2013., Census, 2001., Sarkar, 2011**) with a conserved gene pool of their own. In West Bengal, they are mainly distributed in the forest villages of dooars region of Jalpaiguri and Coochbehar districts. Historically they are considered the primitive inhabitants of the region who remained isolated from other neighboring populations due to strict endogamy. According to H.H. Risley, Rabhas belong to the Indo-mongoloid stock (**Risley, 1891**), having a unique genetic constitution. Thus, there is an urgency to undertake genetic diversity studies in this group.

3.2.3 Gurkha

Indian Gurkhas constitute a community of Nepali speaking people, populating the Eastern and North-Eastern states of the country, mainly West Bengal and Sikkim (**Chatterjee, 1974**) with sizeable populations in the states of Meghalaya, Nagaland, Manipur, Tripura, Mizoram, Arunachal Pradesh and as well as in Assam. In West Bengal, they are distributed in Terai and Dooars, as well as the hilly regions of the northern part of the state. Apart from India, they are found in Nepal and to some extent in Bhutan. They are a mixture of various clans and ethnic groups which include Bahun (Brahmins), Chhetri, Thakuri, Gurung, Magar, Newar, Tamang, Rai (Khambu), Limbu (Subba), Sherpa, Yolmo and many more (**Roy, 2012**). Each of them has their own languages, but Nepali language has become the common binding thread of all the Gurkha castes and clans.

3.2.4 Muslim

Bengali Muslims inhabiting in West Bengal represent the second-largest ethnic Muslim community in the world, after the Arabs (**Eaton, 1993)** native to modern-day Bangladesh and the eastern states of India, including West Bengal and Assam. They speak Bengali dialects and have strong cultural similarities with the Bengali Hindus, thereby increasing the cultural richness of West Bengal. They are the second-largest community as also the largest minority group of the state, Bengali Muslims comprises 27.01% of the total population of West Bengal (**Census, 2011**).

Table 2: Demographic profile of the studied populations

	Populations			
	Gurkha	**Muslim**	**Rabha**	**Rajbanshi**
Sample size	**125**	**140**	**50**	**85**
Total	**400 sample**			
Mean Age (Yr)	**31 yrs**	**34 yrs**	**29 yrs**	**28 yrs**
Linguistic Family	**Tibeto-Burman**	**Indo- Aryan**	**Tibeto-Burman**	**Indo- Aryan**

3.3 RA Based Study

3.3.1 Study population

A total number of 110 Rheumatoid Arthritis patients were included in this study. Samples were collected from an authorized diagnostic laboratory of Siliguri and from North Bengal Medical College and Hospital (NBMCH, Sushrutnagar, West Bengal, India) under the guidance of medical practitioners. The diagnosis of RA was made by the physician based on the medical and clinical history, physical examinations and symptoms of the disease and most importantly, their fulfillment of the American College of Rheumatology criteria 2010 (Aletaha, 2010). The inclusion criteria for the subjects in the patient group for the study included the following:

[1] Resident of Siliguri and adjoining areas of the sub-Himalayan region of West Bengal, India.

[2] Subjects fulfilling the ACR criteria 2010.

[3] Subjects having the symptoms for ≥1 year duration.

[4] Patient should be above 18 years of age.

[5] Patients capable of giving informed consent for becoming a part of the study.

According to ACR criteria 2010 (**Aletaha, 2010**), patient is considered to have definite RA if he/she has 1 or more swollen joint(s) and having ≥6 score on the classification criteria which include joint distribution, serology reports, disease duration and measures of acute phase

reactants. Furthermore, evaluation of the disease was also done based on DAS28 score system for confirmation. Most of the patients belonged to stage II and III as was also evident from the clinical evaluations and other relevant data.

However, there are certain exclusion criteria that were followed in the study. These are listed below:

[1] Patients having a history or currently suffering from primary inflammatory joint diseases or primary autoimmune diseases other than RA.

[2] Patients suffering from known HIV or hepatitis B/C infection.

[3] Individuals having latent TB infection unless they have completed adequate antibiotic prophylaxis.

[4] Reported cases of malignancy (other than basal cell carcinoma) within the last 10 years

[5] Patients with demyelinating disease.

[6] Known history of recent drug or alcohol abuse

[7] Patients with poor tolerability of vein puncture or patients with lack of adequate venous access for required blood sampling during the study period.

[8] Patients currently involved in other clinical trial(s) involving an investigational medicinal product.

[9] Other severe acute or chronic medical or psychiatric condition, or laboratory abnormality, which according to the investigator, may impart excess risk associated with study participation, or which would render the patient inappropriate for becoming a part of the study.

3.3.2 Sample collection strategy

The demographic profile and other ethnical and familial information were filled in a detailed questionnaire (**Appendix-A**). Based on the questionnaire, only unrelated subjects were considered eligible to participate in the study. Three- generation pedigree charts were prepared to

assure un-relatedness in all the samples. The patients were also asked to complete the Health Assessment Questionnaire (HAQ) (**Appendix B**) for their inclusion in the study.

5 ml of whole blood was then obtained from the volunteers with their informed consent by vein puncture method. 3 ml of the sample was allowed to stand for serum preparation while the remaining 2 ml was stored in EDTA vacutainer tubes for genotyping study at -20°C until use. The investigation was approved by the Human Ethics Committee of the Department of Zoology, University of North Bengal, India.

Two diagnostic tests were performed for the confirmation of the positive samples affected with rheumatoid arthritis. These were RF titre assay and anti-CCP test.

3.3.3 RF titre assay

The assay is based on the immunological agglutination principle with enhancement of the reaction by latex. In this immunoturbidimetric assay, latex bound heat inactivated IgG (antigen) reacts with the RF antibodies in the sample to form antigen antibody complexes which, following agglutination, are measured turbid metrically. The Assay was performed with COBAS INTEGRA Rheumatoid factors II assay kit (Cat. No. 20764574 322) using COBAS INTEGRA 400 plus analyzer (Roche, Germany). Manufacturers‘ instructions were followed for quantitative estimation of RA. The lower detection limit of the test was 10.00 IU/ml. The measuring range of the assay was 10-130 IU/ml. However, samples with higher values of RA were measured using post dilution rerun. The normal reference cut off value for RF titre in the serum was considered to be up to 20 IU/ml as per the laboratory standardizations and guidelines.

3.3.4 Anti-CCP estimation

Serum samples were subjected to anti-CCP assay using commercially available Elecsys anti-CCP assay on the Cobas e 411 Analyzer (Roche Diagnostics, Mannheim, Germany). **3gen Diagnostics Pvt. Ltd., Siliguri, India, is sincerely acknowledged for providing the access to the instrument and in sharing their expertise in executing the assay.** The Elecsys Anti-CCP immunoassay is a two-step IgG-capture test principle immunoassay with streptavidin-coated microparticles and electrochemiluminescence detection. The procedure was conducted according to the manufacturer‘s recommendations. Results were determined using a calibration curve that

is generated specifically on each instrument by a 2-point calibration and a master curve provided with the reagent bar code. Anti-CCP antibodies were measured in U/mL and the results were considered positive at a cut off value of ≥17 U/ml.

3.4.1 Disease samples of Typhoid patients

Patients were also selected those are affected with the *S. typhi*, because the population in this region is also susceptible to typhoid due to different environmental hazards. As TLRs are highly associated with the typhoid fever, discussed in the review of literature part, the study of the association of TLRs with the disease has been performed.

3.4.2 Collection of the samples

Samples were collected from the district hospitals and from the pathological laboratory by vein punctured method. All blood samples were collected in an EDTA container and stored in – 20°C.

3.4.3 Criteria for collection of samples

Samples were collected from the patients with their proper consent and all diagnostic criteria were checked for the disease conformations.

3.5 Collection of the samples from typhoid patients

3.5.1 Selection of Patients

Typhoid patients were selected on the basis of the specific symptoms that had been found during the typhoid fever in Siliguri region. Typhoid patients were screened by Widal test positive result carried out by serum agglutination test. The serum agglutination test was done against *S. typhi* ―O‖ and ―H‖ antigens using Salmonella antigen kit (Beacon diagnostic Pvt. Ltd, India). The serum antibody titre of 1: 80 or above was considered positive result for the typhoid fever caused by *S. typhi* according to the manufacturer instruction (**Appendix-C) (Table 15 in result section).**

3.5.2 Sample Collection

3 ml blood was collected by vein puncture from typhoid patients (n= 44) during December 2014 to June 2016 from Siliguri and adjacent areas. Healthy control subjects (n= 70) were taken from

the same hospitals. A detailed clinical report was taken from the patients who were admitted in the hospitals and primary health care of Siliguri and adjacent areas with gastro-intestinal problems (**Appendix-D)** (**Appendix-E)**.

Samples were collected in presence of the expert physicians and collected in the EDTA vial. The samples were then kept in -20°C. All the donors were informed regarding the purpose of the study before the collection of the blood samples and written consents were provided by the volunteers. The investigation was approved by the Human ethics committee of the Department of Zoology, University of North Bengal **(Zoo/4133/2011)** and was performed in accordance with the Declaration of Helsinki, 1975.

3.6 Disease Sample of HIV patients

3mL blood was collected by vein puncture from HIV patients (n= 55) during December 2015 to June 2016 from Siliguri and adjacent areas. Healthy control subjects (n= 70) were taken from the same hospitals. A detailed clinical report of CD4+ results were taken from the patients who were admitted in the hospitals of Siliguri and adjacent areas **(Table 18 in result section).**

Samples were collected in presence of the expert physicians and collected in the EDTA vial. The samples were then kept it in -20°C. All the donors were informed regarding the purpose of the study before the collection of the blood samples and written consents were provided by the volunteers. The investigation was approved by the Human ethics committee of the Department of Zoology, University of North Bengal (Zoo/4133/2011) and was performed in accordance with the Declaration of Helsinki, 1975.

3.6.1 Selection of patients

HIV patients were selected based on the specific symptoms that had been found during the disease progression in the HIV positive patients from Hospitals of Siliguri region. Positive HIV patients were selected on the basis of their viral infection and counting of CD4+ cells within the range of 156- 756 $\times 10^6$ cells/L.

Table 3: Demographic profile of the Disease samples

	Selected disease		
	Rheumatoid Arthritis	**Typhoid**	**Human Immunodeficiency Virus**
Sample size	**110**	**44**	**55**
Control size	**100**	**70**	**70**
Total sample size		**209**	

3.7 Laboratory Analysis

Laboratory analysis includes extraction of genomic DNA from venous blood samples for ten TLR genes.

3.7.1 Extraction of genomic DNA

3.7.1.1 DNA extraction

The extraction procedure was standardized both for fresh and frozen blood samples and slight modification has been made according to Guha *et. al.,* 2017 (**Appendix -F**).

1. 500µl of blood sample was transferred from the vacutainer to an eppendorf tube. In case of frozen blood, the sample was thawed at room temperature for 20-30 minutes before transferring the blood to the eppendorf tube.

2. Plasma was aspirated out carefully by centrifuging the sample at 6000 rpm for 7 minutes at 4°C.

3. 1 ml of RLB was added to the precipitate, mixed gently and was allowed to stand at room temperature for 1-2 minutes.

4. The mixture was then centrifuged at 6000 rpm for 6 minutes at room temperature.

5. The supernatant was discarded. This step may be repeated 1-2 times until a white coloured pellet is obtained.

6. 500 µl of pre warmed DNA extraction buffer was added to the pellet followed by 30ul of 10% SDS and 2ul of B-Mercaptoethanol respectively and mixed gently. The mixture was then incubated at 56-60 °C for 1 hour. .

7. Chloroform: isoamyl alcohol (24:1) was added to the mixture after incubation and shaken well. The mixture was then centrifuged at 12000rpm for 12 min at 4 °C.

8. The supernatant was pipetted out in another fresh sterilized centrifuge tube containing chilled ethanol. The tube was shaken for a while until fine white threads appeared in the solution. The sample tube may be kept at -20 °C for 20 minutes instead of shaking.

9. The sample was then centrifuged at 12000 rpm for 12 minutes at 4 °C.

The supernatant was discarded without disturbing the pellet and 500 µl of 90% alcohol was added to it.

10. The sample was then centrifuged at 12000 rpm for 12 minutes at 4

°C. Step 10 and 11 were repeated with 500 µl of 70% alcohol.

11. The supernatant was discarded and the pellet was allowed to dry at 37 °C.

12. The pellet was then dissolved overnight in 100 µl of TE buffer.

13. The DNA solution was then stored at -20 °C for future use.

3.7.1.2 Characterization of DNA

3.7.1.3 The integrity

The integrity of high molecular weight DNA is an important factor, which should be considered during extraction step. Integrity was checked by electrophoresis on 1% agarose prepared in 1x TBE buffer containing Ethidium bromide. The high molecular weight genomic DNA appeared as a single band near the well.

3.7.1.4 The concentration

DNA was quantified by measuring the optical density at 260/280 nm. 5ul of stock genomic DNA was taken and 995ul of water was added (Dilution factor D.F- 200), mixed well and O.D was taken at 260 nm in a spectrophotometer (name of this). DNA concentration of the sample was calculated as follows:

1 OD = 50UG/ML OF dsDNA

XOD= X x 50ug/ ml of ds DNA

The original DNA solution was diluted by a factor, DF= 200

The concentration of DNA in the original stock DNA

= X x 50 x DF ug/ml

= X x 50 x 200ug/ml

= 10,000 x X ug/ml

E. g. if OD of the diluted sample= 0.016

DNA concentration = 10,000 x 0.016 ug/ml

= 160 ug/ml

3.7.1.5 Purity of DNA

Purity of DNA was determined by taking the O.D. of the samples at 280nm for protein concentration and at 260 nm for DNA concentration. The ratio OD260/280 was calculated. DNA samples having the ratio of 1.5 or above were considered good. In case the ratio was less than 1.2 then the extraction process was repeated. DNA samples were checked and compared with the known standard size lambda DNA. Any RNA contamination was checked by measuring the OD value in spectrophotometer. Samples where large amount of RNA contamination was seen treated with RNAseA at 37°c for 2hrs followed by reextraction with phenol chloroform and isoamyl alcohol mixture.

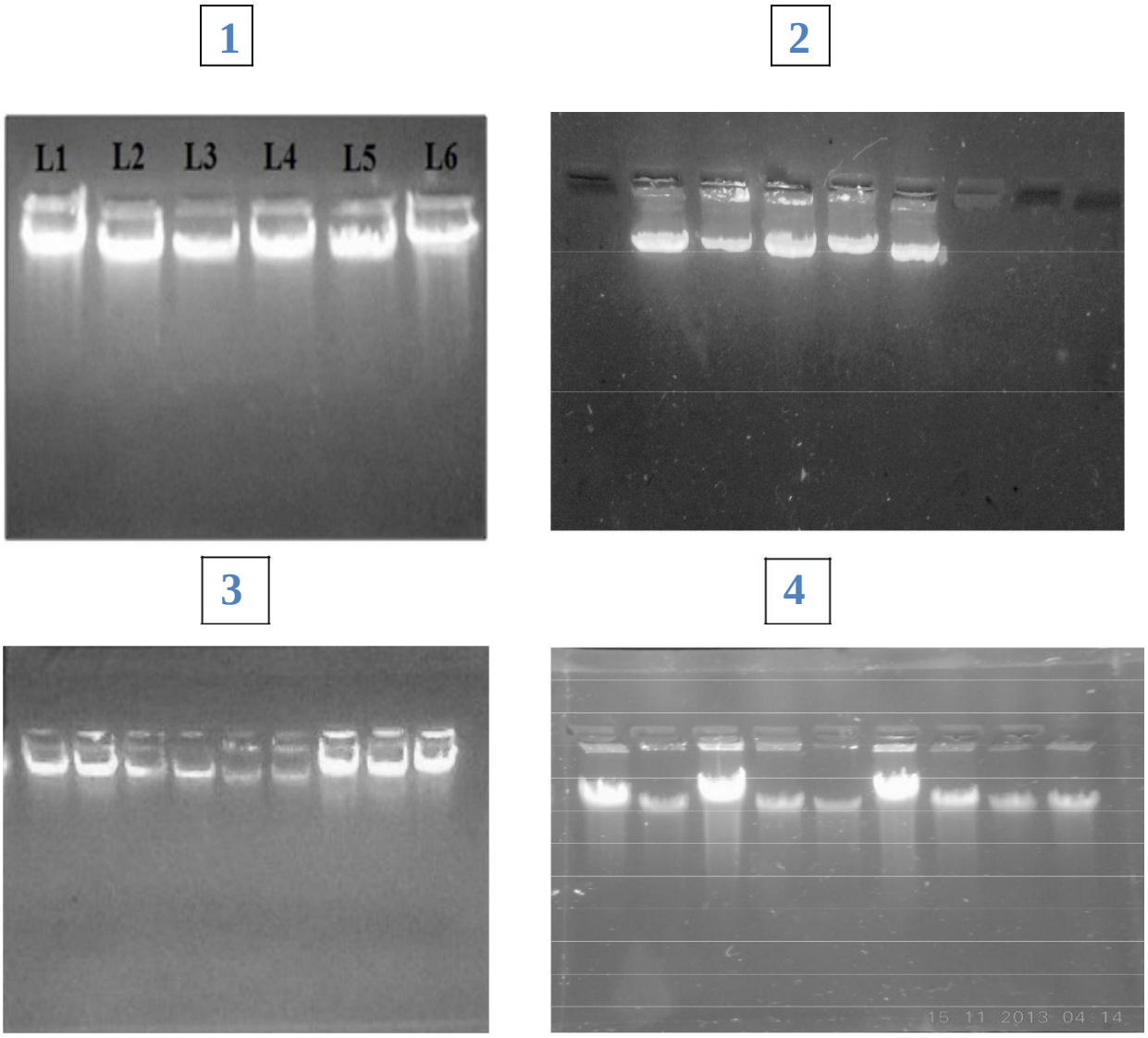

Fig 6: 1.2% agarose gel picture showing genomic DNA of different populations (1.Muslim, 2.Rabha, 3.Gurkha) and (4) HIV disease sample

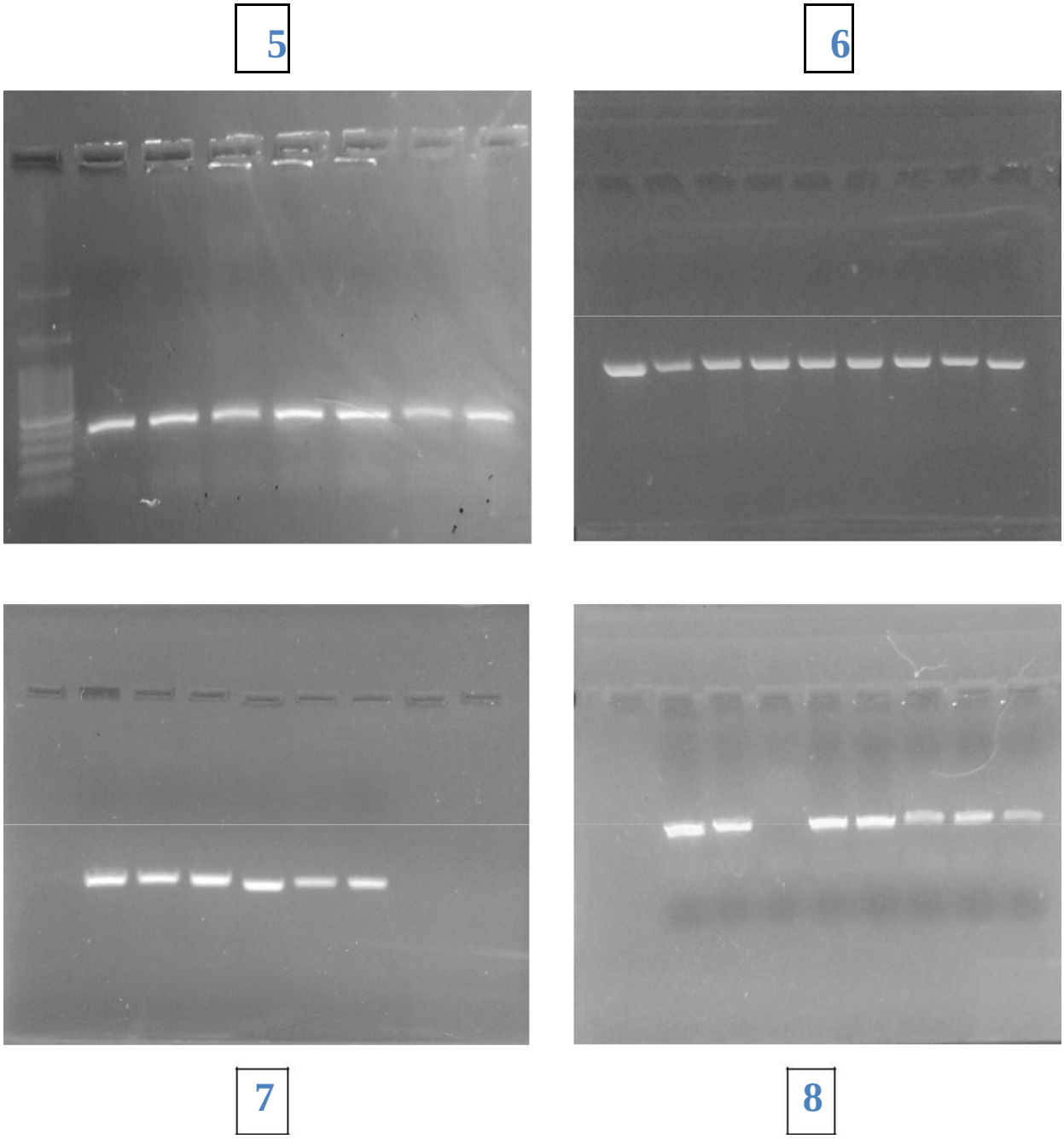

Fig 7: 1% agarose gel of TLR bands (after PCR) in case of (5) Rheumatoid arthritis, (6) Human Immunodeficiency virus, and (7) (8) Typhoid fever in different disease patients.

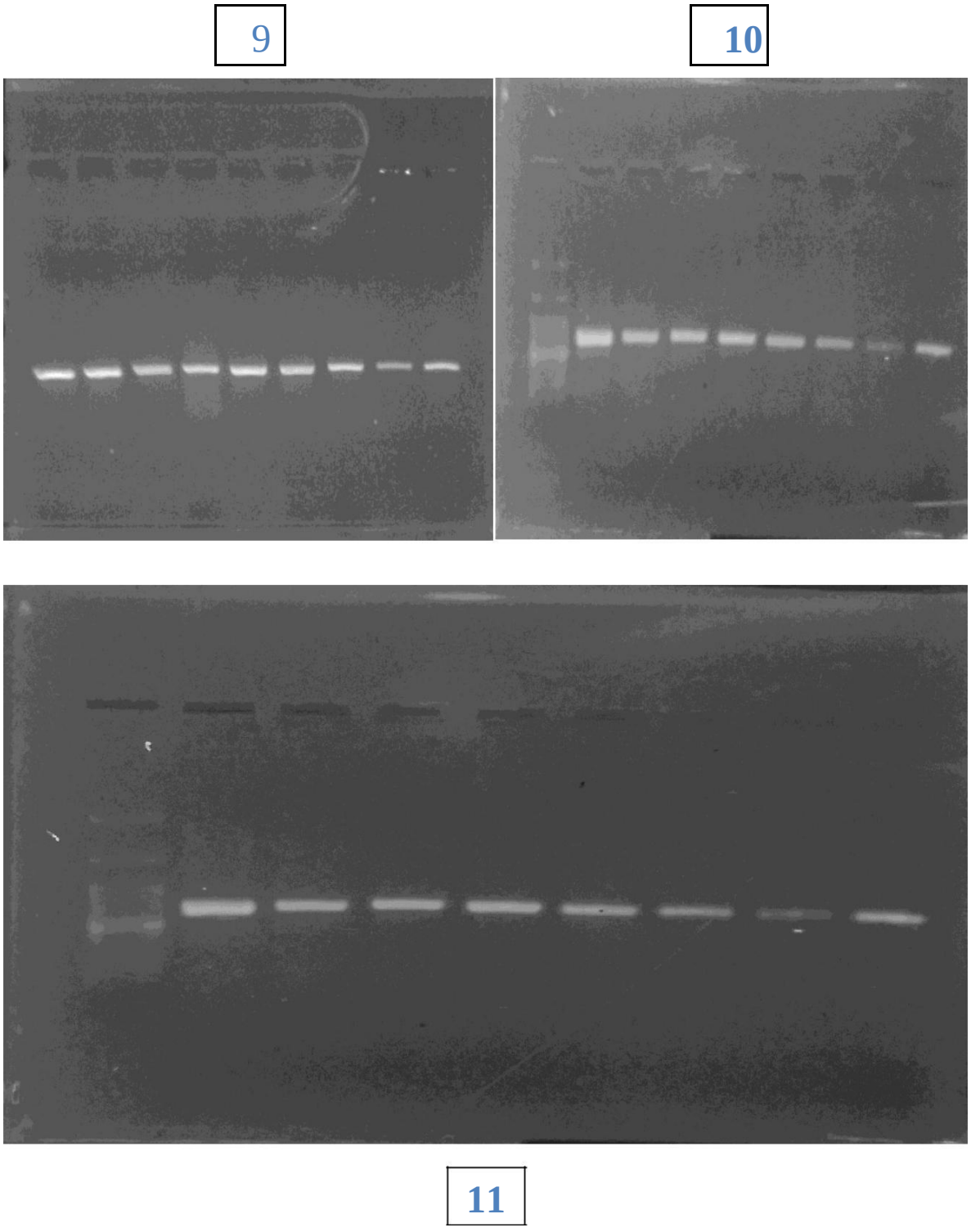

Fig 8: 1% Agarose gel picture of TLR4 genes (after PCR) in different populations of North Bengal region of India

3.7.1.6 Storage of DNA

Samples which were in regular use kept at 4°C. Rests of the samples were stored at the -20°C.

3.8 Gene specific analysis

Sequence specific primer based analysis was done for the present study. Conserved exon sequences from ten human TLR genes were chosen using NCBI data server. Initial PCR amplifications of TLR genes provided enough data for further analysis. PCR was carried out in thermostable PCR tubes with 25ul of PCR mixture that consisted of 5ul of 10X buffer (Appendix) 1.4 – 1.7ul of $MgCl_2$(25mM), 2ul of dNTPs (100mM), 1.3- 1.5ul of forward and reverse primers and 0.2-0.3ul of Taq DNA polymerase (5U/ul), and 1.5- 2.0ul DNA. PCR mixture was overlaid with 50ul of autoclaved light mineral oil to prevent evaporation during the high temperature cycles. The amplification was done using automated thermo cycler at successive incubation steps for denaturation, annealing and extension. PCR cycling conditions for all ten human TLR genes are shown in **Table 4.**

3.8.1 Primer designing

Primers flanking the region of interest were taken in each PCR. For the generic amplification, primers were chosen to amplify all known exon sequences at loci and were complementary to sequence shared by all the TLR gene sequences. The list of all the primers is given in **Table 4**. All the ten human TLR genes were designed using PRIMER BLAST software.

Table 4: Table 1: List of primers for the 10 TLR alleles in human.

Genes	Forward Primers (5‘-3‘)	Reverse Primers (3‘-5‘)	Product size(bp)	GC content(%)	Tm
TLR1	TCAACCAGGAATTGGAATAC	AGTTCCAGATTTGCTACAGT	382	40	52
TLR2	GGATGGTTGTGCTTTTAAGTACTG	AAGATCCCAACTAGACAAAGACTG	2671	41.67	55.5
TLR3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	792	44/40	57
TLR4	TTCTTCTAACTTCCTCTCCTGTG	TTAGCTGTTCGGCTCTACTATGG	1087	43/47	58.6
TLR5	CATTGTATGCACTGTCACTC	CCACCACCATGATGAGAGCA	446	45/55	57.6
TLR6	ACAACCCTTTAGGATAGCCACTG	AAACTCACAATAGGATGGCAGG	398	47.83	56.9
TLR7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG	545	44	50.5
TLR8	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG	637	45.83	58.4
TLR9	TCTAGGGGCTGAATGTGACC	ACAACCCGTCACTGTTGCTT	1106	55	59.8
TLR10	GTCGAAGACCCAATATACAG	ATTAAGCAATAGAACCGATG	954	45/35	52
Growth Hormone (Positive control)	CTTCCCAACCATTCCCTTA	CGGATTTCTGTTGTGTTTC	424	47/42	50.3

3.8.2 Amplification Check

Mini gel electrophoresis apparatus was used for separation of amplified PCR products. The amplified product was run on 1% agarose gel with a current strength of 30mAmps until the tracking dye travelled to 3cms from the origin. TBE buffer was used as tank buffer and DNA marker of 100bp size. The bands were visualized using UV- trans- illuminator (Bangalore Genei). The band size ranges between 430bp to 1200bp according to the primer used. The photographs were taken and permanent records were on experiment sheet.

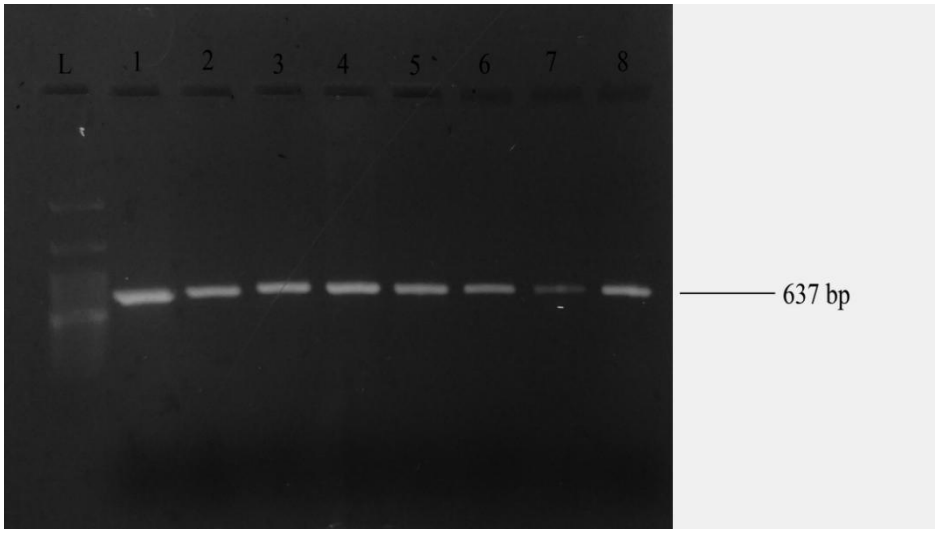

Figure 9: 1% agarose gel electrophoresis (after PCR) to show the presence of TLR8 gene in 8 Rajbanshi Individuals. The marker used in Lane 1 is Lambda DNA-Hind III double digested ΦX174 DNA.

3.9 Statistical analysis

3.9.1 Gene frequency

Gene frequency was calculated by direct counting method from the observed number of alleles at a locus divided by the number of gametes. The missing values were excluded from such estimation.

3.9.2 Genotypic frequency

Genotypic frequency (GF) was obtained from the observed number of a given genotype at each locus. Missing values were excluded from such estimation.

3.9.3 Chi- square and G^2 tests

In most of the past studies the goodness of fit tests such as Chi- square or G Square test were usually employed, when determining the difference between the observed genotypic frequencies and those expected under Hardy- Weinberg Equilibrium. Such tests were often found inaccurate when one or more genotypes had low expected counts. For these reasons, the use of G^2 or χ^2 is recommended only for preliminary analysis and that was followed in the present study.

Computer program POPGENE was used to compute the genotypic frequencies under random mating using the algorithm by Levene (1949).

3.9.4 Exact test

Haldane (1954) described the use of an exact test in 1954 and this forms the basis of our test. The procedure described in Guo and Thompson using a test analogous to Fischer‘s exact test on a 2x2 contingency table ; but extended to a triangular contingency table of arbitrary size was used. The test was done using a modified version of Markowitz Random walk algorithm described by Guo and Thompson (1992).

The test assumes that the gene frequencies are given. A contingency table is first built. The k x k entries of the table is the observed gene frequencies and k is the number of alleles.

This has been followed for the analysis of disease samples.

3.9.5 Gene diversity Analysis

Nei (1973) defined gene diversity for a single locus as the heterozygosity expected under HWE, disregarding the actual genotype frequencies in the population. With this definition, it was shown by Nei that

$H_T = H_S + D_{ST}$

Where D_{ST} is the inter population gene diversity,

H_T is the expected heterozygosity in the total population and is the average expected heterozygosity in the sub populations.

$H_T = H_S + D_{ST}$

$1 = H_S/ H_T + D_{ST} / H_T$

$D_{ST}/ H_T = 1- H_S/ H_T$

$G_{ST} = 1- H_S/ H_T$

$G_{ST} = 1- H_S/H_T$

G_{ST} is called the co- efficient of gene differentiation. H_S, H_T and G_{ST} were calculated from the average expected heterozygosity at the loci studied and their mean values over loci using the above formula. G_{ST} denotes the extant of gene diversity between populations with respect to the total population and H_S is the gene diversity attributable to that between individuals within subpopulations.

3.9.6 Genetic distances

With the help of computer program PHYLIP, (version) sub program GENDIST, Nei‘s (1972) genetic distance was computed from gene frequencies of the respective populations like Gurkha, Muslim, Rabha and Rajbanshi. The measure assumes that all the differences between populations arise from genetic drift. Nei‘s distance is formulated for an infinite iso-alleleles model of mutation, in which there is a rate of neutral mutation and each mutant is considered to be a completely new allele. It is assumed that all loci have the same rate of neutral mutation, and that the genetic variability in the population is at equilibrium between mutation and genetic drift, with the effective population size of each population remaining constant.

Nei‘s distance is given by

$$D = -\ln \frac{\sum_l \sum_u X_u Y_u}{\sqrt{\left(\sum_l \sum_u X_u^2\right)\left(\sum_l \sum_u Y_u^2\right)}}$$

Where m is summation over loci, i over alleles at the m th locus and where, P1mi is the frequency of the I th allele at the m th locus in population 1. Subject to the above assumptions, Nei‘s genetic distance is expected, for a sample of sufficient number of equivalent loci, to rise linearly with time. This measure has been observed to work reasonably well in case of closely related groups within species.

3.9.7 Sensitivity and Specificity test for disease

3.9.7.1 Sensitivity:

Probability that a test result will be positive when the disease is present (true positive rate).

= a / (a+b)

3.9.7.2 Specificity:

Probability is a test result shows negative when the disease is not present (true negative rate).

= d / (c+d)

3.9.7.3 Positive likelihood ratio:

Ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease:

= True positive rate / False positive rate = Sensitivity / (1-Specificity)

3.9.7.4 Negative likelihood ratio:

Ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease:

= False negative rate / True negative rate = (1-Sensitivity) / Specificity

3.9.7.5 Positive predictive value: probability that the disease is present when the test is positive.

= a / (a+c)

3.9.7.6 Negative predictive value: probability that the disease is not present when the test is negative.

= d / (b+d)

3.9.7.7 Test Score calculation:

	Disease	**Not Disease**
Positive	True Positive (A)	False Positive (B)
Negative	False Negative (C)	True Negative (D)

3.9.7.8 Calculating ODD ratio

The odds ratio (OR), its standard error and 95% confidence interval are calculated according to Altman, 1991.

The odds ratio is:

$$OR = \frac{a/b}{c/d}$$

$$= \frac{a \times d}{b \times c}$$

With the standard error of the log odds ratio being

$$SE [\ln (OR)] = \sqrt{1/a + 1/b + 1/c + 1/d}$$

and 95% confidence interval

95% CI= exp (ln (OR) – 1.96 x SE {ln (OR)}) to exp (ln (OR) + 1.96 x SE { ln(OR)})

Cases with positive (bad) outcome

Number in exposed group: **a**

Number in control group: **c**

Cases with negative (good) outcome

Number in exposed group: **b**

Number in control group: **d**

3.9.7.9 Calculating Relative Risk

The relative risk (RR), its standard error and 95% confidence interval are calculated according to Altman, 1991.

The relative risk or risk ratio

$$RR = \frac{a/(a+b)}{c/(c+d)}$$

Standard error of the log relative risk

$$SE \{\ln (RR)\} = \sqrt{1/a + 1/c - 1/a+c - 1/c+d}$$

95% confidence interval

95% CI= exp (ln (RR) – 1.96 x SE { ln (RR)}) to exp (ln (RR) + 1.96 x SE { ln(RR)})

3.9.7.10 Relative Risk calculation

Exposed group:

Number with bad (positive) outcome: a

Number with good (negative) outcome: b

Control group:

Number with bad (positive) outcome: c

Number with good (negative) outcome: d

CHAPTER 4

RESULTS

4. RESULTS

4.1 TLR gene frequencies among four different populations

The carrier or observed frequencies (OFs) and the estimated gene frequencies of the 10 TLR genes in our four studied populations have been shown in **Table 1**. All the 10 TLR loci have been detected in each and every population. The observed frequencies of all known TLR genes estimated in Gurkha, Muslim, Rabha and in Rajbanshi populations respectively are represented in **Table 5**. It has been observed that the frequency of TLR4 was very high among the Rabhas, while TLR5 was found to be the least frequent among the studied genes. It has also been observed that among the 10 TLR genes, TLR4 has the highest frequency among the Gurkha‘s (0.968) while TLR5 (0.971) was found to be the highest among the Muslims (**Fig. 14**). Apart from TLR2, TLR5 gene also showed low frequency in the Rabha population. Another interesting observation reported from the study was the low frequency of the TLR2 gene in all the studied populations. The gene frequencies of all the 10 TLR genes in the three populations were also calculated and presented in **Table 5**. It has been observed that in Rajbanshi population, the frequency of TLR8 (0.894) was highest followed by TLR6 (0.882) and TLR9 (0.882) respectively.

Table 5: Observed carrier frequencies (OFs) and the estimated gene frequencies of the 10 TLR genes in the four study populations.

	RAJBANSHI(RA)	GURKHA(G)	RABHA(R)	MUSLIM
TLR1	0.847	0.928	0.760	0.886
TLR2	0.176	0.608	0.600	0.571
TLR3	0.729	0.856	0.740	0.943
TLR4	0.788	0.968	0.900	0.557
TLR5	0.847	0.928	0.460	0.971
TLR6	0.882	0.896	0.640	0.907
TLR7	0.870	0.864	0.680	0.943
TLR8	0.894	0.824	0.700	0.829
TLR9	0.882	0.784	0.820	0.793
TLR10	0.376	0.832	0.540	0.793

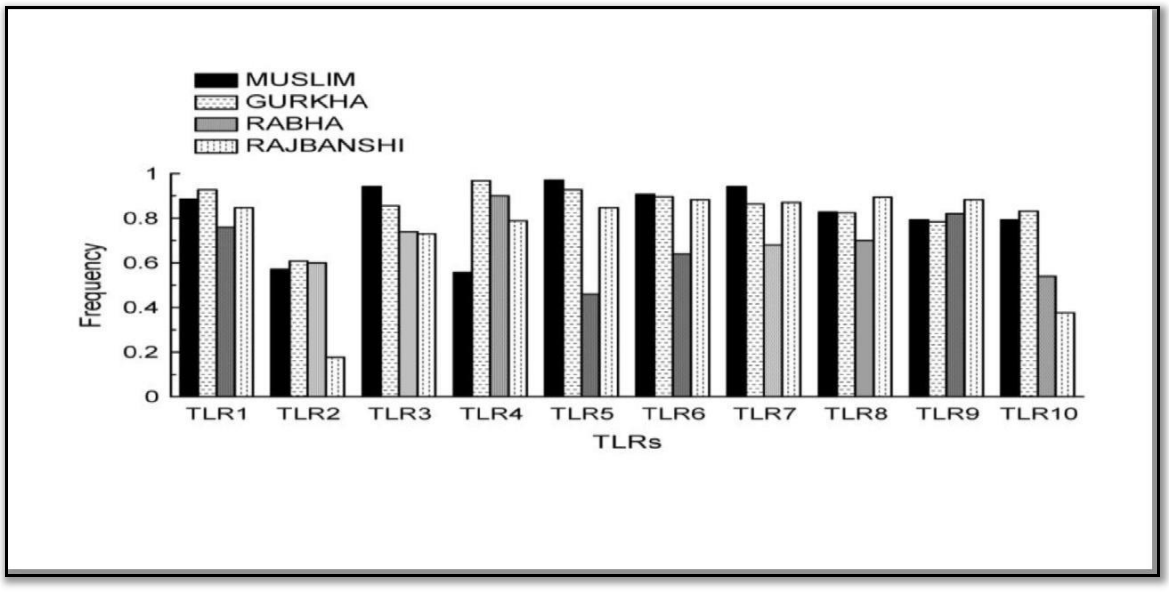

Fig.10- Frequency graph of ten TLR genes of the four populations in the North Bengal region (Kyplot ver-2.0).

4.2. Study of heterogeneity among the local population(s).

4.2.1 Chi-Square Test

Chi-square analyses (χ^2) have been performed to compare the differences in carrier frequencies (OF) of TLR genes among the four populations **(Table 6)**. Significant differences were observed in case of 9 out of 10 TLR loci among all the four populations except for TLR9. No significant difference was found for TLR9 in any of the comparisons among four populations. Mean unbiased genetic diversity of TLR genes in the three populations was calculated to be 0.240 ± 0.038 for Gurkhas, 0.258±0.049 for Muslims and 0.410±0.033 for Rabhas. It is observed that there are no significant differences between Rajbanshi and Gurkha when compared with the other two populations. There are no significant differences found for TLR1 and TLR9 when compared between the Rajbanshis and other three populations. When compared Rajbanshi with other three populations it was found that non significant cases were observed only for TLR1 and in TLR9 but significant results has been found in case of other TLRs.

Table 6. Chi square (χ^2) values of different populations and their comparison (Kyplot beta ver-2.0).

	RAXG	RAXM	RAXR	MXG	GXR	RXM
TLR1	2.723	0.401	1.056	0.9314	8.020**	3.687
TLR2	36.63***	32.22***	23.54***	0.229	0.005	0.034
TLR3	4.385*	18.52***	0.004	4.691*	2.549	13.427***
TLR4	15.569***	11.33***	2.047	57.426***	2.134	17.499***
TLR5	2.723	9.99**	20.80***	1.820	45.051***	66.880***
TLR6	0.007	0.135	9.822**	0.009	14.349 ***	17.349
TLR7	0.004	2.700	6.005*	3.927*	6.745**	20.745***
TLR8	1.458	1.330	6.841**	0.0041	2.591	2.986
TLR9	2.728	2.364	0.562	0.0006	0.1057	0.0424
TLR10	44.031***	37.81***	2.789	0.4299	14.6650***	10.612**

*** P< 0.05, ** P< 0.01, *** P< 0.001 N.S- Non significant**

4.2.2 Principal Component Analyses

Principal Component Analyses (PCA) was performed based on the OFs of the ten TLR genes to investigate the genetic relationship as well as the structure of our studied populations **(Fig. 15)**. Frequencies of ten TLR genes were considered in the analyses. The score plot shown in **figure 1** was computed based on first two components, wherein the first and the second component accounted for 56.17% and 31.94% variability respectively. It was evident from the positions occupied by different populations based on their TLR gene frequencies were in accordance with their geographical proximities.

From the Principal Component Analyses it is evident that the four populations occupied three different quadrants of the score plot, whereas the Muslims occupied the upper right quadrant, Gurkhas occupied the same upper right quadrant but lower than Muslim and the Rabhas occupied the lower right quadrant of the plot. Rajbanshi population occupied the upper left quadrant position in the plot. Gurkha and Muslim remain quiet closer to each other in the PCA plot.

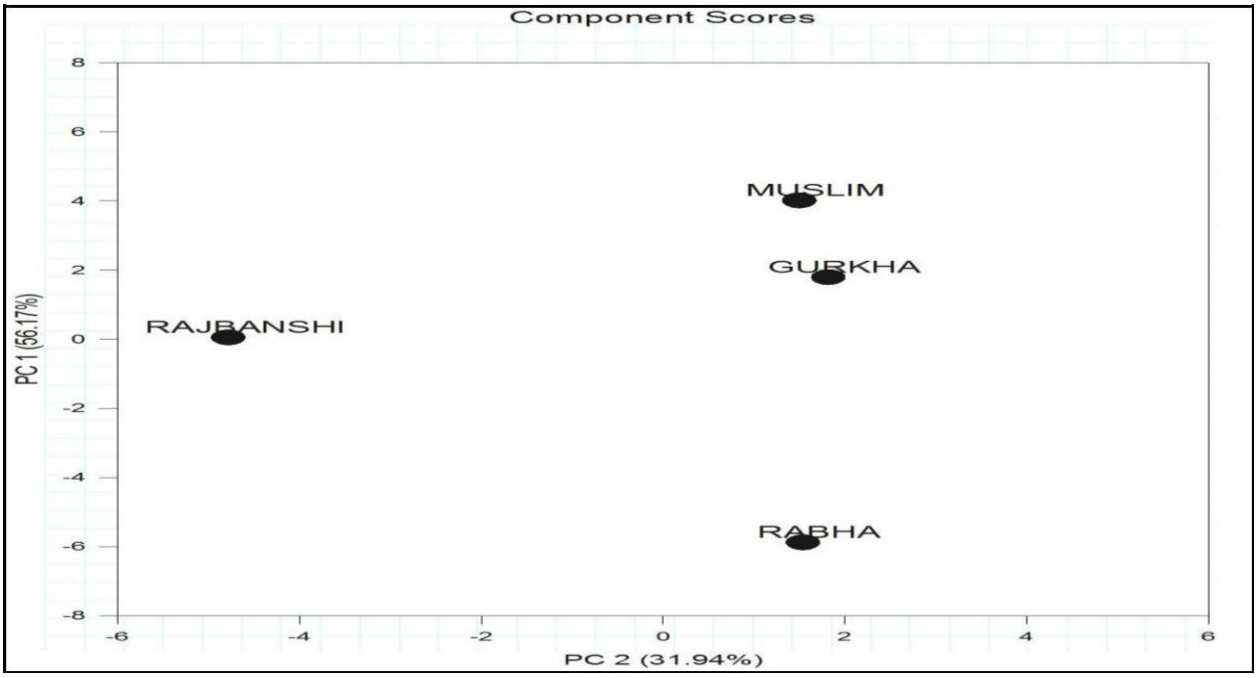

Fig. 11- Principle Component Analyses (PCA) based of the 10 TLR genes in the four ethnic populations of North Bengal (Minitab ver-6).

4.2.3 Genetic distance calculation

Genetic distances represented the measures of Nei genetic distances of the studied populations in the region of North Bengal areas. Nei's genetic distance was also calculated between the populations (**Table 7**) and it is observed that the Gurkha-Muslim genetic distance (0.052) was considerably lesser than the Gurkha-Rabha genetic distance (0.0973). The genetic distance is found to be the highest among the Muslims and the Rabhas (0.1557). The genetic distance between Rajbanshi- Gurkha is 0.0685, between Rajbanshi- Muslim is 0.0694, and between Rajbanshi- Rabha is 0.0745. When considered the Rajbanshi population, least genetic distance is observed among Rajbanshi and Gurkha and greater among Rajbanshi and Rabha (**Fig. 16**).

Table 7: Nei's Genetic distances of four populations using POPGENE (Ver- 1.32) software.

POP ID	RAJBANSHI	RABHA	MUSLIM	GURKHA
RAJBANSHI	**0.000**			
RABHA	0.0745	**0.000**		
MUSLIM	0.0694	0.1557	**0.000**	
GURKHA	0.0685	0.0973	0.0526	**0.000**

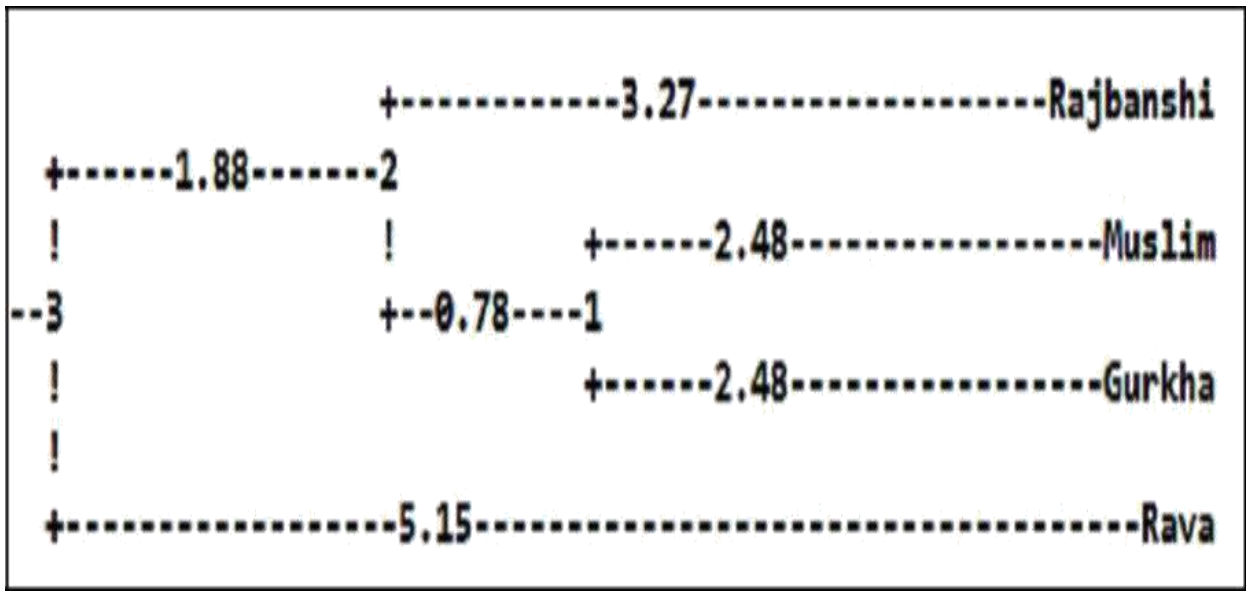

Fig. 12- Neighbour joining tree showing relationship among Rajbanshi and three other populations (POPGENE ver- 1.32 and Phylip ver-3.5)

Frequency, Distribution and Association study of TLR genes among Rheumatoid arthritis, Typhoid fever and Human Immunodeficiency Virus patients

4.3 Association of TLR genes among Rheumatoid arthritis patients

4.3.1 Diagnostic and Demographic profile

Demographic profile of patients and control group has been compared. Out of 110 positive samples of rheumatoid patients, 28 were men and 82 female patients. The healthy control samples were also collected from the Siliguri area with proper consent from the individuals. The average age of the patients group was 47 years whereas in case of control group it was 44 years.

Diagnostic data were also collected for the conformation of the positive samples for the rheumatoid arthritis. The selected diagnostic tests were anti-CCP and RF titre . The median range of anti-CCP in the patients is 182.7 (16.5 to 504.93) but in case of control group the range is very low 10.8(8.2 to 13.1). In case of RF titre the median range is 142.3 (41.20 to 198.0) for RA patients. But low value has been detected for the control group 16.8 (12 to 20.5). These two diagnostic tests were selected as major criteria for the positive samples in case of RA (**Table 8**).

Table 8: Demographic data and clinical characteristics of RA patients and control groups

			RA group (n=110)	Control group (n=100)
Gender n (%)	Male		28 (25.5)	57 (57%)
	Female		82 (74.5)	43(43%)
Age (Years)	Median (Range)		47 (39-54.5)	44(38-50)
Disease duration (Years)	Mean (SD)		5.6± 2.4	-----
Anti CCP (IU/ml)	{Median (IQR)}		182.7 (16.5 to 504.93)	10.8(8.2 to 13.1)
	Positive cases	N (%)	84 (76.4 %)	5 (3.3%)
	Negative Cases	N (%)	26 (23.6%)	95 (96.7%)
RF titre (IU/ml)	{Median (IQR)}		142.3 (41.20 to 198.0)	16.8 (12 to 20.5)
	Positive cases	N (%)	86 (78.2)	42 (27.8%)
	Negative Cases	N (%)	24 (21.8)	109 (72.2%)

(Guha *et. al.*, 2018)

4.3.2 Gene frequency and Chi-square analysis

Observed frequency data of ten TLR genes from 110 rheumatoid arthritis patients were analyzed. It has been found that in rheumatoid patients TLR1 (0.95) and TLR8 (0.95) are highly up regulated **(Fig. 17)**. It has also been found that TLR3, TLR6 and TLR9 are also very high with the frequency of 0.90, 0.92 and 0.82 (**Table 9**). Chi- square analyses for significant data were observed for TLR6 and TLR8 and TLR9 (for 95% significant). In case of other TLRs like TLR2, TLR4, TLR5, and TLR10 have been found some significant results for the association with the disease. On the other hand TLR1 and TLR7 have showed the non-significant result.

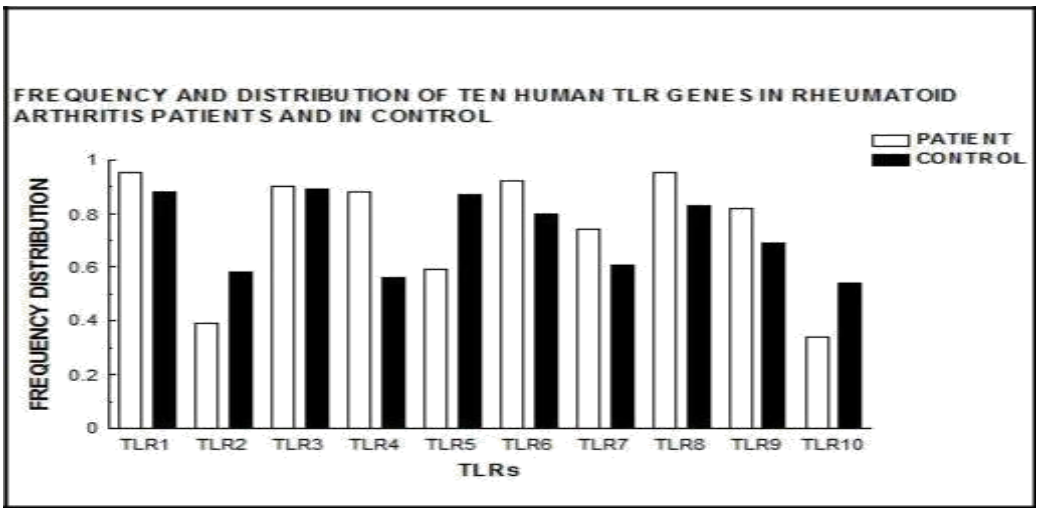

Fig 13. Frequency graph of ten TLR genes of rheumatoid arthritis patients (Kyplot ver-2.0).

Table 9: Gene frequencies of the 10 TLR genes among Rheumatoid arthritis patients and with the control

Gene	Patient	Control
TLR1	0.766	0.653
TLR2	0.219	0.351
TLR3	0.683	0.668
TLR4	0.656	0.336
TLR5	0.360	0.639
TLR6	0.713	0.552
TLR7	0.486	0.375
TLR8	0.766	0.587
TLR9	0.573	0.443
TLR10	0.185	0.321

Table 10: Observed frequencies of the 10 TLR genes in the Rheumatoid arthritis patients and control.

	PATIENTS	CONTROLS	χ^2	Relative risk	p value
TLR1	**0.95**	**0.88**	2.089N.S	1.07	0.09
TLR2	**0.39**	**0.58**	**6.764****	0.67	0.007
TLR3	**0.90**	**0.89**	0.0001	1.01	0.81
TLR4	**0.88**	**0.56**	**25.829*****	**1.57**	<0.0001
TLR5	**0.59**	**0.87**	**19.037*****	0.67	<0.0001
TLR6	**0.92**	**0.80**	**5.193***	1.14	0.01
TLR7	**0.74**	**0.61**	3.264N.S	**1.20**	0.05
TLR8	**0.95**	**0.83**	**6.024***	**1.13**	0.01
TLR9	**0.82**	**0.69**	**4.009***	**1.18**	0.03
TLR10	**0.34**	**0.54**	**8.035****	0.62	0.003

*** P< 0.05, ** P< 0.01, *** P< 0.001 N.S- Non significant**

4.3.3 Relative risk calculation

Relative risk utilizes the probability of an event occurring in one group compared to the other group. The relative risks for different TLRs were calculated and represented in Table 10. The relative risks for TLR4 (RR- 1.57, p- <0.0001), TLR7 (RR- 1.20, p- 0.05) TLR8 (RR- 1.13, p- 0.01), TLR9 (RR- 1.18, p- 0.03) are very high whereas door line association is found in case of TLR1 (1.07, p- 0.09) and TLR3 (1.01, p- 0.81). Low association with the disease is observed in case of TLR2 (RR- 0.67), TLR5 (RR- 0.67) and TLR10 (RR-0.62). Higher value of relative risk (<1) signify the positive association with the disease whereas lower value define the weak association with the disease.

ANOVA test for comparing among the control and patient groups and the p- value is 0.05, two tailed t-test value was assumed to be 0.80.

4.3.4 Odd ratio/ Risk ratio calculation

The odd ratio and 95% confidence interval for ten different TLRs in rheumatoid patients showed that in case of TLR1 (odd- 2.36, CI-1.21-6.49,p- 0.09), TLR4 (odd-5.86, CI- 1.06-3.84,p- <0.0001), TLR6 (odd- 2.80, CI- 0.85-6.55,p- 0.01), TLR8 (odd- 3.55, CI- 0.45-2.69,p- 0.01) and TLR9 (odd- 2.02, CI- 2.90-11.81,p- 0.03) show high associations whereas TLR2 (odd- 0.46, CI- 0.99-3.20,p- 0.006), TLR5 (odd- 0.21, CI- 0.24-0.75,p- <0.0001) and TLR10 (odd- 0.43, CI- 0.10-0.43,p- 0.003) show lower association among the patients and control samples (**Table 11**).

Table 11: Risk ratio and odd ratio for ten different TLRs in association with Rheumatoid arthritis

	Risk ratio	Odd ratio	Confidence intervals (95%)	p value
TLR1	1.07	**2.36**	1.21-6.49	0.09
TLR2	0.67	0.46	0.99-3.20	0.006
TLR3	1.01	1.11	1.33-9.40	0.81
TLR4	**1.57**	**5.86**	1.06-3.84	<0.0001
TLR5	0.67	**0.21**	0.24-0.75	<0.0001
TLR6	1.14	**2.80**	0.85-6.55	0.01
TLR7	**1.20**	1.78	0.26-0.80	0.05
TLR8	**1.13**	**3.55**	0.45-2.69	0.01
TLR9	**1.18**	**2.02**	2.90-11.81	0.03
TLR10	0.62	0.43	0.10-0.43	0.003

4.3.5 Sensitivity and Specificity

The prevalence of the disease in the patients was estimated by the diagnostic test based on Bayer‘s theorem. Sensitivity is the probability that a test will indicate disease among the individuals. Specificity is the fraction of those without disease. The sensitivity is found very high in case of TLR1 (94.55), TLR3 (90.00), TLR6 (91.82) and TLR8 (94.55) (**Table 12**). A low sensitivity is observed in case of TLR2 (39.09), TLR5 (59.09), and TLR10 (33.64) which signified the low prevalence of the disease among the patients. The positive predicted value is the probability that a subject is diseased, given a positive test result. Negative predicted value is the probability that a subject is healthy, given a negative test result. PPV was found highest for TLR4 (63.40), TLR7 (57.04) and TLR9 (56.60).

Table 12: Diagnostic test values for Rheumatoid arthritis patients based on bayer’s theorm.

	SENSITIVITY	SPECIFICITY	PPV	NPV
TLR1	**94.55**	12.00	54.17	66.67
TLR2	39.09	42.00	42.57	38.53
TLR3	**90.00**	11.00	52.66	50.00
TLR4	88.18	44.00	**63.40**	77.19
TLR5	59.09	13.00	42.76	22.41
TLR6	**91.82**	20.00	55.80	68.97
TLR7	73.64	39.00	**57.04**	57.35
TLR8	**94.55**	17.00	55.61	73.91
TLR9	81.82	31.00	**56.60**	60.78
TLR10	33.64	46.00	40.66	38.06

PPV- Positive predicted value, NPV- Negative predicted value

4.4 Association of TLR genes among typhoid fever patients

4.4.1 Observed frequency and Chi-square analysis

Observed frequency data of ten TLR genes from 44 typhoid patients were analyzed. It has been found that in typhoid patients TLR1 (0.977) and TLR6 (0.977) are highly up regulated **(Fig. 18)**. TLR4 and TLR5 are also very high with the frequency of 0.909 and 0.931 (**Table 13**). Chi-square analyses for significant data have been observed for TLR8 and TLR10.

Table 13: Observed frequencies of the 10 TLR genes in the control and patients of typhoid fever.

	PATIENTS	CONTROLS	χ^2	Relative risk	p value
TLR1	0.977	0.885	1.982	1.10	0.04
TLR2	0.295	0.171	1.757	1.72	0.12
TLR3	0.886	0.728	3.153	1.21	0.03
TLR4	0.909	0.8	1.667	1.13	0.09
TLR5	0.931	0.871	0.503	1.06	0.27
TLR6	0.977	0.9	1.429	1.08	0.07
TLR7	0.863	0.914	0.296	0.94	0.41
TLR8	0.772	0.928	4.459*	0.83	0.03
TLR9	0.840	0.9	0.413	0.93	0.37
TLR10	0.681	0.342	11.128***	1.98	0.0004

*** P< 0.05, ** P< 0.01, *** P< 0.001 N.S- Non significant**

4.4.2 Relative risk calculation

The relative risks for different TLRs were calculated **(Table 13)**. The relative risks for TLR7 (RR- 0.94, p- 0.41), TLR8 (RR- 0.83, p- 0.03) and TLR9 (RR- 0.93, p- 0.37) are very low whereas door line association is observed in case of TLR1 (1.10, p- **0.04**), TLR5 (1.06, p- 0.27) and TLR6 (1.08, p- 0.07). On the other hand slightly higher association is observed in case of TLR2 (1.72, p- 0.12), TLR3 (1.21, p- 0.03), TLR4 (1.13, p- 0.09) and TLR10 (1.98, p- 0.0004).

4.4.3 Odd ratio/ Risk ratio calculation

Fischer's exact test for probability showed significant association for TLR8 (p= 0.022, <0.05) and TLR10 (p= 0.0005, >0.001). When calculated the odd ratio and 95% confidence interval for ten different TLRs, in typhoid patients it has been found that in case of TLR2 (odd- 2.02, CI- 0.82-4.97,p- 0.12), TLR4 (odd-2.5, CI- 0.76-8.16,p- 0.12), and TLR5 (odd- 2.01, CI- 0.51-7.89,p- 0.31) shows high associations, whereas TLR7 (odd- 0.59, CI- 0.17-1.97,p- 0.39), TLR8 (odd- 0.26, CI- 0.08-0.82,p- **0.02**) and TLR9 (odd- 0.58, CI- 0.19-1.80,p- 0.35) shows lower association among the patients and control samples (**Table 14**).

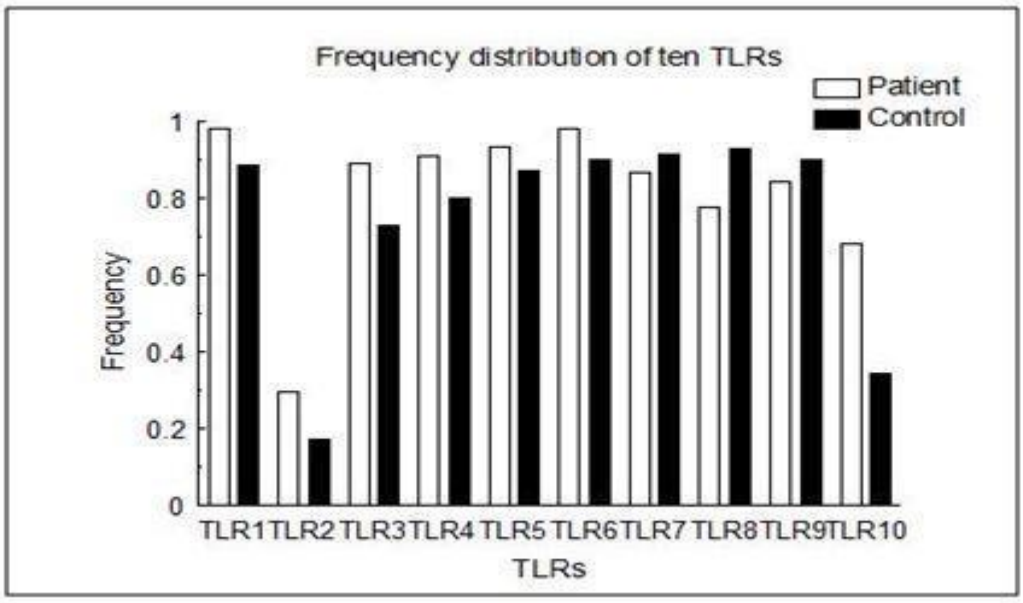

Fig 14. Frequency graph of ten TLR genes of typhoid patients (Kyplot ver-2.0)

The "O" antigen test value for Widal test was considered positive where the values above 1: 80 were marked as a positive sample (**Table 15**). Chi-square analyses ($\chi 2$) were performed to compare the differences in carrier frequencies (OF) of TLR genes among the patients and control samples. Significant differences are observed in case of TLR8 and TLR10. No significant differences are found among the other eight TLRs **(Table 13)**. ANOVA test for comparing

among the control and patient groups and the p- value is 0007 (> 0.001), two tailed t-test value was assumed to be 0.521.

Table 14: Risk ratio and odd ratio for ten different TLRs in association with typhoid fever.

	Risk ratio	Odd ratio	Confidence intervals
TLR1	1.10	**5.54**	0.66-45
TLR2	1.72	2.02	0.82-4.97
TLR3	1.21	2.90	0.99-8.46
TLR4	1.13	2.5	0.76-8.16
TLR5	1.06	2.01	0.51-7.89
TLR6	1.08	**4.77**	0.56-40
TLR7	0.94	0.59	0.17-1.97
TLR8	0.83	0.26	0.08-0.82
TLR9	0.93	0.58	0.19-1.80
TLR10	1.98	**4.10**	1.83-9.17

4.4.4 Sensitivity and Specificity

The prevalence of the disease in the patients was estimated by the diagnostic test based on Bayer‘s theorem. The sensitivity was found very high in case of TLR1 (97.73), TLR4 (90.91), TLR5 (93.18) and TLR6 (97.73) (**Table 16**). A low sensitivity is estimated in case of TLR2, TLR8, and TLR10 which signified the low prevalence of the disease in the patients.

Table 16: Diagnostic test values for typhoid patients based on baye's theorm.

	SENSITIVITY	SPECIFICITY	PPV	NPV
TLR1	97.73	11.43	40.95	88.89
TLR2	29.55	82.86	52.00	65.17
TLR3	88.64	27.14	43.33	79.17
TLR4	90.91	20.00	41.67	77.78
TLR5	93.18	12.86	40.20	75.00
TLR6	97.73	10.00	40.57	87.50
TLR7	86.36	8.57	37.20	50.00
TLR8	77.27	7.14	34.34	33.33
TLR9	84.09	10.00	37.00	50.00
TLR10	68.18	65.71	55.56	76.67

PPV- Positive predicted value, NPV- Negative predicted value

Table 15: Widal test result of the positive typhoid samples

SAMPLE NO.	Age	Sex	Widal test result
1	17	F	—O‖- 1:160 —H‖- 1:20
2	32	M	—O‖- 1:160 —H‖- 1:40
3	19	F	—O‖- 1:160 —H‖- 1:20 —AH‖- 1:20
4	21	F	—O‖- 1:160 —H‖- 1:40

5	32	M	—O‖- 1:160 —H‖- 1:40
6	23	M	—O‖- 1:320 —H‖- 1:40
7	45	F	—O‖- 1:40 —H‖- 1:80 —AH‖- 1:20 —BH‖- 1:20
8	24	M	—O‖- 1:80 —H‖- 1:40 —AH‖- 1:20
9	27	M	—O‖- 1:160 —H‖- 1:80
10	03	F	—O‖- 1:160 —H‖- 1:160
11	26	F	—O‖- 1:160 —H‖- 1:80
12	38	M	—O‖- 1:160 —H‖- 1:80
13	10	M	—O‖- 1:80 —H‖- 1:20
14	19	F	—O‖- 1:160 —H‖- 1:80
15	18	M	—O‖- 1:320 —H‖- 1:160
16	55	M	—O‖- 1:160 —H‖- 1:80
17	22	F	—O‖- 1:320 —H‖- 1:80

18	23	F	—O‖- 1:160 —H‖- 1:80
19	25	M	—O‖- 1:160 —H‖- 1:80
20	74	F	—O‖- 1:80
21	20	F	—O‖- 1:80
22	28	M	—O‖- 1:160 —H‖- 1:40
23	27	M	—O‖- 1:80
24	70	F	—O‖- 1:80
25	30	F	—O‖- 1:20 —H‖- 1:40 —AH‖- 1:160
26	42	F	—O‖- 1:20 —H‖- 1:40 —AH‖- 1:160
27	22	M	—O‖ – 1:80 —H‖- 1:20
28	70	M	—O‖- 1:40 —H‖- 1:20 —AH‖- 1:80
29	23	F	—O‖- 1:80 —H‖- 1:20
30	4	F	—O‖- 1:80
31	40	F	—O‖- 1:160 —H‖- 1:80
32	12	M	—O‖- 1:80
33	17	F	—O‖- 1:160 —H‖- 1:80

34	19	M	—O‖- 1:160 —H‖- 1:40
35	21	F	—O‖- 1:160 —H‖- 1:80
36	55	M	—O‖- 1:160 —H‖- 1:320
37	45	F	—O‖-1:160 —H‖-1:80
38	25	M	—O‖-1:80
39	76	M	—O‖-1:80 —H‖-1:240
40	60	F	—O‖-1:160
41	35	F	—AH‖- 1:160
42	22	F	—O‖- 1:160
43	42	F	—O‖- 1:80 —H‖- 1:160
44	09	F	—O‖- 1:320 —H‖- 1:160

4.5 Association of TLR genes among HIV+ patients

4.5.1 Observed frequency and Chi-square analysis

Observed frequency data of ten TLR genes from 55 typhoid patients were analyzed. It has been observed that in HIV+ patients, TLR8 (0.809) and TLR9 (0.865) are highly up regulated **(Fig. 19)**. TLR1 (0.766) and TLR6 (0.766) are also very high among HIV patients (**Table 17**). Chi-square analyses for significant data are observed for TLR2, TLR4, TLR8 and TLR9. Different samples were confirmed by the CD4+ marker test results (**Table 18**).

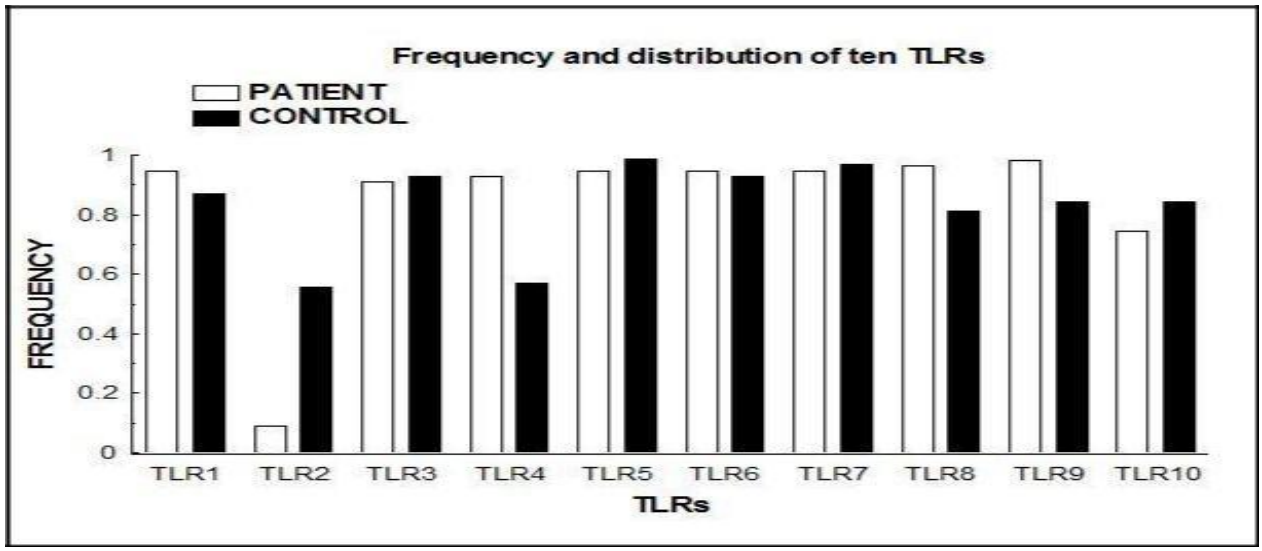

Fig 15 Frequency graph of ten TLR genes was of HIV positive patients.

4.5.2 Relative risk calculation

The relative risks for different TLRs were calculated among HIV+ patients **(Table 17)**. The relative risks for TLR4 (RR- **1.62**, p- 0.41), TLR8 (RR- **1.18**, p- **0.007**) and TLR9 (RR- **1.16**, p- **0.005**) are very high, whereas door line association is found in case of TLR1 (1.08, p- 0.14) and TLR6 (1.01, p- 0.69). On the other hand low association is observed in case of TLR2 (0.16, p- < 0.0001) and TLR10 (0.88, p- 0.19).

ANOVA was performed for patient and control group and the p-value is observed 0.04 (< 0.05). Two-tailed t-test for p-value is assumed to be 0.93 for patient and control group.

Table 17: Gene frequencies of the 10 Human TLR genes in the control and patients and measurement of relative risk.

	Patients	Controls	χ^2	Relative risk	P value
TLR1	0.766	0.641	1.185	1.08	0.14
TLR2	0.046	0.334	27.343***	0.16	< 0.0001
TLR3	0.698	0.732	0.004	0.97	0.69
TLR4	0.730	0.345	17.939***	**1.62**	< 0.0001
TLR5	0.766	0.880	0.573	0.95	0.23
TLR6	0.766	0.732	0.0002	1.01	0.69
TLR7	0.766	0.830	0.076	0.97	0.47
TLR8	0.809	0.569	5.168*	**1.18**	**0.007**
TLR9	0.865	0.603	5.345*	**1.16**	**0.005**
TLR10	0.495	0.603	1.268	0.88	0.19

*** P< 0.05, ** P< 0.01, *** P< 0.001**

4.5.3 Odd ratio/ Risk ratio calculation

Fischer‘s exact test for probability showed significant association for TLR4 (p= 0.00001, >0.001), TLR8 (p= 0.01, >0.05) and TLR9 (p= 0.01, >0.05). The odd ratio and 95% confidence interval for ten different TLRs in HIV+ patients documented that in case of TLR4 the odd is 9.56 and CI is 3.11-29.37 for TLR8 odd is 6.04 and CI is 1.30-28.05 for TLR9 odd ratio is 10.06 and CI- 1 is 25-80.60 indicate high associations, whereas TLR2 (odd- 0.07, CI- 0.02- 0.22), TLR5 (odd- 0.25, CI- 0.02-2.48) and TLR7 (odd- 0.50, CI- 0.08-3.16) shows lower association among the patients and control samples. The risk ratio for TLR4 is 1.6227, TLR8 is 1.1834, and for TLR9 value is 1.1649 shows slightly higher association for the disease (**Table 19**).

Table 19: Risk ratio and odd ratio for ten different TLRs in association with HIV patient.

	Odd Ratio	Confidence interval	Risk ratio	P value
TLR1	2.55	0.65 - 9.94	1.0849	0.22
TLR2	0.07	0.02- 0.22	0.1632	5.22
TLR3	0.76	0.21- 2.80	0.979	0.74
TLR4	**9.56**	3.11-29.37	**1.6227**	0.00001
TLR5	0.25	0.02-2.48	0.9592	0.31
TLR6	1.33	0.30-5.84	1.0182	0.73
TLR7	0.50	0.08-3.16	0.9733	0.65
TLR8	**6.04**	1.30-28.05	**1.1834**	0.01
TLR9	**10.06**	1.25-80.60	**1.1649**	0.01
TLR10	0.54	0.22-1.32	0.8844	0.25

4.5.4 Sensitivity and Specificity

The prevalence of the disease in the patients was estimated by the diagnostic test based on Bayer‘s theorem. The sensitivity is found very high in case of TLR8 (96.36) and TLR9 (98.18) (**Table 19**). A low sensitivity is observed in case of TLR2 (9.09) and TLR10 (74.55) which signify the low prevalence of the disease among the patients.

Table 20: Diagnostic test values for HIV patients based on baye's theorm.

	SENSITIVITY	SPECIFICITY	PPV	NPV
TLR1	94.55	12.86	46.02	75.00
TLR2	**9.09**	44.29	11.36	38.27
TLR3	90.91	7.14	43.48	50.00
TLR4	92.73	42.86	56.04	88.24
TLR5	94.55	1.43	42.98	25.00
TLR6	94.55	7.14	44.44	62.50
TLR7	94.55	2.86	43.33	40.00
TLR8	**96.36**	18.57	48.18	86.67
TLR9	**98.18**	15.71	47.79	91.67
TLR10	74.55	15.71	41.00	44.00

PPV- Positive predicted value, NPV- Negative predicted value

Table 18: CD4+marker test result of HIV patients

SAMPLE NO.	CD4+ RESULT
1	190
2	330
3	232
4	310
5	**156**
6	410
7	340
8	202
9	**756**
10	285
11	612
12	292
13	432
14	190

15	300
16	390
17	465
18	580
19	212
20	200
21	191
22	187
23	580
24	211
25	602
26	209
27	222
28	245
29	280
30	310
31	300
32	340
33	187
34	192
35	190
36	198
37	214
38	210
39	210
40	222
41	210
42	250
43	261
44	238

45	274
46	303
47	300
48	390
49	401
50	300
51	357
52	339
53	192
54	207
55	219

CHAPTER 5

DISCUSSION

5. Discussion

This section has been divided and elaborated into four parts namely: frequency and distribution study of TLR genes in four different populations in North Bengal region of India, frequency, distribution and association study of Rheumatoid arthritis among the patients of Siliguri and adjoining areas, frequency, distribution and association study of Typhoid fever among the patients of Siliguri and adjoining areas, and frequency, distribution and association study of HIV among the patients of Siliguri and adjoining areas.

5.1 Frequency and distribution study of TLR genes in four different populations in North Bengal region of India

This section has been divided again into four categories, each corresponding to a particular population group.

5.1.1 Rajbanshi

Modern Indian populations have originated from two ancestral populations ―Ancestral North Indians‖ who are genetically close to Middle Eastern, central Asians and Europeans while and the other ―Ancestral South Indians‖ who have shown proximity to East- Asians lineage (**Reich, 2009**). Rajbanshi populations are the indigenous ethnic caste population of Eastern Terai and can also be found in Assam, Bengal and Bihar states of India (**Gupta, 2012**). Even today, most of the Rajbanshis are found to have inhabited in Assam, Meghalaya, Tripura, Nagaland, and Manipur also (**Sreshtha, 2009)**.

Rajbanshi people having Indo-European linguistic background (**Shrestha, 2009**) are abundantly present in Terai and Dooars region (outermost sub-Himalayan zones) of India. Many opinions have been put forwarded regarding their origin. However, the most accepted theory stated that the Bodo people entered India during the initial period of the Bikram Sambat and got settled along the bank of the Brahmaputra River. Later, they gradually migrated to Assam and to North and East Bengal. According to historical evidences, the Rajbanshis was the original descendents of the _Koches‘ in the 16th Century in Kamata region with the establishment of the Koch Kingdom by Koch king. This kingdom later came to be known as the kingdom of Kochbihar during the colonial period. Although connected to mongoloid stock, the mighty king is said to

have adopted Hindu religion and culture (**Shrestha, 2009**). Thus historical evidences clearly point towards the influence of Sino-Tibetan lineage on the Rajbanshis. However, according to Sir H. H. Risley, Rajbanshis are of Dravidian origin with considerable mongoloid admixture (**Risley, 1891**).

The frequency and distribution study of ten human TLR genes among Rajbanshi population revealed that in Rajbanshi population, the frequency of TLR8 (0.894) was highest followed by TLR6 (0.882) and TLR9 (0.882). It has been observed that in this population allergy and inflammatory diseases are common and they are also susceptible for viral diseases. Typhoid is also a common disease occurs in Rajbanshi population. It has been observed that TLR5, a cell surface receptor is very frequent among this population. It has also been found that the expression of TLR9 is very high in Rajbanshi population which is expressed when viral infections occur in the individuals.

The Indian population exhibits enormous diversity in its genetic structure which is not only reflected in its diverse cultural and linguistic backgrounds but also renders difficulty in explaining the overall health and disease conditions in different population subgroups (**Tamang *et. al.*, 2012)**. A combined inter-disciplinary approach or method is much needed to explain and understand the disease-associated genetic variants in the populations and their susceptibility (**Tamang *et. al.*, 2012**). The TLR profile of a population in alliance with the surrounding environment plays a complex role in disease pathogenesis (**Cook *et. al.*, 2004., Netea, 2012., Liu *et. al.*, 2012**). Phylogenetic analysis reveals that Rajbanshi population shows close proximity with Gurkha and Muslim and Rabha population remain distantly related with the Rajbanshi. Genetic distance also shows the same structure and relationship with Gurkha and Muslim and a distant relationship with Rabha. In PCA plot it has been found that Rajbanshi takes a different plot in the score plot area where they remain close with the Muslim and Gurkha whereas, Rabha remain distant from Rajbanshi. Although, study based on KIR (**Guha *et. al.*, 2013**) genes and HLA (**Agarwal *et. al.*, 2008**) markers significantly proved that there is Tibetian influence on Rajbnashi population.

5.1.2 Rabha

The Rabha population is a very small ethnic tribal population inhabitating the Eastern Terai and Dooars regions of northern part of West Bengal (**Balakrishnan, 1978**) (**Data highlights, 2001**). Historical evidences suggest their East-Asian origin (**Mitra, 1953**). They have their own socio-cultural and linguistic heritage and are considered as an important tribal population of the state as well as the country. Nei's genetic distance and REML based phylogenetic analysis reveals that Rabha population occupied the lower right quadrant of the plot, whereas Gurkha and Muslim occupied the upper right quadrant in the score plot area. Rabha shows the closeness with the Gurkha population which is in agreement with other study done by using HLA marker. However, interestingly Rajbanshi and Rabha show the distant relationship among each other (**Das *et al.*, 2016**).

5.1.3 Gurkha

Gurkha population constitutes the major inhabitants of the hilly region of North Bengal. They are very hard working and courageous people. They have unique cultures and traditions which make them an important subject of population genetics study. They are also mixture of Indo-Aryan castes and Mongoloid-featured clans. Their main language in this region is Nepali. Genetic diversity analysis proved that there is a considerable proximity of Gurkha population with East-Asian lineages. They have shown tendency to remain in close proximity to the NEAs and SEAs. Neighbour joining tree and genetic distance analysis proves the close proximity of Gurkha with the Muslim population in case of TLR genes as environmental factors strongly influences the TLR gene diversity among different populations of the world with different ethnic background. The PCA plot also shows the close proximity of Gurkha with the Muslim as the Muslim population of Northern part of West Bengal has strong affinities with the Bangladeshi Muslim due to infiltration. On the other hand Gurkha population shows Sino- Tibetian lineage on their genetic affinity. So, the proximity has been occurred due to convergent evolution of TLR genes due to their environmental pathogen present in their surroundings and strong infectious disease sometimes help in convergent evolution of two different ethnic populations (**Laayouni, 2014**). For chi-square analysis TLR8 and TLR9 shows no significant values when compared with Rajbanshi, Muslim and Rabha. Genetic distance shows the closeness of Rajbanshi with the Gurkha population but distant with the Rabha because the Rajbanshi share the same

environment. In recent times they are mixed heterogeneous group in the hilly region of northern part of West Bengal which is also the reason for the admixture of the population with other groups in this region.

5.1.4 Muslim

Another population having a very interesting historical background is the Muslim population of West Bengal constituting 27% of the total population of the state (**Data highlights, 2001**). Recent studies have documented the admixture of the Indian Muslim populations with the local Hindu residents resulting in differential ancestral patterns in different parts of India. (**Papiha, 1996., Robb, 2002**., **Eaaswarkhanth *et. al.*, 2009**). Historical evidences suggested the genetic affinities of the Muslims of West Bengal. During the Partition of Bengal in 1947 divided the erstwhile British Indian province of Bengal between India and Pakistan. The western part of Bengal with predominantly Hindu population came to be known as West Bengal and became a province of India, while the eastern part with predominantly Muslim population became a province of Pakistan and later became the independent country of Bangladesh after the 1971 Bangladesh Liberation War. Thus, after partition, considerable minorities of Muslims were left in West Bengal, which inflated to 27% of the state population at present time. Furthermore, infiltrations by refugees from Bangladesh added to the present population of Muslims in Bengal. Therefore, it can be mentioned that the present Muslim population of West Bengal share ancestral links to present Bangladeshi Muslims. Study on 15 STR autosomal loci suggested the Muslim in India have diverse influences of local Hindu population and with other lineages like East-Asian, Middle-East and from Europe. Y- Chromosomal study on different Muslim population from different parts of India suggested that Muslim populations in general are genetically closer to their non-Muslim geographical neighbors than to other Muslims in India, and that there is a highly significant correlation between genetics and geography (**Gutala *et. al.*, 2006**). Thus, it can be said that the Muslim population from the northern part of Bengal may exhibit differences in their genetic make-up from other Muslim populations of India. So, it is possible that Muslims in the northern part of West Bengal have different genetic affinity with others. It can also be possible that different Muslim populations from other parts of India was colonized in Bengal and mixed with the local Muslim population. Moreover, northern part of West Bengal is a corridor of North-East India where Tibeto-Burma speaking group are

maximum and it may be possible that there is a chance of the influence of Tibeto- Burma speaking group on Muslim (**Debnath, 2011**).

It has been observed that close proximity has been found with Gurkha and Muslim population in the northern part of West Bengal in respect to their TLR genes. Genetic distance study and score plot showing the same result. It can also be observe that the Rajbanshi population is very close to the Muslim in this region. Chi- square analysis for ten TLR genes showed non- significant result for TLR1 and TLR9 when compared with other three populations in this region. It is also predicted from the result that as the TLR genes are the main markers for innate immune system and dependent on the conserved antigens, it influences the diversity and ethnicity of four studied populations in this North Bengal region. Environmental selection of TLR genes among the population influences the distribution pattern of the above mentioned population. On the other hand Gurkha and Muslim show less distance between them although they belong to two different lineages due to convergent evolution of TLR genes among them and also for the sharing of the same environment between them (**Laayouni, 2014**).

5.2 Frequency, distribution and association study of Rheumatoid arthritis among the patients of Siliguri and adjoining areas

Clinical diagnosis of RA is based on the classification criteria and guidelines from the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR) (**Arnett *et al.*, 1988**). Anti-CCP and RF are the two main factors for assessing rheumatoid arthritis. Anti-CCP is an autoantibody produced by the immune system that increases the inflammation in joints of the patients with rheumatoid arthritis. The specific cause of production of anti-CCP is due to the association of genetic and environmental factors. There are many studies that prove anti-CCP antibodies serve as a powerful serologic marker for early diagnosis of RA and prognostic prediction of joint destruction (**Mimori, 2005**).

On the other hand RF factor is also a very common diagnostic factor for prognosis of rheumatoid arthritis. This factor can also be found in case of non rheumatic patient due to various inflammatory diseases but the sensitivity in that case is very low. The frequency of RF antibody in case of rheumatoid arthritis is very high (70-90%) rather than in other arthritis diseases like juvenile idiopathic arthritis (5%), and psoriatic arthritis (<15%) (**Newkirk, 2002**). Genetic and

environmental conditions are also responsible for the worldwide variability in distribution of RFs. Their highest prevalence (up to 30%) has been observed in case of North American Indians tribes (**Jacobsson *et. al.*, 1993).** But different test worldwide suggested that the RF value for RA has sensitivity of 60-90% whereas the specificity is over 80% **(Ingegnoli *et. al.*, 2013)**. It has also been found that diagnostic value of only anti-CCP or RF is not sufficient for RA testing or diagnosis but the collaboration of both this factor induces the disease progression in RA patients. In this case the sensitivity of this test is over 90% alone **(Ingegnoli *et. al.*, 2013)**.

Toll like receptors are expressed by synovial cells within the joints of RA patients and a variety of endogenous TLR ligands are expressed (**Huang and Pope, 2009**). TLR1, TLR2, TLR4, TLR5 and TLR6 are highly expressed on the cell surface and recognize the antigens found on the surface of the pathogen. On the other hand TLR3, TLR7, TLR8 and TLR9 found on the endosomal membrane and antigen must be taken up by the cell. Upon binding to the ligand, TLRs interact with the different adaptor proteins and leads to the activation of resulting cytokines. Different toll like receptor proteins are highly expressed in case of RA patients. Expressions of TLR2 and TLR4 on peripheral blood monocytes have been documented in case of RA patients. TLR3 and TLR7 are also expressed in synovial tissue of RA patients. It has also been observed that in case of early as well as longstanding RA patients these two TLRs are highly expressed (**Huang and Pope, 2009**).

Cytokine plays a vital role in pathogenesis of RA. Synovial environment in case of RA patients produce different types of cytokines which increases the inflammation. Macrophages are the major contributors for the production of cytokines like TNF-α, IL-1, and IL-6. The production of this cytokines creates an environment which supports the differentiation of Th17 and also suppresses the regulatory T cell in that area of inflammation. B-lymphocytes present in the joints also helps in progression of the disease by producing proinflammatory cytokines and generate the autoantibodies (**Thwaites *et. al.*, 2014).** TLRs are also been observed in the synovial membrane of RA patient which also play a certain role for the pathogenesis of the disease. Signalling via TLR induces the production of different cytokines that has been observed in many other cases in rheumatic arthritic conditions.

It has been found that some of the TLRs like TLR1, TLR3, TLR6 and TLR8 showed high frequency in the patient. TLR1 and TLR6 are present in the cell surface and after recognition of

the bacterial, viral or fungal infection induce pro-inflammatory gene expression in the body via MyD88 dependent pathway. These two TLRs mainly recognize the diacyl and triacyl lipopetides as their antigen in the cell surface. On the other hand TLR3 and TLR8 present in the cell compartment which can recognize single and double stranded RNA. TLR3 signalling pathway occurs via TRIF dependent adaptor molecules mainly responsible for the production of interferons. Different small molecules that have been produced during the inflammation are recognized by the TLRs present inside the cell compartment.

It has been found that not only those above mentioned TLR showing high frequency but TLR4 and TLR9 the main TLRs present in the human chromosome also show the high frequency compared to the control subject. It is documented from the previous data that TLR9 is highly expressed due to the autoimmune disorder. This probably causes in case of rheumatoid arthritic patients too. The relative risk for the disease is also high in case of TLR4, TLR7 and TLR9.

High degree of odd ratio for the association with the disease is also found in case of TLR1, TLR4, TLR6, TLR8 and for TLR9. The highest odd has been found in case of TLR4 in the patients of Siliguri and adjoining region. It has been shown that in Chinese Han population certain polymorphic variation in the exon region of TLR4 contributed to RA pathogenesis which supports our data. The anti-CCP positive and RF positive patients with certain mutation in the TLR4 gene associated with the blunted receptor activity and diminished inflammatory response in humans **(Wang *et. al.*, 2017)**. The TLRs present inside the cell compartment are also responsible for the progression of the disease due to the production of various antigens during disease progression. So, the data also revealed that the different TLRs present in the cell compartment show a very high frequency.

It has been postulated from the different studies that expression of TLR2, TLR3 and TLR7 are significantly up regulated in RA synovial fibroblast tissue in case of RA patients but high expression of TLR4 has also been detected on macrophages present in the RA synovium **(Goh and Midwood, 2012**). Our data suggested that the high elevation of TLR4 in the RA patient, but door line association found in case of TLR3 and TLR7. Although TLR7 showing the much higher association rather than TLR3. Here it has also been found that risk ratio for TLR7 is high than any other TLR except TLR4. So it can be easily predicted that TLR7 plays a vital role for the severity of the disease in RA patients.

It has also been documented that induction of TLR3 by certain RNA molecules that is released by the necrotic cells of synovial tissue activates synovial fibroblast in case of RA patient (**Brentano *et. al.*, 2005**). So, TLR3 is also a potential inducer for the production of different kinds of cytokines and dysfunction of this receptor in the endosomal compartment might responsible for creating autoimmune diseases in human. No such significant study has been done on the role of TLR8 and rheumatoid arthritis but it has been experimentally proved that TNF-α has been secreted by the induction of certain single stranded RNA molecules in the RA patient (**Huang and Pope, 2009**). The high odd ration for TLR8 and also the high sensitivity (94.55) highlighted that TLR8 plays a crucial role in the pathogenesis of the disease.

TLR9 expressed on the endosomal compartment and sense the CpG DNA and unmethylated DNA present in the cell compartment. Activation by the different types of necrotic DNA causes release of different types of proinflammatory cytokines like TNF-α, IL-1β, IL-6 etc. Significant association has been found in case of TLR9 in RA patients and also high odd ratio signified the association with the disease. The sensitivity for the positive association with the disease has also been found.

In our study, it was observed that door line association has been found in case of TLR1 and TLR6 in association with the risk factor but the high odd ratio of this two TLR defined their role in case of RA pathogenesis. Due to the presence of different antigens released during the disease condition, may increase their expression in the patients. Although no sufficient data have been found for the profound role of TLR1, TLR5 and TLR6 in case of RA but the frequencies of TLR2 and TLR10 are found very low in the patients. Sensitivity or true positive cases has been found for TLR1 and TLR8. Association with the disease has also been calculated for other cell surface as well as for the endosomal TLR which is also responsible for the disease pathogenesis.

In case of rheumatic patients, certain TLRs play a vital role for the pathogenesis of the disease. Due the presence of certain antigens which is released by the different cell types activates the TLR receptors which again via different signalling proteins produce certain proinflammatory cytokines that induce the disease progression in the patients. Ten different TLRs are present in the human chromosome but all the different TLRs do not play a specific role as an inducer for the disease. Screening of the ten human TLR genes among the patients of North Bengal region tells about s the overall scenario of the role of TLRs and their frequency pattern which help us to

analyze further role of other different TLRs in case of RA. Certain polymorphic variety has also played a specific role for the susceptibility of the disease in a particular population which carry those alleles or specific alleles which might resistant for the disease.

5.3 Frequency, distribution and association study of Typhoid fever among the patients of Siliguri and adjoining areas

Bacteria are able to overcome the species barriers and adapt to new hosts is central to the understanding of both the origin of infectious diseases and the emergence of new diseases. The genetic analysis of typhoid fever caused by *Salmonella typhi* can serve as a useful model for studying host adaptation mechanisms, because these pathogens are physiologically well characterized and lend themselves to genetic analysis in different populations in the world (**Baumler *et. al*., 1998**). Enteric fever such as typhoid fever is a major human bacterial infection in India. Although the disease is not common in urbanized countries but it remains an important and persistent health problem in developing nations like India. Hospital-based surveys and reports from India indicate that enteric fever is a major public health problem in our country, with *Salmonella enterica serovar typhi* (*S. typhi*) the most common pathogenic agent. In recent times the number of *S. typhi* infected cases are increasing because risk factors such as poor sanitation, lack of safe drinking water supply and low socio economic conditions in resource-poor countries are amplified by the evolution of multidrug resistant salmonellae with reduced susceptibility to different drugs failure cases as ported in India (**Kanungo *et. al.,* 2008**).

The role of TLRs in typhoid fever has not been extensively studied in India especially in northern part of West Bengal where the health problems become the major issues related to the tea gardens. Some of the studies have been proven regarding the association of TLRs with the typhoid fever in India (**Sivaji *et. al.,* 2015., Sivaji *et. al.,* 2016**). Association study among the Malay population on TLR4 polymorphism confers a higher risk factor for typhoid infection (**Bhuvanendran *et. al.,* 2011**). According to **Dunstan *et. al.,*** (2005) premature stop codon of TLR5 polymorphism suggested no association with the typhoid fever caused due to *S. typhi*. TLR5 might not play an important role in TLR-stimulated innate immune responses to human infection with *Salmonella enterica serovar typhi*. Initiation of these responses may rely on other TLRs that recognize different bacterial ligands (**Dunstan *et. al.,* 2005**).

It has been found that the frequencies of some of the TLRs like TLR1, TLR4, TLR5 and TLR6 were very high in compare to healthy controls. The different antigens produced by *S. typhi* elevated the TLR expression in typhoid patients. Recognition of different antigens like vi-capsule, flagellin, LPS and other antigens definitely activated the signaling pathways for the production of different cytokines in the human. The interaction between TLRs and Pathogen associated molecular patterns (PAMPs) produced from the *S. typhi* increases the formation of inflammosome. It brings the neutrophil and macrophages and induces the production of pro-inflammatory cytokines like interleukin (IL)-6, IL-1b, tumor necrosis factor (TNF)-a, and interferon-gamma (IFN)-c (**De Jong *et. al.,* 2012**).

It has been primarily focused on the overall TLRs frequency distribution patterns among the typhoid patients in this region which was not been previously studied. Chi- square analysis reveals the significant values for different TLRs which positively associated with the disease. Correlation study also shows the close association with the patient and the control values for all ten human TLRs.

Positive association was found for TLR1 and TLR6 with the disease in respect to their odd ratio which was very highly associated with the disease and TLRs. Door line association has been found among the patients in comparison to their relative risk and risk ratio for the *S. typhi* infected patients. It signifies the positive relationship of the disease among typhoid patients in respect to their TLRs. Increased level of TLR1, TLR4, TLR5, and TLR6 expression in the cells proves that antigens from *S. typhi* highly increased the frequency pattern of those TLRs in course of the disease. It has been now established that TLR5 which recognizes the flagellin protein present in the bacteria and plays a significant role in case of typhoid fever. During the contamination of bacterial infection, the expression level of this TLR gene becomes maximum in most of the patients. According to **Hue *et. al.,*** (2009) TLR4 mainly recognizes the LPS, extent genetic variation within the TLR4 gene involved in defense against typhoid fever in Vietnamese population (**Hue *et. al.,* 2009**).

Sensitivity test for TLR1, TLR4, TLR5, and TLR6 are very high in typhoid positive patients which signify the prevalence of the disease in the population. The predictive values of any diagnostic test are related to its disease prediction ability. The low positive predicted values (PPV) are found when compared to the negative predicted values (NPV). It has also been proven

that the flagellin protein from the bacteria increases the expression of TLR5 in positive cases and multiplies the disease susceptibility among patients.

5.4 Frequency, distribution and association study of HIV+ patients among the patients of Siliguri and adjacent areas.

The major innate recognition system for viral/ bacterial invaders in vertebrates as well as in human is now thought to be the Toll-like receptor family. TLR genes are descended from similar receptors (Toll) originally found in Drosophila and share a Toll/ IL-1R (TIR) domain required for intracellular signaling as well as an extracellular region containing leucine-rich repeats (**Bafica *et. al.,* 2004**). The first report for association of TLR signaling and HIV stimulation came from studies in which LPS was shown to stimulate the viral LTR activity in chloramphenicol acetyl-transferase reporter transfected monocyte and macrophage-like cell lines via a mechanism associated with NF-kb activation (**Pomerantz *et. al.,* 1990**). NF-κB is necessary for IL-6 and tumor necrosis factor (TNF) production, IFN-β requires both NF-κB and IRF3, while IRF7 is required for IFN-α production (**Takeuchi and Akira, 2009**). In case of HIV+ patients, it has been reported that polymorphism in TLR3 gene (Leu412Phe) has a protective role against the disease (**Huik *et. al.,* 2013**). In another case, two variants found in TLR4 (Asp299Gly, Thr399Ile) which recognizes lipopolysaccharide (LPS) as their ligand are associated with the increased infection risk in HIV+ patients (**Papadopoulos *et. al.,* 2010**). According to **Martinelli *et. al.,*** (2007) pDCs, which normally secretes the IFN- gamma and activates the natural killer cell, are also suppressed due to gp120 viral envelope protein. The viral envelope protein inhibits the TLR9 mediated induction of proinflammatory cytokines in pDCs (**Martinelli *et. al.,* 2007**). Different polymorphic variants of TLR genes related to the susceptibility or resistance to the HIV depend on the ethnicity among different populations of the world (**Mackelprang *et. al.,* 2014**).

In our study, we have found drastic increasing of TLR4, TLR8 and TLR9 in HIV+ patients. TLR4 mainly recognizes endotoxin (LPS) as their ligands. HIV is an enveloped retrovirus which uses RNA as their genetic material and used reverse transcriptase and DNA integration in host cells to replicate. The envelope protein complex of HIV-1 is synthesized as a polyprotein (gp160) that is cleaved intracellularly to a heterodimer of surface subunit gp120 and trans-

membrane subunit gp41, are non-covalently linked (**McCune *et. al.,* 1988**). TLR4 binds to the gp120 protein of HIV and trigger proinflammatory cytokine production via activation of NF-κB. In this study the higher odd ratio and the relative risk for the disease indicates the ongoing promotion of the disease (**Nazli *et. al.,* 2013**). On the other hand function of TLR9 has been suppressed by gp120 protein. It also suppresses the function of pDCs cells and IFN-α where TLR9 expresses (**Martinelli *et. al.,* 2007**). TLR9 also expresses in the cell compartment like in endosomal compartment where they successfully recognizes the ssRNA, CpG oligonucleotides and express constitutively (**Carty and Bowie, 2010**). Certain polymorphic variation in TLR9 (1635A/G and +1174G/A) increases the susceptibility for the disease (**Bochud *et. al.,* 2007**) It has been also found that it is same for higher level of odd ratio and relative risk and thereby it can be suggested that TLR9 constitutively express in the cell. The sensitivity is also very high near 100% for TLR9 where the disease is positive for number of samples in our study. Another TLR also important in case of HIV is TLR8 which recognizes single stranded viral RNA and mainly express in myeloid DCs and in monocytes/ macrophages in human. The odd ratio and relative risk are showing the higher values in case of the disease. Sensitivity is also very high in case of TLR8. TLRs mainly expressed during HIV, produce type-I interferon cytokines via TLR signaling pathway. Significant data were also found for TLR2, TLR4, TLR8 and TLR9. P value was also considered for significance in case of TLR4, TLR8 and TLR9 which indicates the positive correlation of the disease with the TLR markers.

CHAPTER 6

Summary and Conclusion

6. Summary and Conclusion

6.1 Population- based study

Selection and convergent evolution are the two main forces which shape the TLR genes in their respective environment. Populations, which share their environment and inhabited in a particular region face a strong selection pressure due to the presence of pathogen in their surrounding environment. Also the Muslim population, very close to Gurkha in respect of the presence of TLRs, faces the same environmental condition. As a result, selection of specific TLR genes of the immune system shaping the population in respect of their ethnicity. On the other hand, convergent evolution of TLR genes has occurred due to the sharing of similar environment. Although Gurkha and Muslim belong to two different lineages, but their TLR distribution is same due to the sharing of similar environmental conditions. Rajbanshi, Gurkha and Rabha belong to the same East-Asian lineage, but considerably differ in their TLR distribution. This striking observation may infer the impact of environmental selection on the distribution of TLR genes. Such influences of the environment on TLR distribution may depend on the constant presence of specific pathogens in respective environment. Thus, it may be assumed that TLR genes play a significant role in shaping the genetic ancestry of the above mentioned populations from North Bengal region of India as well as in determining the disease exposure in these populations.

Among Rajbanshi population, high frequency of TLR8 and TLR9 is observed. So, it can inferred that this population, mainly inhabited in the tea garden areas of Terai and Dooars region of North Bengal, may be susceptible to some viral diseases. In Gurkha population, mainly inhabited in the hilly region of North Bengal, TLR4 and TLR5 are present with high frequency. It means that they might come in contact with the bacterial infections. A survey report on this population conveys their susceptibility for bacterial infections. On the other hand, TLR3, TLR5, and TLR7 are present with high frequency. Probably they get infected both with bacterial and viral diseases as we have found that occurrence of HIV infection among Muslim population is much higher than other populations in this region. In Rabha population the frequency of TLR4 is highest, which indicates that the population is susceptible to LPS and other bacterial antigens. A report is

already published out of the present work on the susceptibility of chronic gastro-intestinal diseases among Rabha population.

Phylogenetic and genetic distance assessment based on ten human TLR loci revealed that Muslim and Gurkha population are close to each other. Rabha is distantly related with the other three populations. Genetic distance analysis also proves the closeness of Gurkha and Muslim. The distances between Rajbanshi -Gurkha and Rajbanshi- Muslim are also close.

Conclusively, the combined picture represented the result of the analysis of TLR genes infer that:

1. The studied population now-a-days is very much a mixed population in this region.

2. The substantial variation of the studied population has been seen in this region in respect of their TLR genes.

3. Environmental selection acts on this population in respect to their TLR genes. Due to the sharing of the same environmental conditions it is very much conclusive to say that they come close not due to their ethnicity but in respect to their TLR genes which is very interesting.

4. On the other side, it has also been quiet interesting that convergent evolution occurs among the four populations in this region. Convergent evolution occurs in the TLR genes shapes the population irrespective of their ethnicity.

5. Gurkha, Muslim and Rabha belong to the Mongoloid origin as it has been reported in various studies on other markers like HLA and KIR. The deviation of this finding occurs in respect of the TLR genes, because TLRs are considered as the main markers of the innate immunity. It also depends on the environmental pathogen for their proper functioning. This is the main reason why the populations irrespective of their ethnicity merge into a common line.

It has been concluded that the population in this region are now a mixed population. They all share their gene pool among each other now- a- days. Genetic structure of Muslim population of this region has received the gene flow from the neighboring country, Bangladesh but there is also a considerable admixture of Tibeto Burman element in Muslim population of this region and that differentiates from the other Muslim population of India. In Gurkha population, gene flow occurs between Nepali speaking Gurkha and the population of other region. The admixture of Rajbanshi

of this region with the local Bengali population cannot be ignored. The Rabha population is restricted in some particular area in North Bengal region and not mixed with the other local population. So, the gene pool is restricted on them because of their endogamous character.

The present study on these four populations unveiled the curtain of the frequency and distribution in respect of their TLR genes. Environmental selection of some specific TLR genes among different population and convergent evolution shapes the population of this region into a peculiar nature irrespective of their ethnicity. This study is one of the primary and first hand report on population based study of TLR genes. So, further study is needed to reveal their genetic background in respect of their TLR genes and to know hoe TLR genes act on them in different conditions. One needs to lift up the curtain and try to find out their immune status and other polymorphic variation in the TLR gens which might help to build the knowledge how TLR helps to fight against various diseases.

6.2 Rheumatoid arthritis- based study

Rheumatoid arthritis is a systemic inflammatory autoimmune disease, characterized by chronic, erosive polyarthritis and by the presence of various autoantibodies in serum and synovial fluid. The main diagnostic tests are anti-CCP and RF titre assay for RA. The disease is caused due to the genetical and environmental factors. Different study suggested the association of toll like receptor with RA. Various polymorphic varieties present in the TLR genes in different population worldwide demonstrate their association with RA.

The findings of this study can be summarized as below:

1. The concentration of anti-CCP and the titre are very high in majority of the patients.

2. The frequency of TLR1, TLR6 and TLR8 are highest among patients compared to the control group. Low frequency is observed for TLR2, TLR5 and TLR10. It is evident from the study that the TLRs, those are present in high frequency mostly related with the RA , also recognize the antigens produced during inflammation in the joints.

3. The high odd ratio for TLR1, TLR4, TLR6, TLR8 and TLR9 determines the association with the disease which is also very much significant.

4. Relative risks are also very high for TLR4, TLR6, TLR7, TLR8 and TLR9. Door line association has been found for TLR1 and TLR3. It also signifies the positive relationship of TLRs with the disease.

5. High level of sensitivity for some of the TLRs deciphers the positive association with the disease.

6.3 Typhoid –based study

Typhoid fever is one of the most common diseases among the tea garden areas of this region due to the unhygienic conditions, lack of sanitization and lack of proper drinking water in this region. The disease is not very common in urbanized areas but frequently found in the rural especially in the tea garden areas. One needs to study the genetic nature of TLR genes and their frequency distribution in Siliguri and adjoining areas.

The findings of this study can be summarized as below:

1. The frequency graph clearly indicates that TLR1, 5 and TLR6 are highly up regulated, more or less consistent with the previously published reports. Widal test result is also very high in majority of the samples.

2. The door line association found for the respective TLRs indicates the positive association of the disease with the TLRs.

3. The sensitivity factor also high for TLR 1, 5 and 6 which shows the positive association of the disease among the population of the area.

4. Odd ratio among the groups also very high which indicates the susceptibility for the predisposition of the disease.

6.4 HIV –based study

Human immunodeficiency virus is a major causative agent for the acquired syndrome disease worldwide which demolish the immune system of individuals. There are no proper medications or vaccine to cure the disease because of the frequent mutation in the genome of the virus. Present study investigated the association of HIV+ patients with TLR variants in Siliguri region.

The findings of this study can be summarized as below:

1. The frequency graph clearly indicates that TLR8 and 9 are highly up regulated which are more or less in agreement with the previously published reports.

2. The positive associations in case of their relative risk are also found for the respective TLRs, indicates the positive association of the disease with the TLRs.

3. The sensitivity factor also high for TLR4, 8 and 9 shows the positive association of the disease among the population of the area.

4. Odd ratio among the groups also highly up-regulated indicates the susceptibility for the predisposition of the disease.

In this present work, the main goal was to determine the genetic predisposition in three main diseases and the role of TLR genes in the disease pathogenesis in multi ethnic populations in Siliguri and adjoining areas. Current findings on the risk factor of three diseases based on TLR gene profile provide a compact knowledge on the genetic basis of the diseases. Further study is needed to illuminate the role of single nucleotide polymorphism of TLR genes and susceptibility/resistant for the diseases in Sub- Himalayan region of West Bengal.

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INDICES APPENDICES

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Indices

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Appendix A

Health Assessment Questionnaire HAQ (D1)

Name:		
Age:	Sex:	
Date:	Telephone:	
Address:		
Height(cm):	Weight (kg):	Disease Duration (yrs):

HAQ Score=	Without any difficulty (0)	With some difficulty (1)	With much difficulty(2)	Unable to do (3)
1.Dress yourself, including tying Sari/salwar/dhoti/ doing buttons?				
2. Get in and out of bed?				
3. Lift a full cup or glass to your mouth?				
4. Walk outdoors on flat ground?				
5. Wash and dry your entire body?				
6. Squat in the toilet or sit cross-legged on the floor?				
7. Bend down to pick up				

clothing from the floor?				
8. Turn a tap on and off?				
9.Get in and out of auto rickshaw/ manual rickshaw/car?				
10. walk three kilometers?				
11. Shop in a vegetable market?				
12.Climb a flight of stairs?				

Physicians global assessment (0-100)	
Subject Global Assessment VAS (0-100)	
Subject Pain Assessment VAS (0-100)	
Tender joint Count	
Swollen joint Count	
DAS28 (ESR) (0-3)	
CRP (mg/dl)	
ESR (mm/h)	
RF titre (+/-)	
MTXdosage and usage duration	
other drugs dosage	
other drugs usage duration	

Appendix B

General Questionnaire

Cellular Immunology laboratory,

Department of Zoology, University of North Bengal,

Raja Rammohunpur,

Siliguri- 734013.

General Information:

Serial No.	
Name	
Father's /Mother's/ Husband's Name	
Address	
Phone	
Date of Birth	
Marital Status	
Sex	

Pedigree Status/ relatedness:

Diseases, if any	
Duration of Disease	
Significant Past History of Disease and infection	
Treatments / Medicines taken	

Comments:

Lab in Charge

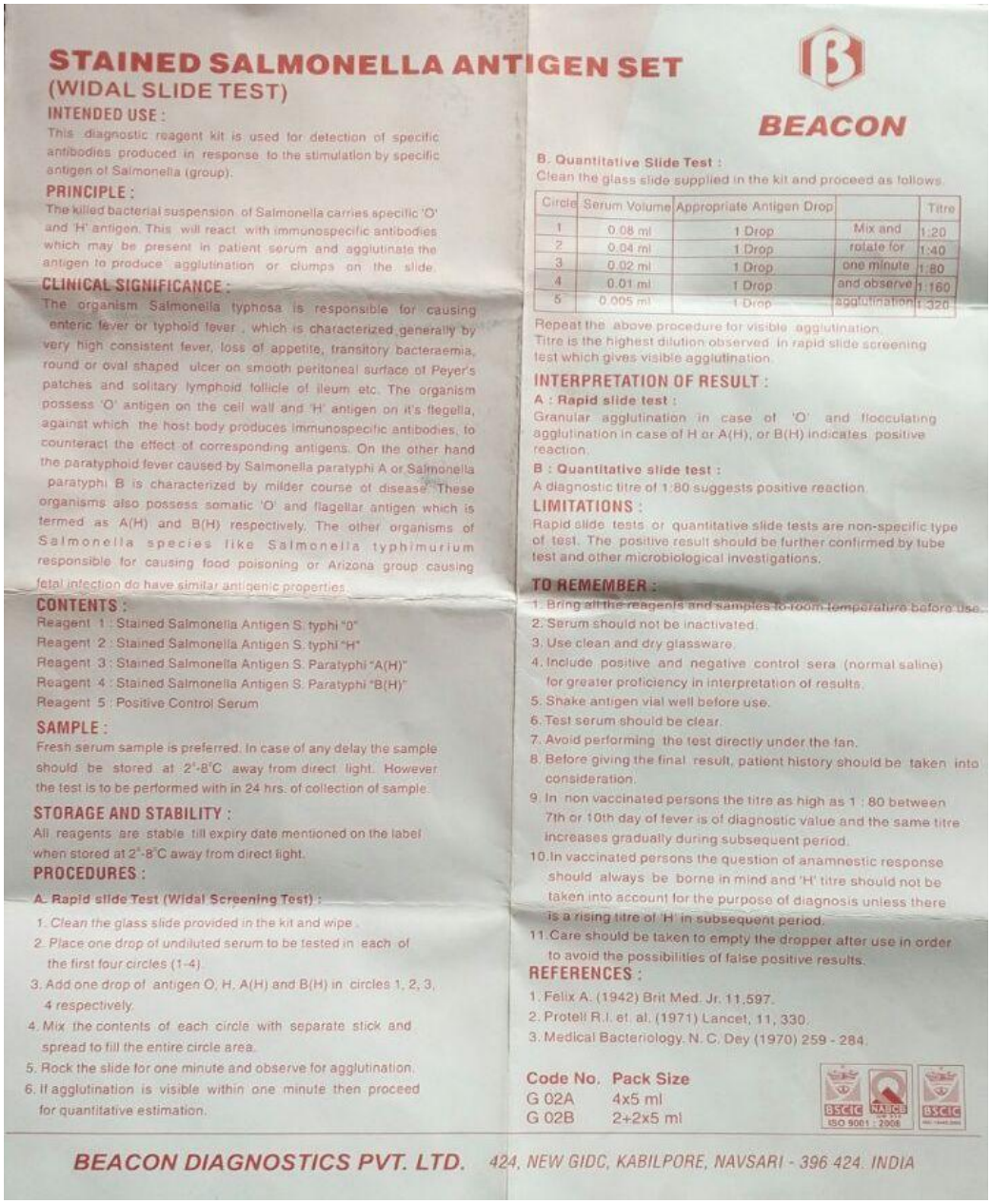
Cellular Immunology laboratory,

Department of Zoology

Date-

Appendix C

Stained Salmonella antigen set manual



STAINED SALMONELLA ANTIGEN SET
(WIDAL SLIDE TEST)

BEACON

INTENDED USE :

This diagnostic reagent kit is used for detection of specific antibodies produced in response to the stimulation by specific antigen of Salmonella (group).

PRINCIPLE :

The killed bacterial suspension of Salmonella carries specific 'O' and 'H' antigen. This will react with immunospecific antibodies which may be present in patient serum and agglutinate the antigen to produce agglutination or clumps on the slide.

CLINICAL SIGNIFICANCE :

The organism Salmonella typhosa is responsible for causing enteric fever or typhoid fever , which is characterized generally by very high consistent fever, loss of appetite, transitory bacteraemia, round or oval shaped ulcer on smooth peritoneal surface of Peyer's patches and solitary lymphoid follicle of ileum etc. The organism possess 'O' antigen on the cell wall and 'H' antigen on it's flegella, against which the host body produces immunospecific antibodies, to counteract the effect of corresponding antigens. On the other hand the paratyphoid fever caused by Salmonella paratyphi A or Salmonella paratyphi B is characterized by milder course of disease. These organisms also possess somatic 'O' and flagellar antigen which is termed as A(H) and B(H) respectively. The other organisms of Salmonella species like Salmonella typhimurium responsible for causing food poisoning or Arizona group causing fetal infection do have similar antigenic properties.

CONTENTS :

Reagent 1 : Stained Salmonella Antigen S. typhi "0"
Reagent 2 : Stained Salmonella Antigen S. typhi "H"
Reagent 3 : Stained Salmonella Antigen S. Paratyphi "A(H)"
Reagent 4 : Stained Salmonella Antigen S. Paratyphi "B(H)"
Reagent 5 : Positive Control Serum

SAMPLE :

Fresh serum sample is preferred. In case of any delay the sample should be stored at 2°-8°C away from direct light. However the test is to be performed with in 24 hrs. of collection of sample.

STORAGE AND STABILITY :

All reagents are stable till expiry date mentioned on the label when stored at 2°-8°C away from direct light.

PROCEDURES :

A. Rapid slide Test (Widal Screening Test) :

1. Clean the glass slide provided in the kit and wipe .
2. Place one drop of undiluted serum to be tested in each of the first four circles (1-4).
3. Add one drop of antigen O, H, A(H) and B(H) in circles 1, 2, 3, 4 respectively.
4. Mix the contents of each circle with separate stick and spread to fill the entire circle area.
5. Rock the slide for one minute and observe for agglutination.
6. If agglutination is visible within one minute then proceed for quantitative estimation.

B. Quantitative Slide Test :

Clean the glass slide supplied in the kit and proceed as follows.

Circle	Serum Volume	Appropriate Antigen Drop		Titre
1	0.08 ml	1 Drop	Mix and	1:20
2	0.04 ml	1 Drop	rotate for	1:40
3	0.02 ml	1 Drop	one minute	1:80
4	0.01 ml	1 Drop	and observe	1:160
5	0.005 ml	1 Drop	agglutination	1:320

Repeat the above procedure for visible agglutination.
Titre is the highest dilution observed in rapid slide screening test which gives visible agglutination.

INTERPRETATION OF RESULT :

A : Rapid slide test :

Granular agglutination in case of 'O' and flocculating agglutination in case of H or A(H), or B(H) indicates positive reaction.

B : Quantitative slide test :

A diagnostic titre of 1:80 suggests positive reaction.

LIMITATIONS :

Rapid slide tests or quantitative slide tests are non-specific type of test. The positive result should be further confirmed by tube test and other microbiological investigations.

TO REMEMBER :

1. Bring all the reagents and samples to room temperature before use.
2. Serum should not be inactivated.
3. Use clean and dry glassware.
4. Include positive and negative control sera (normal saline) for greater proficiency in interpretation of results.
5. Shake antigen vial well before use.
6. Test serum should be clear.
7. Avoid performing the test directly under the fan.
8. Before giving the final result, patient history should be taken into consideration.
9. In non vaccinated persons the titre as high as 1 : 80 between 7th or 10th day of fever is of diagnostic value and the same titre increases gradually during subsequent period.
10. In vaccinated persons the question of anamnestic response should always be borne in mind and 'H' titre should not be taken into account for the purpose of diagnosis unless there is a rising titre of 'H' in subsequent period.
11. Care should be taken to empty the dropper after use in order to avoid the possibilities of false positive results.

REFERENCES :

1. Felix A. (1942) Brit Med. Jr. 11.597.
2. Protell R.I. et. al. (1971) Lancet, 11, 330.
3. Medical Bacteriology. N. C. Dey (1970) 259 - 284.

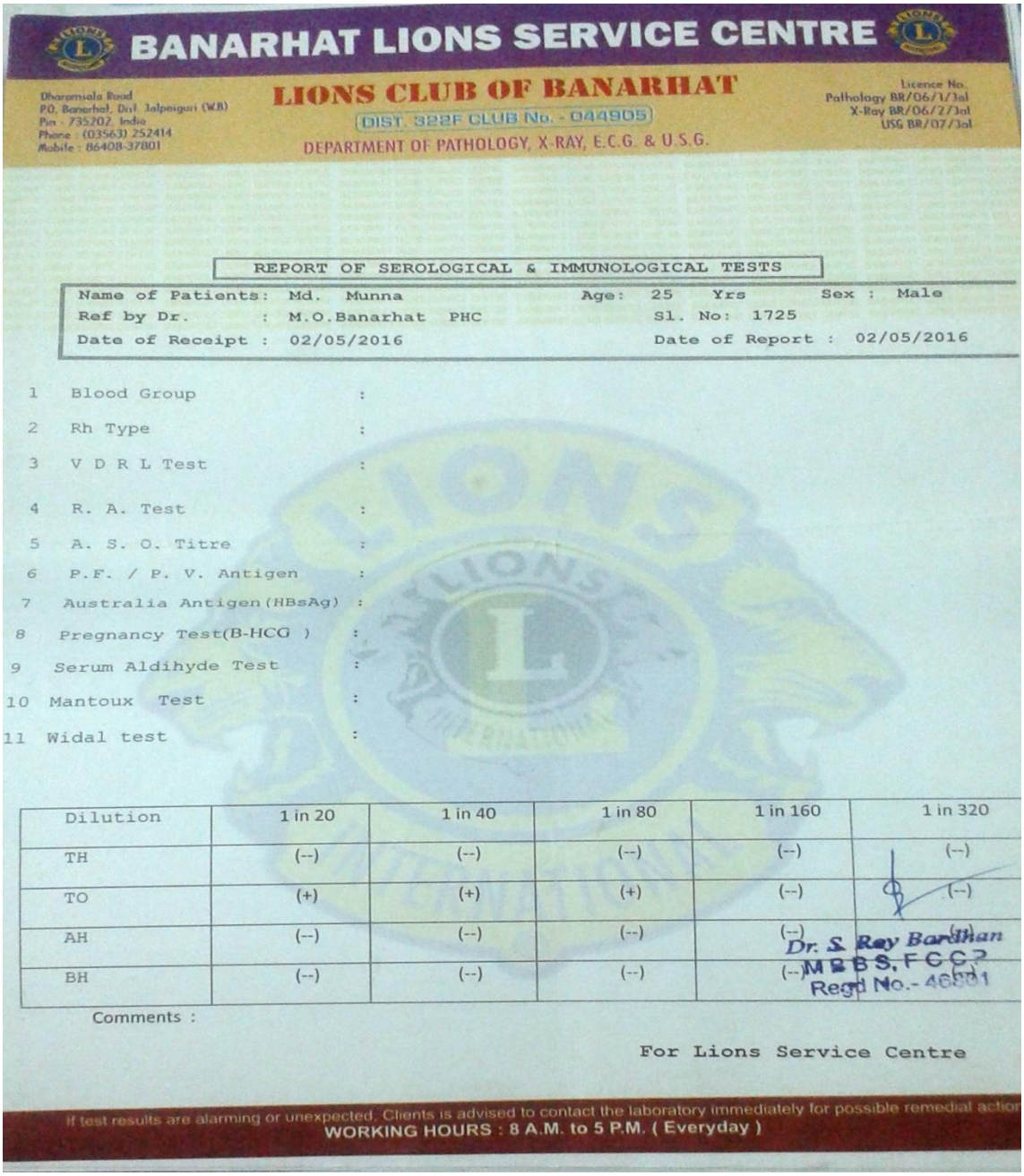
Code No.	Pack Size
G 02A	4x5 ml
G 02B	2+2x5 ml

BSCIC NABCB ISO 9001 : 2008 BSCIC

BEACON DIAGNOSTICS PVT. LTD. 424, NEW GIDC, KABILPORE, NAVSARI - 396 424. INDIA

Appendix D

Widal test result of a patient



BANARHAT LIONS SERVICE CENTRE

Dharamsala Road
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Mobile : 86408-37801

LIONS CLUB OF BANARHAT
DIST. 322F CLUB No. - 044905
DEPARTMENT OF PATHOLOGY, X-RAY, E.C.G. & U.S.G.

Licence No.
Pathology BR/06/1/Jal
X-Ray BR/06/2/Jal
USG BR/07/Jal

REPORT OF SEROLOGICAL & IMMUNOLOGICAL TESTS

Name of Patients: Md. Munna Age: 25 Yrs Sex : Male
Ref by Dr. : M.O.Banarhat PHC Sl. No: 1725
Date of Receipt : 02/05/2016 Date of Report : 02/05/2016

1 Blood Group :
2 Rh Type :
3 V D R L Test :
4 R. A. Test :
5 A. S. O. Titre :
6 P.F. / P. V. Antigen :
7 Australia Antigen(HBsAg) :
8 Pregnancy Test(B-HCG) :
9 Serum Aldihyde Test :
10 Mantoux Test :
11 Widal test :

Dilution	1 in 20	1 in 40	1 in 80	1 in 160	1 in 320
TH	(--)	(--)	(--)	(--)	(--)
TO	(+)	(+)	(+)	(--)	(--)
AH	(--)	(--)	(--)	(--)	(--)
BH	(--)	(--)	(--)	(--)	(--)

Comments :

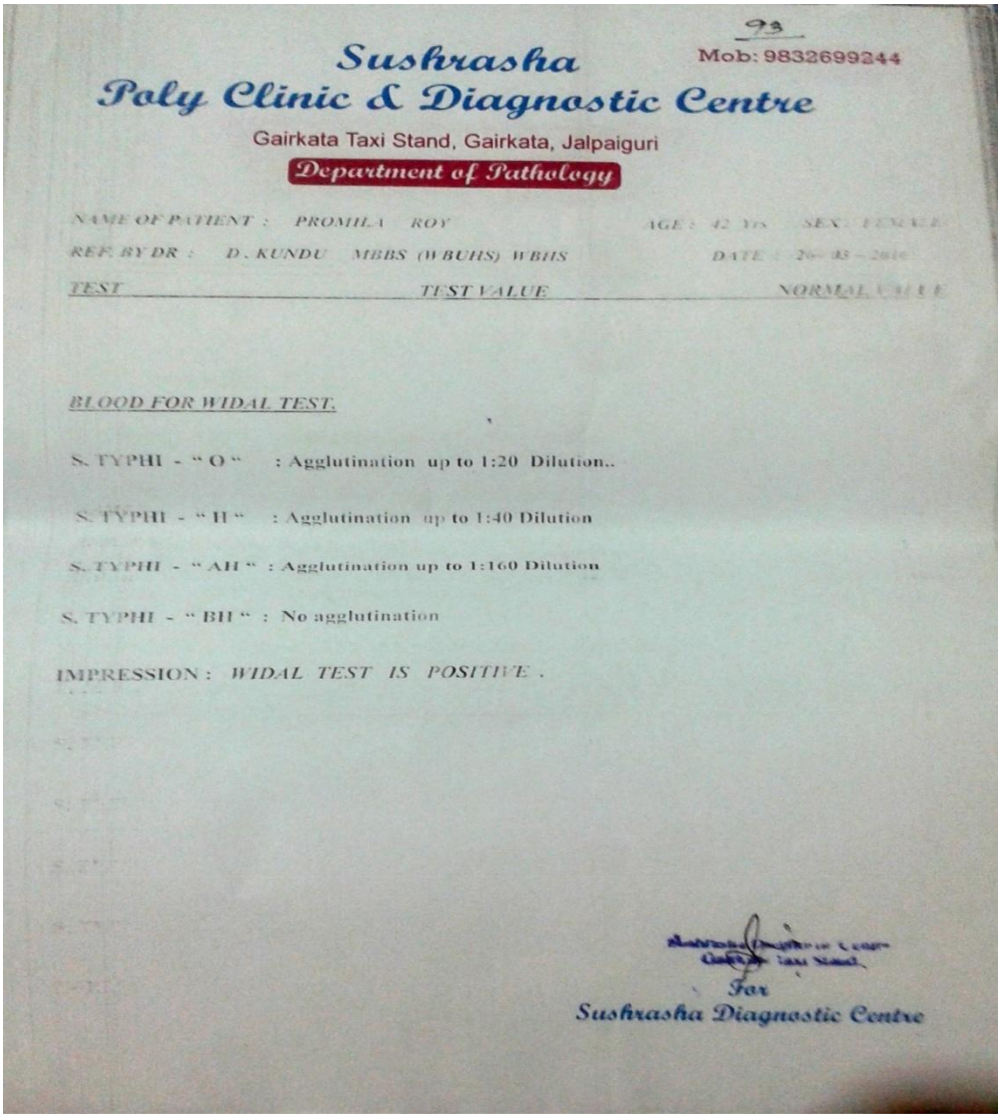
Dr. S. Roy Bardhan
MBBS, FCCP
Regd No.- 46881

For Lions Service Centre

If test results are alarming or unexpected, Clients is advised to contact the laboratory immediately for possible remedial action
WORKING HOURS : 8 A.M. to 5 P.M. (Everyday)

APPENDIX E

Widal test positive result



93

Sushrasha
Poly Clinic & Diagnostic Centre

Mob: 9832699244

Gairkata Taxi Stand, Gairkata, Jalpaiguri

Department of Pathology

NAME OF PATIENT : PROMILA ROY AGE : 42 Yrs SEX : FEMALE

REF. BY DR : D. KUNDU MBBS (WBUHS) WBHS DATE : 26-03-2016

TEST TEST VALUE NORMAL VALUE

BLOOD FOR WIDAL TEST.

S. TYPHI - " O " : Agglutination up to 1:20 Dilution..

S. TYPHI - " H " : Agglutination up to 1:40 Dilution

S. TYPHI - " AH " : Agglutination up to 1:160 Dilution

S. TYPHI - " BH " : No agglutination

IMPRESSION : WIDAL TEST IS POSITIVE .

For
Sushrasha Diagnostic Centre

Appendix F

Chemicals, reagents and kits

RBC lysis Buffer (RLB)

0.155 mol/L NH_4CL (Himedia),

10 mmol/L $KHCO_3$ (Himedia) and

0.1 mol/L EDTA (Na_2) (MERCK) in 1000 ml of distilled H_2O. The pH was adjusted to 7.6.

10 % SDS

Dissolve 1 g SDS (Himedia) in 10 ml of distilled water.

Extraction Buffer

1.5mol/L Tris (MERCK) pH 7.6

0.4 mol/L disodium salt of ethylenediaminetetra acetic acid (Na_2EDTA) (Merck, Germany),

2.5mol/L NaCl (MERCK)

2% Cetyl trimethyl ammonium bromide (CTAB) (Merck, Germany) 850ml H_2O. Adjust the pH to 8.0 and make the final volume to 1 litre.

6. β-Mercaptoethanol

7. Chloroform: Isoamyl alcohol (24:1)

8. Deoxyribonucleotide Triphoshate (dNTPs) set: Bangalore Genei, India.

The deoxyribonucleotide triphoshates are the monomers of DNA polymer consisting of dATP, dCTP, dGTP and dTTP. The dNTPs are used at saturation concentration in PCR amplification of DNA.

9. PCR Buffer with MgCl2 (Bangalore Genei, India)

The PCR buffer is optimized for use in PCR experiment. Generally the PCR buffer is supplied along with Taq polymerase by the commercial companies.

10. Ethidium Bromide (Gibco BRL, USA)

Dissolve 0.5 ug in 1 ml of TBE buffer

11. Gel Loading Dye/Solution

0.05%Bromophenol Blue - 50 mg
4.0% Sucrose - 20g
0.1 M EDTA - 1.46g
0.5% SDS - 250 mg

Dissolve EDTA in 25ml of distilled water by adjusting the pH to 8.0 with 5 N NaOH and add Bromophenol blue. Once dissolved, add sucrose and finally SDS. Adjust the final volume to 50 ml and stir at 80^{oC} to make the solution viscous. 1 volume of gel loading solution is optimal to 1-4 volume of sample. Bromophenol bleu serves as the tracking dye while sucrose adds density and facilitaties sample loading. EDTA is including terminating the action of intrinsic DNAase activity. SDS helps to dissociate DNA – Protien complexes, which can otherwise interfere the electrophoresis.

12. Taq DNA Polymerase (Bangalore Genei India)

13. 10X TBE Buffer

0.9M TRIS - 109.06g
0.02M EDTA - 7.44g
0.9M boric Acid - 55.647 g

Dissolve in 1000 ml of distilled water and store at 4^{0C} Prepare 1X as working buffer.

13. TE Buffer/Solution

1mM TRIS - 121.16 g

0.1mM EDTA - 37.224 g

Dissolve in 950 ml distilled water and adust pH to 7.5. Adjust the final volume to 1000 ml adding distilled water.

14. Phosphate Buffered Saline (PBS), pH 7.2 (Himedia, India)

APPENDIX G

Publications

Das A. Guha P. Chaudhuri TK. 2017. Diversity of Ten TLR Genes in Some ethnic populations of North Bengal region of India. ***IJPBS***. 7:255-62.

Das A. Guha P. Chaudhuri TK.2017. Role of Toll like Receptors in bacterial and viral diseases-A systemic approach. ***EJMHG***. 18: 373- 379.

Das A, Guha P, Chaudhuri, TK. 2016. Environmental selection influences the diversity of TLR genes in Ethnic Rajbanshi population of North Bengal Region of India. ***JGEB.*** **14:241-45.**

Guha P. **Das A**. Dutta S. Chaudhuri TK.2017. A Rapid and Efficient DNA Extraction Protocol from Fresh and Frozen Human Blood Samples. ***JCLA***. 32: 1-7.

Das A, Guha P, Chaudhuri T.K. 2016. Comparative sequence analysis of TLR2 TLR4 and TLR9 genes among selected vertebrates-A meta- analysis. ***Int J Pharm Pharm Sci.*** 8:180-185.

Guha P, **Das A**, Dutta S, Bhattacharjee S, Chaudhuri TK.2015. Study of genetic diversity of KIR and TLR in the Rabhas, an endogamous primitive tribe of India. ***Hum Immunol***. 76: 789– 794.

Guha P. Paul S. **Das A**. Halder, B. Bhattacharjee S. Chaudhuri, T.K. 2014. Analyses of Human and Rat Clinical Parameters in Rheumatoid Arthritis Raise the Possibility of Use of Crude Aloe vera Gel in Disease Amelioration. ***Immunome Res****.10:2.2-7.*

Guha, P. Srivastava, SK. **Das, A**. Bhattacharjee, S. Halder, B. Chaudhuri, TK. 2013. Comparative Analyses of the ABO, KIR, and HLA loci among the Rajbanshi of North Bengal region, India. ***Annals of Pharma research***. 1: 18-24.

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Research Article | Biological Sciences | Open Access | UGC Approved | MCI Approved Journal

GENETIC DIVERSITY OF TEN TLR GENES IN SOME ETHNIC POPULATIONS OF NORTH BENGAL REGION OF INDIA-A SYSTEMIC STUDY

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***Corresponding Author Email: dr_tkc_nbu@rediffmail.com**

ABSTRACT

Objectives: *Toll like Receptors (TLRs) are conserved transmembrane proteins that recognize Pathogen Associated Molecular patterns (PAMPs) and initiate innate immune system functions.* ***Method:*** *Hereby, we have aimed to study the diversity and frequency distribution of 10 TLR genes in the three ethnic populations of North Bengal namely Rabha, Gurkha and Muslim. We took 315 samples of which 125 Gurkhas, 140 Muslims and 50 Rabha samples. PCR-SSP was done for all the ten human TLR genes. We have constructed the phylogenetic tree, Principal component analysis (PCA) and Genetic distance for all three populations.* ***Results:*** *We have documented the highest frequency of TLR4 among the Rabhas (0.90) and Gurkhas (0.96) while TLR5 (0.97) have the highest frequency among the Muslims. Based on TLR frequencies the genetic distances were calculated which revealed that Rabha and Muslim are distantly related (0.089) while Gurkha and Muslim (0.023) are much closer. This observation is interesting as both Rabha and Gurkha are East-Asian origin while Muslim belongs to the Middle East lineage.* ***Conclusion:*** *This may be because of the effect of the environment in combination with the pathogens present in that environment, as TLR is mainly responsible to participate in innate immune response. Convergent evolution also plays a significant role in shaping the three populations in this region inspite of their different ethnicity. This study is a pioneering report on population based TLR frequency distribution in North Bengal region.*

KEY WORDS

Toll Like Receptors, Pathogen Associated Molecular Pattern, Genetic distance, East-Asian, Principal Component analysis.

INTRODUCTION

Infectious pathogens act as the major force for generating selection pressure in human evolutionary history [1]. Migrations of humans to different parts of the world resulted in the exposure of the immune response genes to local pathogenic antigens and thus get modified as per environmental demands. Thus, pressures exerted by the local pathogens cause positive selections of some genetic markers in the population for developing protection against the pathogens [1]. TLRs are genetically conserved pattern recognition receptors (PRR) which are capable of recognizing diverse sets of conserved antigens [2]. Ten members constitute this receptor family in both human with their respective genes located in different chromosomes of the human genome [3]. Majority of the TLR molecules are cell surface receptors which includes TLR1, 2 4 6 8 and 10 respectively, while others are endosomal in nature (TLR 3, 7 and 9). The members of the TLR family are capable of recognizing different conserved antigens like lipopolysaccharide, flagellin, CpG DNA and even double stranded RNA [2] and may also regulate the susceptibility of a population to pathogenic invasions and disease progression [4] [5].

Indian population comprises of various religions, tribes and castes each having their unique socio-cultural and ethnic background, most of which are strictly endogamous [6]. The Indian subcontinent has experienced several human migration events. One such major event includes the migration of Indo-European-speaking people from West Eurasia whose admixture with indigenous Dravidian populations led to the subsequent establishment of the Hindu caste system [7]. Such extensive admixture and enormous genetic diversity among the Indians make them primary target for genetic diversity analyses [7] [8].

The Rabha population is a very small ethnic tribal population inhabitating the Eastern Terai and Dooars regions of northern part of West Bengal [8] [9]. Historical evidences suggest their East-Asian origin [10]. They have their own socio-cultural and linguistic heritage and are considered as an important tribal population of the state as well as the country. On the other hand, Gurkhas constitute the major inhabitants of the hilly region of North Bengal. They are very hard working and courageous people. They have unique cultures and traditions which make them an important subject of population genetics study. Another population having a very interesting historical background is the Muslim population of West Bengal constituting 27% of the total population of the state [9]. Recent studies have documented the admixture of the Indian Muslim populations with the local Hindu residents resulting in differential ancestral patterns in different parts of India [11] [12] [13].

In this study, we have aimed to study the TLR genetic profile in Gurkha, Rabha and Muslim populations and analyzed the role of TLRs in selection and phylogenetic analyses, if any.

MATERIAL AND METHODS

1 Study populations

The study population consisted of 125 Gurkhas, 140 Muslims and 50 Rabha samples. All blood samples were collected from Darjeeling, Coochbehar and Jalpaiguri districts (26° 20'- 27° 03' N and 88° 18'- 89° 29' E) of Northern West Bengal, India. The samples were collected on the basis of their ethnicity, caste and health conditions. Individuals having three generations of common pedigree were excluded from the analyses. All the donors were informed regarding the purpose of the study before the collection of the blood samples and written consents were provided by the volunteers. The investigation was approved by the Human ethics committee of the Department of Zoology, University of North Bengal (Zoo/4133/2011) and performed in accordance with the Declaration of Helsinki, 1964. 3 mL of blood sample was taken from each volunteer by vein puncture method under the guidance of a medical practitioner and was stored in EDTA containing vials at -20°C until use.

2 DNA extraction and TLR specific PCR- SSP typing

Genomic DNA was extracted from the samples by the standard Phenol- Chloroform extraction method with slight modifications. This was followed by PCR-SSP typing for all the 10 TLR genes [14] **(Table no. 1)**. Primers were designed based on the conserved sites of the ten human TLR genes using NCBI BLAST http://www.ncbi.nlm.nih.gov/tools/primer-blast/.

Primers were supplied by the Integrated DNA Technologies, Inc, Iowa, USA. Each PCR reaction mixture of 25µL volume contained 5X PCR buffer (Promega Corporation, Agora, Fitchburg Center, Fitchburg, Wisconsin), 5 µL of 10mM dNTPs, 1.5µL of 25mM MgCl2, 1.5µL of primers, and 1-1.5 U of Taq DNA polymerase. DNA samples were checked before use for their 260/280 absorbance and 1.5-2ul of 100ng DNA samples were then added to the PCR mixture. The reaction conditions for PCR consisted of an initial denaturation step of 94°C for 3 minutes followed by 30 cycles of 94° C for 30 s, 56.9° C for 50 s and 72°C for 1minutes and final extension of 72^{0}C for10 minutes respectively with slight modifications of annealing temperature for different primer sets. The PCR products were then analyzed on ethidium bromide prestained 2% agarose gel by electrophoresis. Samples were then visualized on UV transilluminator. All the lanes of the product loaded gel showed a control band, except for the negative control lane. The reactions were repeated in case of false reactions where no control bands were found.

3 Statistical analyses

Statistical data were analysed in Kyplot, MS-excel, GenAlEx (ver-6.5), SPSS (Ver-15.0) and MINITAB (Ver-6). Observed frequencies were performed by direct counting the number of the gene present by the total number of the sample. Gene frequencies and Chi- square data were also calculated. Correlation studies were carried out among the three populations. Euclidean

distance based hierarchical cluster analysis was performed from the observed frequency data. Principal component analysis (PCA) score plot and neighbour joining (NJ) tree was constructed using SPSS (ver. 15.0).

Table No. 1: List of forward and reverse primers for the 10 TLRs in human.

Genes	Forward Primers (5'-3')	Reverse Primers (3'-5')	Product size(bp)	GC content (%)
TLR1	TCAACCAGGAATTGGAATAC	AGTTCCAGATTTGCTACAGT	382	40
TLR2	GGATGGTTGTGCTTTTAAGTACTG	AAGATCCCAACTAGACAAAGACTG	2671	41.67
TLR3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	792	44/40
TLR4	TTCTTCTAACTTCCTCTCCTGTG	TTAGCTGTTCGGCTCTACTATGG	1087	
TLR5	CATTGTATGCACTGTCACTC	CCACCACCATGATGAGAGCA	446	45/55
TLR6	ACAACCCTTTAGGATAGCCACTG	AAACTCACAATAGGATGGCAGG	398	47.83
TLR7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG	545	44
TLR8	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG	637	45.83
TLR9	TCTAGGGGCTGAATGTGACC	ACAACCCGTCACTGTTGCTT	1106	55
TLR10	GTCGAAGACCCAATATACAG	ATTAAGCAATAGAACCGATG	954	45/35
Growth Hormone (Positive control)	CTTCCCAACCATTCCCTTA	CGGATTTCTGTTGTGTTTC	424	47/42

RESULTS AND DISCUSSION

The observed frequencies of all known TLR genes estimated in Gurkha, Muslim and Rabha populations respectively are represented in **(Table no. 2).** In one of our previous studies, we have screened TLR1-5 genes in the Rabha population [15]. It was observed that TLR4 was found in very high frequency among the Rabhas, while TLR5 was found to be the least frequent among the studied genes [14]. It has been observed that among the 10 TLR genes, TLR4 has the highest frequency among the Gurkhas and the Rabhas while TLR5 was found to be the highest among the Muslims **(Fig. 1).** When compared among the three populations, it was observed that TLR5 has the highest calculated frequency value (0.971) in the Muslim population followed by TLR4 in the Gurkha population (0.968). In contrast, it was interestingly observed that TLR4 has the lowest frequency in the Muslims (0.557) while TLR5 gene was the least frequent among the Rabhas. Apart from TLR2, TLR5 gene also showed low frequency in the Rabha population. Another interesting observation reported from our study was the low frequency of the TLR2 gene in all the studied populations. The gene frequencies of all the 10 TLR genes in the three populations were also calculated and presented in **(Table no. 3)**. Chi-square analyses ($\chi 2$) were performed to compare the differences in carrier frequencies (OF) of TLR genes among the three populations **(Table no. 2).** It was found that no significant differences were observed for 7 out of 10 TLR loci among the Gurkhas and the Muslims which outnumbered the non-significant cases in Gurkhas vs. Rabhas and Rabha vs. Muslims comparisons respectively. No significant differences were found for TLR2, 8 and 9 in any of the comparisons. Mean unbiased genetic diversity of TLR genes in the three populations was calculated to be 0.240 ± 0.038 for Gurkhas, 0.258±0.049 for Muslims and 0.410±0.033 for Rabhas respectively. Hierarchical cluster analysis was also performed followed by the construction of a neighbour joining tree based on the Euclidean distances calculated from the observed frequencies of the TLR genes in the above-mentioned populations, as shown in **(Fig 2a)**. It was quite surprising to see from the tree that the Gurkhas clustered with the Muslims while the Rabha population occupied a different branch of the tree.

Table No. 2: Observed frequencies of the 10 TLR genes in the three populations. χ^2 values were also mentioned where each gene was compared between two populations for any statistical differences.

	Muslims (M)	Gurkha (G)	Rabha (R)	MXG	GXR	RXM
TLR1	0.886	0.928	0.760	0.9314	8.020**	3.687
TLR2	0.571	0.608	0.600	0.229	0.005	0.034
TLR3	0.943	0.856	0.740	4.691*	2.549	13.427***
TLR4	0.557	0.968	0.900	57.426***	2.134	17.499***
TLR5	0.971	0.928	0.460	1.820	45.051***	66.880***
TLR6	0.907	0.896	0.640	0.009	14.349 ***	17.349
TLR7	0.943	0.864	0.680	3.927*	6.745**	20.745***
TLR8	0.829	0.824	0.700	0.0041	2.591	2.986
TLR9	0.793	0.784	0.820	0.0006	0.1057	0.0424
TLR10	0.793	0.832	0.540	0.4299	14.6650***	10.612**

*** P< 0.05, ** P< 0.01, *** P< 0.001**

Table No. 3: Gene frequencies of the 10 TLR genes. The gene frequencies were calculated from the observed frequencies of the 10 TLR genes using the formula 1-√ {1-f}, where f is the observed frequency.

Gene	Muslim	Gurkha	Rabha
TLR1	**0.662**	**0.732**	**0.510**
TLR2	**0.345**	**0.374**	**0.368**
TLR3	**0.761**	**0.621**	**0.490**
TLR4	**0.335**	**0.821**	**0.684**
TLR5	**0.831**	**0.732**	**0.265**
TLR6	**0.695**	**0.678**	**0.400**
TLR7	**0.761**	**0.631**	**0.434**
TLR8	**0.586**	**0.580**	**0.452**
TLR9	**0.545**	**0.535**	**0.576**
TLR10	**0.545**	**0.590**	**0.322**

Table No. 4: Nei's Genetic distances among the three populations using GenAlEx (ver- 6.5) software.

	Gurkha	Muslim	Rabha
Gurkha	**0.000**		
Muslim	**0.023**	**0.000**	
Rabha	**0.056**	**0.089**	**0.000**

From the Principal Component Analyses **(Fig. 2b)** it was evident that the three populations occupied three different quadrants of the score plot whereby the Muslims occupied the upper left quadrant, Gurkhas occupied the upper right quadrant and the Rabhas occupied the lower right quadrant of the plot. Nei's genetic distance was also calculated between the populations **(Table no. 4)** whereby, it was observed that the Gurkha-Muslim genetic distance (0.023) was considerably lesser than the Gurkha-Rabha genetic distance (0.056). The genetic distance was found to be the highest among the Muslims and the Rabhas (0.089).

Figure1. Radar chart constructed using MS EXCEL demonstrating the visual differences in the observed frequency distribution of the 10 TLR genes in the three ethnic populations of North Bengal.

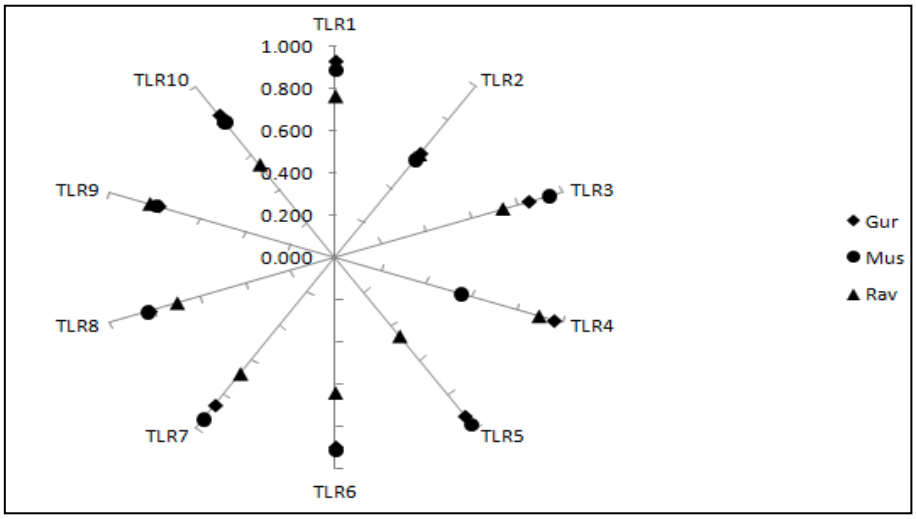
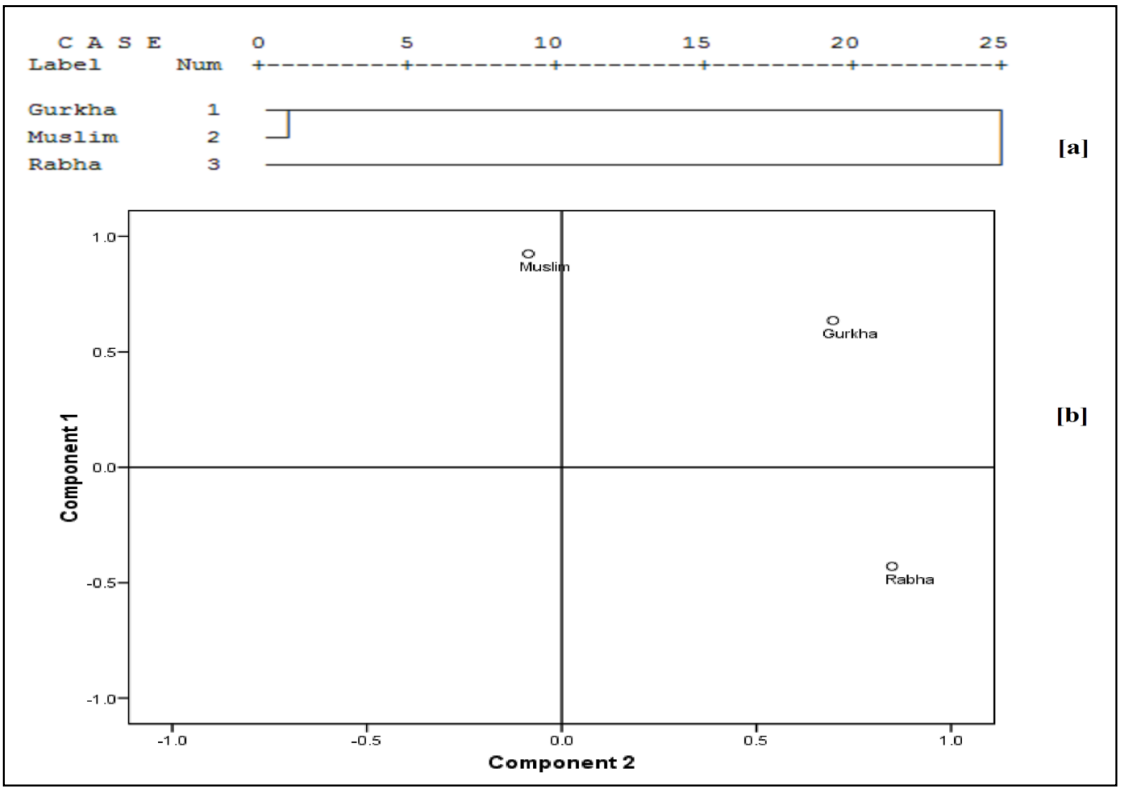


Figure 2. (a) Euclidean distance based Hierarchial cluster analysis was performed using from the observed frequency data by using SPSS (Ver- 15) software. (b) Principle Component Analyses (PCA) based on observed frequencies of the 10 TLR genes in the three ethnic populations of North Bengal constructed with Minitab (ver-6).



The Indian population exhibits enormous diversity in its genetic structure which is not only reflected in its diverse cultural and linguistic backgrounds but also renders difficulty in explaining the overall health and disease conditions in different population subgroups [6]. A combined inter-disciplinary approach or method is much needed to explain and understand the disease-associated genetic variants in the populations and their susceptibility [6]. The TLR profile of a population in alliance with the surrounding environment plays a complex role in disease pathogenesis [5] [16] [17]. In one of our previously published reports, we found that

chronic gastritis and associated stomach problems were very much common in Rabha population. This basic observation led us to speculate that Killer Cell immunoglobulin like receptors (KIR) and Toll like receptors (TLR) may join hand in hand to regulate disease progressions in Rabha population [15]. Based on the frequencies of the TLR4 and TLR5 genes, we assumed that such profile of these genes may indicate an up regulation of IFN-γ production by NK cells which in turn may regulate the prevalence of the Helicobacter pylori negative gastritis [18] [19]. Our assumption was also supported by previously published reports which suggested that the TLR genes play crucial roles in disease pathogenesis [16] [5]. Furthermore, the presence of TLR genes can be documented from the genetic pools of each and every population of the world. Thus, this marker may be considered to clarify the genetic diversity and the relatedness among different populations [11]. However, such preliminary observation requires experimental support. Therefore, in this pioneering study, we have analyzed the frequency of TLR genes in Rabha and two other common population groups of West Bengal, India. Considering the above-mentioned fact, it can be said that this study may help to explore the KIR-TLR connections in disease pathogenesis; because it would always be a judicious decision to explore the distribution pattern of these two gene families in populations before finding their relations and roles in immune responses.

Based on $\chi 2$ analyses it was found that the Gurkha population showed non-significant difference for 7 TLR genes with Muslims in comparison to only 5 TLR genes with the Rabhas, suggesting proximity of the Gurkhas with the Muslim population rather than the Rabhas. A similar observation was also made from the Nei's genetic distance measures where it was seen that the Gurkhas have considerably lesser genetic distance with the Muslim than that of the Rabhas. This observation was also supported by the Neighbour joining dendogram constructed on the basis of Euclidean distances. There is no doubt in the fact that this is a very unlike observation since both Gurkhas and Rabhas are considered to be of East-Asian origin [20] while the Muslims belong to Arabian or Iranian lineages [13]. Thus, the question may be raised regarding the role of TLR in exploring the population phylogenetics and migration pattern. This may be one of the prime reasons why frequency distribution studies based on TLR genes were not conducted earlier in different populations of the world.

Another interesting observation made from our study was that of the TLR4 which has established the predominance over other members of the family, was found in Gurkhas and Rabhas with higher frequencies with no significant differences. In contrary, this gene was found at a very low frequency in the Muslims. On the other hand, TLR5 was present higher frequency in the Muslims and Gurkhas while at a very low frequency in the Rabhas. Such TLR frequency distribution in the three populations suggests the genetic remoteness of the Rabhas with the Muslims while Gurkha occupying an intermediate position. It also suggested the influence of similar environmental exposure on the selection of TLR markers in these populations. This observation was further supported by the Nei's genetic distance measures, Euclidean distance-based NJ tree and PCA score plot. In our study, the three populations have occupied three different quadrants of the score plot and thereby signifying the considerable genetic variability with each other. This was further supported by the mean unbiased diversity measures, whereby it was seen that the Gurkhas showed the lowest value followed by the Muslims and the Rabhas respectively.

According to anthropological evidences, both the Gurkhas and the Rabhas belong to the East Asian origin [10] [15]. However, the TLR profiles in these two populations are quite different. This may have resulted due to the strong influence of the environmental selection on their TLR gene pool. Apart from the selected region of Northern part of West Bengal, the Rabhas are scarcely distributed in the North-Eastern states of India. In contrast, the Gurkhas are distributed over a wide range in the Eastern and North-Eastern part of the country while also being the major population of the neighbouring country of Nepal [20]. Thus, the selection pressure of the surrounding environment was larger on the TLR gene pool of the Gurkha population compared to that of the Rabhas, resulting in the drifting apart of their gene pool from that of the Rabhas.

On the other hand, the Muslim population selected for our study shared the same ancestry with the Bangladeshi Muslims and therefore are distributed over a wide range encompassing the whole of Bengal and Bangladesh. Their TLR gene pool was not only

influenced by inter-regional marriages with Muslims from all over India but also experienced East-Asian influence due to human migration events from neighbouring geographical locations. Furthermore, due to their robust spatial distribution, the selection pressure of the surrounding environment was huge on the TLR gene pool of the Muslim population. Interestingly, it was found that the Muslims population showed genetic proximity to the Gurkhas. This may have occurred due to convergent evolution of TLR genes in these two populations probably due to the selection pressure exerted by the environment which they share [21] [22]. Our speculation has been supported by previously published reports whereby it was suggested that infectious disease like plague may exert influence on the convergent evolution of TLRs in some recent human populations with different genetic ancestry but having exposure to similar environmental condition. Such convergent evolution of TLR was observed among the Romanians and Rroma, which are populations with different origins but sharing the same environment [23].

CONCLUSIONS

In conclusion, it would not be sensible enough to say that TLR gene profile of a population generates sufficient data to establish the genetic connection of the population with other world populations. However, further investigations are required in order to analyze the role of TLR genes in studying population origin and migration events. Furthermore, this study has showed that the TLR gene profile of a population is highly influenced by its ambient environment. Therefore, studies on frequency distribution of TLR genes in different population around the World are very essential since these studies may help us to understand the susceptibility of a disease in a population having a particular genetic makeup and geographical distribution and may also pave the way to further advanced genetic researches for disease eradications.

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Conflict of interest: The authors declare that they have no conflicts of interest.

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ORIGINAL ARTICLE

Environmental selection influences the diversity of TLR genes in ethnic Rajbanshi population of North Bengal Region of India

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KEYWORDS

Toll-like receptors;
Genetic distance;
PCAplot;
Dendrogram

Abstract *Background:* Toll-like receptors are the mediators of the innate immune response to pathogens. In human, this gene family regulates the inflammatory pathways and is associated with the susceptibility to infection.

Subjects and methods: The distribution and the diversity patterns of TLR genes in Rajbanshi population ($n = 85$) who are the inhabitants of the Northern part of West Bengal, have been investigated in the present study. PCR-SSP was done for all the ten TLR genes. We have also constructed the phylogenetic tree principal component analysis and genetic distance for all the four populations.

Results: It has been observed that in Rajbanshi population, the frequency of TLR8 (0.894) is higher and the frequency of TLR2 (0.176) is very low. Dendrogram based analysis, as well as the PCA plot, documented the closeness of Rajbanshi and Gurkha population. However, Rabha is distantly related to Rajbanshi population though evidences suggest their emergence from the same East-Asian lineage. Genetic distances between Rajbanshi–Gurkha and Rajbanshi–Muslim are very much smaller than that of Rajbanshi–Rabha populations.

Interpretations & conclusions: Therefore, it may be concluded that Rajbanshi, Gurkha and Muslims are very much mixed populations and have genetic closeness due to exposure to similar environmental conditions. On the other hand, the Rabhas strictly follow the endogamy and are restricted to a particular region and therefore maintain considerable distances with the other three populations. The data showed some interesting observations which deviate the contemporary thought in respect to the population genetics.

1. Introduction

Human populations around the world are not only extremely diverse but also show wide adaptability to their respective local environment. Such kind of adaptation is needed not only due

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to difference in their food habits but also due to the development of tolerance against the microbial world around us [12]. Genetic markers play a very important role in the study of the gene-disease and gene-environment interactions. Balancing selection is a major factor which shapes the innate immune system. Furthermore, genes of the immune system are under constant evolutionary pressures [5]. The immune-related genes keep on changing during the evolutionary process because of their continuous encounter with the environmental antigens thereby influencing disease susceptibility [1,15]. This study proves how the environmental pathogens influence the modification or change of different markers under different conditions. TLRs are among those markers which can specifically recognize the conserved molecular patterns like Pathogen Associated Molecular Patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [3]. Genetic variations in TLRs may induce or inhibit the susceptibility of some diseases. It was also proved that strong pressures exerted by infectious diseases like plague may influence the convergent evolution of some of the TLRs in some human populations (European and Rroma) in recent times [13]. The present investigation has been aimed to study the frequency pattern of ten TLR genes in Rajbanshi population in North Bengal Region of India. Rajbanshi population is an ethnic caste group found in North Bengal and neighboring areas [11]. Koch and Rajbanshis are actually two different tribes but united by the great king into one and named as Koch-Rajbanshi [11]. They are the inhabitants of Jalpaiguri and Cochbehar districts of North Bengal. Previous studies on HLA and KIR genes revealed the influence of East Asian lineages on the Rajbanshis [14,2,9]. Their main dialect is Bengali in this region and they are mainly Hindu in their religion. However, recently this population has mixed with the Indo-Aryan and with the Dravidians lineages [4]. Herein, the frequency and distribution of 10 TLR genes in the ethnic Rajbanshi population have been studied to find out how the local environmental pressure/selection shapes the TLRs profile of a population.

2. Materials and methods

2.1. Study design

Blood samples were collected (n = 85) from the region of Cooch Behar, Jalpaiguri and adjacent areas of Siliguri where they inhabit mostly (26° 20′–27° 03′ N and 88° 18′–89° 29′ E). The samples were collected on the basis of their ethnicity, caste and health conditions. Individuals having three generations of common pedigree were excluded from the analyses. All the donors were informed regarding the purpose of the study before the collection of the blood samples and written consents were provided by the volunteers. The investigation was approved by the Human ethics committee of the Department of Zoology, University of North Bengal (Zoo/4133/2011).

Blood sample (3 ml) was taken from each volunteer by vein puncture method under the guidance of a medical practitioner and was stored in EDTA containing vials at −20 °C until use. Genomic DNA was extracted from the samples by the standard Phenol–Chloroform extraction method with slight modifications. This was followed by PCR-SSP typing for all the 10 TLRs (Table 1). Primers were designed based on the conserved regions of the 10 different TLR genes using NCBI BLAST http://www.ncbi.nlm.nih.gov/tools/primer-blast/. Primers were supplied by the Integrated DNA Technologies, Inc, Iowa, USA.

2.2. Statistical analysis

Neighbor-joining tree and Nei's genetic distances have been constructed using POPGENE (ver-1.32) and Phylip (ver-3.5). Chi-square calculation was done using Kyplot (ver-2.0). Principal component analysis (PCA) was done using Sigma plot (ver-13).

3. Results

3.1. Frequency calculation

Observed frequencies of 10 TLR genes in Rajbanshi population as well as in other three populations were calculated. It has been observed that in Rajbanshi population, the frequency of TLR8 (0.894) was highest followed by TLR6 (0.882) and TLR9 (0.882) respectively (Fig. 1 and Table 2). When Rajbanshi population was compared with Gurkha, Muslim and Rabha populations of this region, (Communicated elsewhere) it was found that the frequency of TLR8 was highest among the Rajbanshi population. However, the frequency of TLR4 was highest in Gurkha and Rabha populations. Previously TLR1 to TLR5 genes were screened among the Rabha population [10] where it was found that the frequency of TLR4 was calculated as highest and TLR5 was in least frequency. On the other hand, TLR5 was highest among Muslims (Communicated elswhere). Interestingly, the frequency of TLR2 was very low among all the four populations.

3.2. Chi-square analysis

When Chi-square analysis was performed among the four populations, it was found that there are no significant differences between Rajbanshi and Gurkha when compared with the other two populations. There were no significant differences found for TLR1 and TLR9 when compared between the Rajbanshis and other three populations.

3.3. Genetic distance and PCA analysis

Genetic distance based Neighbor-joining dendrogram was constructed and interestingly, it was found that Rajbanshi, Gurkha and Muslim occupied the same cluster wherein Gurkha and Muslim population were grouped together, while the Rabhas occupied a separate cluster (Fig. 2). It was assessed from the principal component analysis (PCA) that Rajbanshi and Rabha populations are quite distantly placed in the plot whereas Muslim and Gurkha are very close to each other (Fig. 3). Nei's genetic distance was calculated by comparing Rajbanshi with three other populations and it has been found that the distance between Rajbanshi and Rabha was 0.0745, between Rajbanshi and Gurkha was 0.0685 and that between Rajbanshi–Muslim was 0.0694 (Table 3).

Table 1 List of forward and reverse primers for the 10 TLRs in human.

Genes	Forward primers (5′–3′)	Reverse primers (3′–5′)	Product size (bp)	GC content (%)	Tm
TLR1	TCAACCAGGAATTGGAATAC	AGTTCCAGATTTGCTACAGT	382	40	52
TLR2	GGATGGTTGTGCTTTTAAGTACTG	AAGATCCCAACTAGACAAAGACTG	2671	41.67	55.5
TLR3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	792	44/40	57
TLR4	TTCTTCTAACTTCCTCTCCTGTG	TTAGCTGTTCGGCTCTACTATGG	1087	43/47	58.6
TLR5	CATTGTATGCACTGTCACTC	CCACCACCATGATGAGAGCA	446	45/55	57.6
TLR6	ACAACCCTTTAGGATAGCCACTG	AAACTCACAATAGGATGGCAGG	398	47.83	56.9
TLR7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG	545	44	50.5
TLR8	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG	637	45.83	58.4
TLR9	TCTAGGGGCTGAATGTGACC	ACAACCCGTCACTGTTGCTT	1106	55	59.8
TLR10	GTCGAAGACCCAATATACAG	ATTAAGCAATAGAACCGATG	954	45/35	52
Growth hormone (positive control)	CTTCCCAACCATTCCCTTA	CGGATTTCTGTTGTGTTTC	424	47/42	50.3

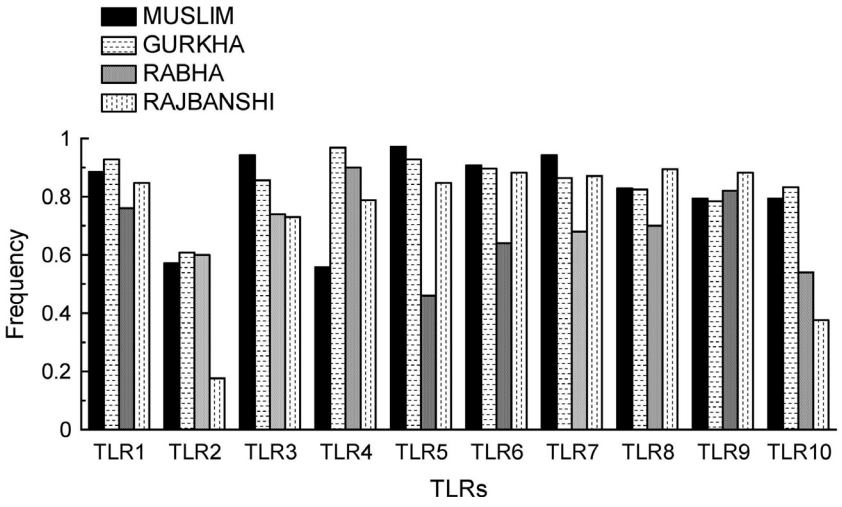

Figure 1 Frequency graph of ten TLR genes was constructed using Kyplot (ver-2.0) of the four populations in the North Bengal region.

Table 2 Observed frequencies of the 10 TLR genes in the four populations. χ^2 values were also mentioned where each gene was compared between two populations for any statistical differences.

	Rajbanshi(RA)	Gurkha(G)	Rabha(R)	Muslim	RAXG	RAXM	RAXR	MXG	GXR	RXM
TLR1	0.847	0.928	0.760	0.886	2.723	0.401	1.056	0.9314	8.020**	3.687
TLR2	0.176	0.608	0.600	0.571	36.63***	32.22***	23.54***	0.229	0.005	0.034
TLR3	0.729	0.856	0.740	0.943	4.385*	18.52***	0.004	4.691*	2.549	13.427***
TLR4	0.788	0.968	0.900	0.557	15.569***	11.33***	2.047	57.426***	2.134	17.499***
TLR5	0.847	0.928	0.460	0.971	2.723	9.99**	20.80***	1.820	45.051***	66.880***
TLR6	0.882	0.896	0.640	0.907	0.007	0.135	9.822**	0.009	14.349***	17.349
TLR7	0.870	0.864	0.680	0.943	0.004	2.700	6.005*	3.927*	6.745**	20.745***
TLR8	0.894	0.824	0.700	0.829	1.458	1.330	6.841**	0.0041	2.591	2.986
TLR9	0.882	0.784	0.820	0.793	2.728	2.364	0.562	0.0006	0.1057	0.0424
TLR10	0.376	0.832	0.540	0.793	44.031***	37.81***	2.789	0.4299	14.6650***	10.612**

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

4. Discussion

Modern Indian populations have originated from two ancestral populations: on one hand "Ancestral North Indians" who are genetically close to Middle Eastern, central Asians and Europeans while on the other hand, "Ancestral South Indians" who have shown proximity to East-Asians lineage [16]. Rajbanshi populations are the indigenous ethnic caste population of Eastern Terai and can also be found in Assam, Bengal and Bihar states of India [11]. Even today, most of the Rajbanshis are found to inhabit in Assam, Meghalaya, Tripura, Nagaland, and Manipur also [18]. Herein we have made an attempt to unveil the TLR profile of this population as it one of the most widely distributed scheduled caste population of Eastern and North Eastern India.

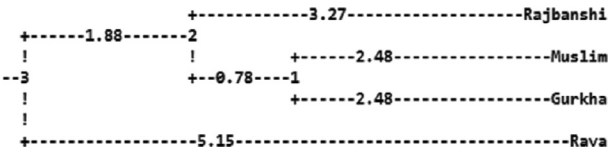

Figure 2 Neighbour joining tree was constructed using POPGENE (ver-1.32) and Phylip (ver-3.5) showing relationship among Rajbanshi and three other populations.

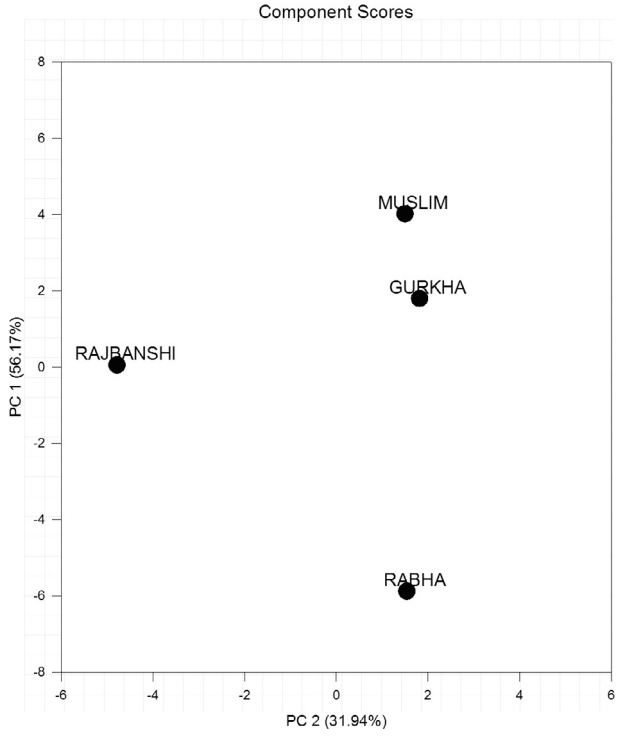

Figure 3 Principal component analyses (PCA) based on observed frequencies of the 10 TLR genes in the four ethnic populations of North Bengal constructed with Sigma plot (ver-13).

Table 3 Nei's genetic distances among the Rajbanshi population using POPGENE (Ver-1.32) software.

POP ID	Rajbanshi	Rabha	Muslim	Gurkha
Rajbanshi	**0.000**			
Rabha	0.0745	**0.000**		
Muslim	0.0694	0.1557	**0.000**	
Gurkha	0.0685	0.0973	0.0526	**0.000**

The bold values $\leq$0.05 signified the similarities of the same populations.

Earlier researches have shown that environmental pathogens have modified the TLR profiles in individuals affected by various diseases like asthma and tuberculosis [19,8]. The selection pressure has also modified the TLR gene pool in various populations in the world [13]. Recent studies have revealed that populations of different genetic ancestry have shown convergent evolution with respect to some of the TLR genes due to the interactions with some infectious diseases like plague in a particular environmental condition [13]. Sometimes polymorphism in the TLRs may also become susceptible or resistant to certain diseases [1,15]. In our previous study, we found that chronic gastritis and associated stomach problems were common in Rabha population and therefore we speculated that there is a strong relationship between KIR and TLR in disease progression [10]. The genetic heterogeneity among the Indian populations have put forward an immense challenge before the researchers of different fields [20]. Subsequently, the strict endogamy practices in the populations of North Bengal along with the evolutionary forces have resulted in higher differences in allele frequencies between the groups, which have remained intact for thousands of years [7,17].

Recently we have screened 10 TLR genes in the ethnic Rajbanshi population of North Bengal and compared with the other three populations of this region. It is interesting to find that the genetic distance between Rajbanshi and Rabha is greater than the Rajbanshi–Gurkha and Rajbanshi–Muslim respectively. But, as per anthropological and genetic records, East Asian lineages have strong influence on the genetic ancestry of Rajbanshi, Rabha and Gurkha respectively [6,9,10] and Muslim population of this region showed their proximity with indigenous non Muslim population along with a small frequency of the Middle East ancestry [20,7]. It was found that the Muslims, the Gurkhas and the Rajbanshis are the residents of the same locality and are therefore exposed to similar environmental effects. On the other hand, the endogamous tribal Rabha population is very much confined in their local environment. Thus the genetic closeness of Rajbanshis with Muslims and distant relationship with Rabha indicated that similar environmental pressure may be responsible for the convergent evolution and selection of the TLR gene pool among the populations inspite of their different genetic ancestry.

The principal component analyses also documented the distant positions of Rabha and Rajbanshi in the score plot while Muslim and Gurkha were close to each other. Researchers all over the world focussed their work on correlating the associations of KIR with various diseases worldwide but concrete study with respect to TLR diversities, distributions and frequencies in different populations of the World are needed on the populations especially in the region of North Bengal where various ethnic endogamous populations are residing. This was also supported by Neighbor-joining dendrogram which suggested the closeness of the Rajbanshis with Gurkhas and Muslims while showing distant relation with the Rabhas. Convergent evolution has occurred in TLR genes among the above-mentioned populations due to the sharing of similar

environmental conditions. It is quite interesting to observe that although the Rajbanshi, Gurkha and Rabha populations have shared ancestry due to their emergence from a common East-Asian stock, there is no similarity in the distribution of TLR genes among them as has been recorded in our present study. However, there exist considerable similarities in the distribution of TLR genes between the Muslim and the Gurkha population who share the same environment but differ considerably in their ethnicity. This striking observation may depict the impact of environmental selection on the distribution of TLR genes. Such influences of the environment on TLR distribution may depend on the constant presence of specific pathogens present in respective environment. Thus, it may be assumed that TLR genes play a significant role in shaping the genetic ancestry of the above mentioned populations from North Bengal region of India as well as in determining disease exposure in these populations.

5. Conclusion

It is also sensible to say that this work is a pioneer work done in the above-mentioned populations on TLRs and diversity study which must be needed around the world on different populations. Such works on TLRs diversity in different populations are very much essential to understand the disease susceptibility and the phenomenon by which local environment creates pressure to change the frequencies and genetic makeup of TLRs.

Authors' contributions

Avishek Das performed the experiments and analyzes the data. Pokhraj Guha analyzed the data. Tapas Kumar Chaudhuri designed the study and analyzed the data.

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Original article

Role of toll like receptors in bacterial and viral diseases – A systemic approach

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ABSTRACT

Background: Toll like receptors are key-receptors of the innate immune system, but their role against bacterial and viral infections are yet to be understood.
Aim: The present study is aimed to investigate the diversity and frequency distribution of 10 TLR genes among typhoid fever and HIV+ patients. In this study, 44 samples were taken from typhoid patients and 55 samples from HIV+ patients.
Patients and methods: Widal test positive samples (>1:80) in case of typhoid and the percentage of CD4+ count in case of HIV+ patient were considered for the PCR-SSP analysis.
Results: We found that the frequencies of TLR1 and TLR6 were highest in typhoid patients, whereas the frequencies of TLR8 and TLR9 displayed higher among HIV+ patients. Chi-square values were significant for TLR8 and TLR10 in the case of typhoid patients, whereas in HIV patients significant values were considered for TLR2, TLR4, TLR8 and TLR9 respectively. The odds ratio calculated highest for TLR1 and TLR6 among typhoid patients. TLR4 and TLR9 calculated were highest odd for HIV+ patients. A door line association of TLRs with the disease was found when the relative risk was calculated for TLR2 (1.72), TLR3 (1.21) and TLR10 (1.98) in bacterial infection, whereas in case of viral infection relative risk was calculated for TLR4 (1.62), TLR8 (1.18) and in TLR9 (1.16).
Conclusion: This study reports the frequency distribution and association of human TLR genes with the bacterial and viral infection in the North Bengal region of India for the first time. It also signified the gene- disease- environment association study in case of infectious diseases and also the risk factors of bacterial and viral infections in this region. It also depicts the role of TLRs in the recognition of the pathogens.

1. Introduction

Free-living organisms have the ability to cope up with the new environment by modifying their gene expression patterns [1]. Extensive variations at the genomic level made the analyses of gene-disease association and their susceptibility possible [2]. The frequency of genes and their alleles vary between different populations in case of different diseases [3]. Till date, slow progress has been observed in the field of genome-wide association studies for the infectious disease in comparison to other diseases. However, some studies involving bacterial and viral diseases contribute substantially to the growing knowledge of the host genetic variations and treatment of the diseases [4,1]. Toll like receptors that regulate both innate and adaptive immune response and polymorphism in the TLR genes has been investigated in case of various diseases [5].

Enteric fever has become an alarming infection nowadays among populations in some areas of India [6]. *Salmonella enterica* serotype *typhi* (S. typhi) is a gram-negative bacterium, restricted in human and cause a wide range of food- and water-borne diseases ranging from self-limiting gastroenteritis to systemic typhoid fever [7]. The occurrence of typhoid fever is less in developing and industrialized countries, but high in the countries of South-East Asia including India [6]. According to Crump et al. (2004) typhoid fever caused over 20 million illnesses and over 200 thousand deaths during the year 2000 [8]. Poor sanitation, lack of safe drinking water supply and low socioeconomic conditions

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has amplified the disease in India, which increases the morbidity and mortality [9].

Primarily, TLR4 and TLR5 play major roles in the activation of immune responses against LPS and flagellin. TLR4 polymorphisms among the Asian Malay population confer a higher risk for typhoid infection in case of *S. typhi* [10]. Genetic association study among the Vietnam population has proven no association of TLR5392STOP stop codon with typhoid fever [11]. Binding site modulation of TLR gene receptors against the lipopolysaccharide (LPS), flagellin or other antigens of *Salmonella typhi* evokes the host immune response during typhoid fever [12].

On the other side susceptibility to the human immunodeficiency virus (HIV) infection and disease progression are variable among populations [13]. A small percentage of 0.2% of the HIV-1 sero-positive patient is able to control the HIV-1 infection over 10 years. The adult HIV prevalence at national level has 0.26% in 2015 [14]. It means that they can maintain a viral load of fewer than 50 copies of HIV-1 RNA per ml [15] more of HIV-1 RNA will accelerate the prevalence of the disease. Infection with human immunodeficiency virus (HIV) results in progressive deterioration of the immune system in untreated patients [16]. Different TLRs expressed on different cell types in the human immune system and up-regulated by the effect of cytokines like IFN-y induces the expression of TLR4 in peripheral blood monocytes [17].

The HIV disease progression can be estimated by measuring marker expression in the course of the disease. The degree of CD4+ T-cell depletion is the most important marker for the detection of HIV [18]. Indeed, the most characteristic feature of HIV is the depletion of the CD4+ T-helper-inducer subset of T cells. The other markers that are also reliable for estimating HIV disease progression include b2m, neopterin, IgG, IgM, anti-p24, anti-gp120, TNF etc. [18].

Several association studies have been reported in case of TLRs with HIV. It has been reported that depletion of CD4+(Th2) cells in HIV positive individuals releases bacterial components that directly activates TLR4 [19]. According to Baenziger et al. (2009), the chronic activation of TLR7 leads to immune dysregulation in murine model which is similar to human [20]. Several other TLRs are also associated with HIV disease progression.

2. Subjects and methods

2.1. Selection of patients for typhoid fever

Typhoid patients were diagnosed by expert doctors of North Bengal Medical College and Hospital, Shushrutnagar, Siliguri (latitude & longitude 26.7271°N, 88.3953°E) on the basis of specific symptoms of typhoid fever. Screening of the typhoid patients were based on the positive results of the Widal test [21]. The serum agglutination test was done against S. typhi "O" and "H" antigens using a Salmonella antigen kit (Beacon diagnostic Pvt. Ltd, Navsari India). The test was performed according to the manufacturer's instruction. The serum antibody titer of 1:80 or above was considered positive for the typhoid fever.

2.2. Selection of patients for human immunodeficiency virus

Fifty-five HIV-infected patients (including 33 women, 22 men and, median age-34) and 70 healthy individuals (47 women, 23 men, a range of 20–52) were included in this study (Table 1). Individuals under any sorts of medication were excluded from the control group (n = 70) in our study. Positive HIV patients were selected based on the viral infection and counting of CD4+ cells within the range of 156–756 $\times$ 10^6 cells/L. Laboratory values for patients who did not receive anti retroviral therapy (ART) had 117–730 $\times$ 10^6 cells/L CD4+ cells per litre, but CD4+ count became 142–890 $\times$ 10^6 cells/L after receiving ART.

Table 1
Demographic characteristics of Typhoid fever patients and HIV+ patients and Healthy donors.

Sex	Typhoid patients	Healthy Donors
Male	18 (40%)	27 (38%)
Female	26 (60%)	43 (62%)
Total	**44**	**70**
Sex	HIV+ patients	Healthy Donors
Male	22(40%)	23(32%)
Female	33(60%)	47(67%)
Total	**55**	**70**

HIV-Human Immunodeficiency Virus.

2.3. Sample collection

3 mL of venous blood was collected from Forty-four typhoid patients between December 2014 to June 2016 from Siliguri and adjoining areas of West Bengal and Seventy healthy control subjects were taken after screening by the doctors. A detailed clinical report was taken from the patients who were admitted to the hospitals and primary health care of Siliguri and adjacent areas with gastro-intestinal problems.

The demographic characteristics of Fifty-five HIV+ patient and seventy healthy donors are represented in Table 1. 5 mL of blood samples were collected from each individual who attended the District Hospital with prior informed consent. Simultaneously, samples were collected from healthy donors after proper examination by the doctors.

The samples were stored in EDTA at −20 °C until use. The investigation was approved by the Human ethics committee of the Department of Zoology, University of North Bengal (Zoo/4133/2011) and performed in accordance with the Declaration of Helsinki, 1975.

2.4. DNA extraction and PCR-SSP typing

Genomic DNA was extracted from the blood samples using the standard Phenol-Chloroform extraction method with slight modifications. DNA integrity was checked in UV-transilluminator. O.D value was taken with 260/280 nm. Value of 1 or above was found as good quality of the DNA (Fig 1a). PCR-SSP typing was done for all the 10 TLRs. The TLR primers were designed using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 2) [22] and procured from the Integrated DNA Technologies, Inc, Iowa, USA. Each 25 μl PCR reaction mixture contained 5X PCR buffer (Promega Corporation, Agora, Fitchburg Center, Fitchburg, Wisconsin), 5 μL of 10 mM dNTPs, 1.5 μL of 25 mM MgCl2, 1.5 μL of primers, and 1–1.5 U of Taq DNA polymerase. 1.5–2 μl of 100 ng DNA samples were then added to the PCR mixture. The reaction conditions for PCR consisted of an initial denaturation step of 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 56.9 °C for 50 s and 72 °C for 1 min, followed by a single final extension of 72 °C for10 min. Slight modifications in the annealing temperatures of different primer sets were made as per the requirement. The PCR products were analyzed using ethidium bromide prestained 1% agarose gel electrophoresis. Samples were then visualized on UV transilluminator. All the lanes of the product loaded gel showed a control band, except for the negative control lane. The reactions were repeated to avoid false reactions where no control bands were found (Fig 1b).

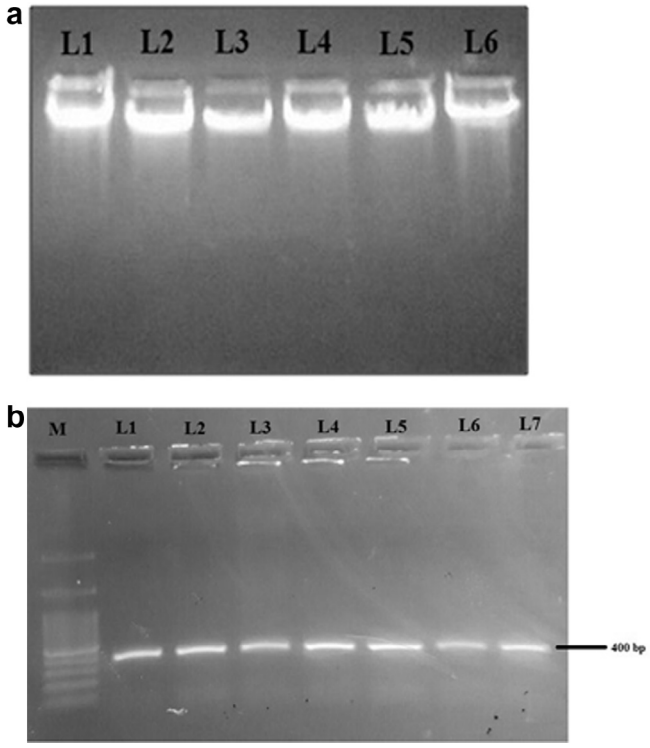

Fig. 1. a-Agarose gel electrophoresis showing results of DNA isolated from typhoid patient's blood samples (L1-L6). b-Agarose gel electrophoresis results showing PCR amplification of TLR5 in HIV+ samples (M-100 bp DNA ladder, L1-L7 positive samples).

3. Statistical analyses

All statistical data were analyzed using SPSS (Ver-16.0) (Armonk, New York, USA), Kyplot (ver-2.0) and MS-Excel programme (Redmond, Washington, USA). Statistical significances were determined using the chi-square test or the Fisher's exact test with p-value <0.05 being considered significant.

4. Results

4.1. Analysis of typhoid patients

Observed frequencies of ten TLR genes from 44 typhoid patients are represented in Table 3a. It has been observed from the table that in case of typhoid patients, both TLR1 and TLR6 have the highest frequency of 0.977, which were followed by TLR 4 and TLR5, having the frequency of 0.909 and 0.931 respectively (Table 3) (Fig 2a). Chi-square analyses (χ^2) were performed to compare the differences in carrier frequencies (OF) of TLR genes among the patients and control samples. Among the 10 TLR loci, significant differences are observed only in case of TLR8 and TLR10 (Table 3). ANOVA was also performed for study the significant differences among control and patient groups which was found 0.0007 ($p < 0.001$) and in case of two-tailed *t*-test, the value was 0.521 for patient and control group.

Fischer's exact test for probability showed significant association for TLR8 (p = 0.022, <0.05) and TLR10 (p = 0.0005, <0.001). When the odd ratio and 95% confidence interval [23–25] for ten different TLRs in typhoid patients were calculated, it has been documented that TLR2 (odd- 2.02, CI- 0.82–4.97, p- 0.12), TLR4 (odd- 2.5, CI- 0.76–8.16, p- 0.12), and TLR5 (odd- 2.01, CI- 0.51–7.89, p- 0.31) showed high associations, whereas TLR7 (odd- 0.59, CI- 0.17–1.97, p- 0.39), TLR8 (odd- 0.26, CI- 0.08–0.82, p- **0.02**) and TLR9 (odd- 0.58, CI- 0.19–1.80, p- 0.35) showed lower association among the patients and control samples (Table 4). The relative risks for different TLRs were calculated. The relative risks for TLR7 (RR- 0.94, p- 0.41), TLR8 (RR- 0.83, p- 0.03) and TLR9 (RR- 0.93, p- 0.37) are very low, whereas door line association is found in case of TLR1 (1.10, p- **0.04**), TLR5 (1.06, p- 0.27) and TLR6 (1.08, p- 0.07). On the other hand a little bit of higher associations are observed in cases of TLR2 (1.72, p- 0.12), TLR3 (1.21, p- 0.03), TLR4 (1.13, p- 0.09) and TLR10 (1.98, p- 0.0004) (Table 4).

The disease prevalence was estimated using diagnostic tests based on Bayer's theorm. The sensitivity was found to be very high in case of TLR1 (97.73), TLR4 (90.91), TLR5 (93.18) and TLR6 (97.73) (Table 5). Low sensitivity was reported in cases of TLR2, TLR8, and TLR10 which signified the low prevalence of the disease in the patients.

4.2. Analysis of HIV patients

Observed frequency data of ten different TLR genes from 55 HIV positive patients were analyzed. It is observed that the gene frequency of TLR8 (0.809) and TLR9 (0.865) are very high (Table 6) (Fig 2b). Chi-square analyses (χ^2) were performed to compare the differences in carrier frequencies (OF) of TLR genes among the patients and control samples. Significant differences are found in case of TLR2, TLR4, TLR8 and TLR9. No significant differences have been observed among the other TLRs (Table 6). ANOVA was also performed for significant difference among the control and patient groups which was 0.04 ($p < 0.05$) and in case of two-tailed *t*-test, the value was 0.93 for patient and control group.

Fischer's exact test for probability showed significant association for TLR4 (p = 0.00001, >0.001) and TLR8 (p = 0.01, <0.01) and TLR9 (p = 0.01, <0.01). When odd ratio and 95% confidence interval [22–24] for ten different TLRs in HIV patients were calculated, high associations are observed in case of TLR4 (odd- 9.56, CI-3.11–29.37, p- < 0.0001), TLR8 (odd-6.04, CI- 1.30–28.05, p- 0.007), and TLR9 (odd- 10.06, CI- 1.25–80.60, p- 0.005), whereas TLR2 (odd- 0.07,

Table 2
List of primers for the 10 TLR alleles in human.

Genes	Forward Primers (5′-3′)	Reverse Primers (3′-5′)	Product size(bp)	GC content (%)
TLR1	TCAACCAGGAATTGGAATAC	AGTTCCAGATTTGCTACAGT	382	40
TLR2	GGATGGTTGTGCTTTTAAGTACTG	AAGATCCCAACTAGACAAAGACTG	2671	41.67
TLR3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	792	44/40
TLR4	TTCTTCTAACTTCCTCTCCTGTG	TTAGCTGTTCGGCTCTACTATGG	1087	43/47
TLR5	CATTGTATGCACTGTCACTC	CCACCACCATGATGAGAGCA	446	45/55
TLR6	ACAACCCTTTAGGATAGCCACTG	AAACTCACAATAGGATGGCAGG	398	47.83
TLR7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG	545	44
TLR8	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG	637	45.83
TLR9	TCTAGGGGCTGAATGTGACC	ACAACCCGTCACTGTTGCTT	1106	55
TLR10	GTCGAAGACCCAATATACAG	ATTAAGCAATAGAACCGATG	954	45/35
Growth Hormone (Positive control)	CTTCCCAACCATTCCCTTA	CGGATTTCTGTTGTGTTTC	424	47/42

Table 3
Observed frequencies of the 10 TLR genes in the control and typhoid patients. χ^2 values were also mentioned where each gene was compared with controls and patients for any statistical differences.

Patients controls			χ^2	Relative risk	p value
TLR1	**0.977**	0.885	1.982	1.10	0.04
TLR2	0.295	0.171	1.757	1.72	0.12
TLR3	0.886	0.728	3.153	1.21	0.03
TLR4	0.909	0.8	1.667	1.13	0.09
TLR5	**0.931**	0.871	0.503	1.06	0.27
TLR6	**0.977**	0.9	1.429	1.08	0.07
TLR7	0.863	0.914	0.296	0.94	0.41
TLR8	0.772	0.928	4.459*	0.83	0.03
TLR9	0.840	0.9	0.413	0.93	0.37
TLR10	0.681	0.342	11.128***	1.98	0.0004

*P < 0.05, **P < 0.01, ***P < 0.001.

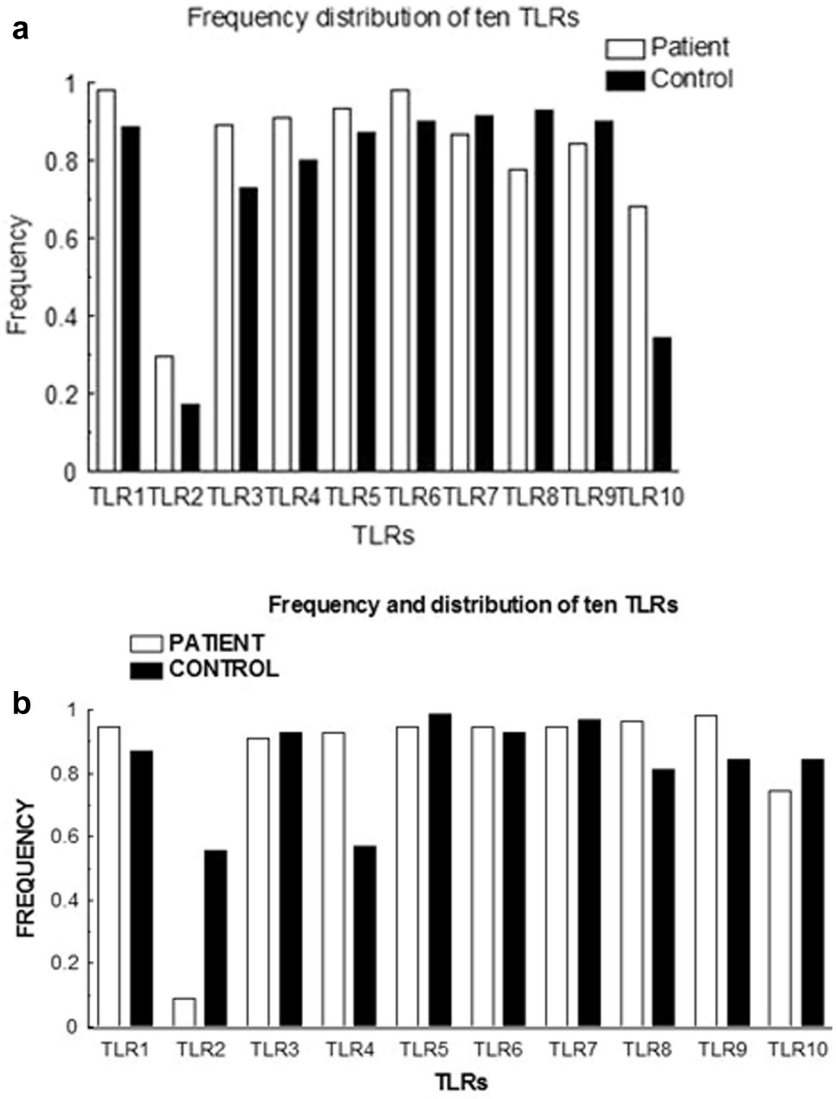

Fig. 2. a-Frequency graph of ten TLR genes was constructed using Kyplot (ver-2.0) of typhoid patients. b-Frequency graph of ten TLR genes was constructed using Kyplot (ver-2.0) of HIV positive patients.

CI- 0.02–0.22, p- < 0.0001), TLR5 (odd- 0.25, CI- 0.02–2.48, p- 0.23) and TLR7 (odd- 0.50, CI- 0.08–3.16, p- 0.47) showed lower association with the disease (Table 7). The relative risks for different TLRs were calculated (Table 6). The relative risks for TLR2 (RR- 0.16), TLR5 (RR- 0.95) and TLR10 (RR- 0.88) are found to be very low, whereas door line associations has been found in case of TLR4 (1.62, p- < 0.0001), TLR8 (1.18, p- 0.007) and TLR9 (1.16, p- 0.005).

The prevalence of the disease in the patients was estimated diagnostically using Bayer's theorem. The sensitivity values are

Table 4
Risk ratio and odd ratio for ten different TLRs in association with typhoid fever.

	Risk ratio	Odd ratio	Confidence intervals
TLR1	1.10	**5.54**	0.66–45
TLR2	1.72	2.02	0.82–4.97
TLR3	1.21	2.90	0.99–8.46
TLR4	1.13	2.5	0.76–8.16
TLR5	1.06	2.01	0.51–7.89
TLR6	1.08	**4.77**	0.56–40
TLR7	0.94	0.59	0.17–1.97
TLR8	0.83	0.26	0.08–0.82
TLR9	0.93	0.58	0.19–1.80
TLR10	1.98	**4.10**	1.83–9.17

Table 5
Diagnostic test values for typhoid patients based on bayer's theorm.

	Sensitivity	Specificity	PPV	NPV
TLR1	97.73	11.43	40.95	88.89
TLR2	29.55	82.86	52.00	65.17
TLR3	88.64	27.14	43.33	79.17
TLR4	90.91	20.00	41.67	77.78
TLR5	93.18	12.86	40.20	75.00
TLR6	97.73	10.00	40.57	87.50
TLR7	86.36	8.57	37.20	50.00
TLR8	77.27	7.14	34.34	33.33
TLR9	84.09	10.00	37.00	50.00
TLR10	68.18	65.71	55.56	76.67

PPV-Positive predicted value, NPV-Negative predicted values.

Table 6
Observed frequencies of the 10 Human TLR genes in the control and HIV+ patients. χ^2 values were also mentioned where each gene was compared with controls and patients for any statistical differences and measurement of relative risk.

	Patients	Controls	χ^2	Relative risk	P value
TLR1	0.766	0.641	1.185	1.08	0.14
TLR2	0.046	0.334	27.343***	0.16	<0.0001
TLR3	0.698	0.732	0.004	0.97	0.69
TLR4	0.730	0.345	17.939***	**1.62**	<0.0001
TLR5	0.766	0.880	0.573	0.95	0.23
TLR6	0.766	0.732	0.0002	1.01	0.69
TLR7	0.766	0.830	0.076	0.97	0.47
TLR8	0.809	0.569	5.168*	**1.18**	**0.007**
TLR9	0.865	0.603	5.345*	**1.16**	**0.005**
TLR10	0.495	0.603	1.268	0.88	0.19

*P < 0.05, **P < 0.01, ***P < 0.001.

Table 7
Risk ratio and odd ratio for ten different TLRs in association with HIV patient.

	Odd Ratio	Confidence interval	Risk ratio	P value
TLR1	2.55	0.65–9.94	1.0849	0.22
TLR2	0.07	0.02–0.22	0.1632	5.22
TLR3	0.76	0.21–2.80	0.979	0.74
TLR4	**9.56**	3.11–29.37	**1.6227**	0.00001
TLR5	0.25	0.02–2.48	0.9592	0.31
TLR6	1.33	0.30–5.84	1.0182	0.73
TLR7	0.50	0.08–3.16	0.9733	0.65
TLR8	**6.04**	1.30–28.05	**1.1834**	0.01
TLR9	**10.06**	1.25–80.60	**1.1649**	0.01
TLR10	0.54	0.22–1.32	0.8844	0.25

found to be very high in cases of TLR8 (96.36), andTLR9 (98.18); however similar sensitivity values have been reported in cases of TLR1, TLR5, TLR6 and TLR7 (Table 8). On the other hand, low specificity values were found in case of TLR5 (1.43), TLR7 (2.86) and little bit of higher specificity in case TLR2 (44.29) are found.

Table 8
Diagnostic test values for HIV patients based on bayer's theorm.

	Sensitivity	Specificity	PPV	NPV
TLR1	94.55	12.86	46.02	75.00
TLR2	**9.09**	44.29	11.36	38.27
TLR3	90.91	7.14	43.48	50.00
TLR4	92.73	42.86	56.04	88.24
TLR5	94.55	1.43	42.98	25.00
TLR6	94.55	7.14	44.44	62.50
TLR7	94.55	2.86	43.33	40.00
TLR8	**96.36**	18.57	48.18	86.67
TLR9	**98.18**	15.71	47.79	91.67
TLR10	74.55	15.71	41.00	44.00

PPV-Positive predicted value, NPV-Negative predicted value.

5. Discussion

Bacteria and viruses have the peculiar ability to overcome species barriers and can adapt in new hosts. This concept helps us to understand the underlying mysteries behind the origin and emergence of infectious diseases. The TLR based genetic analysis in Typhoid and HIV patients may serve as a powerful model for studying mechanisms of host adaptation, because the pathogens responsible for these diseases are physiologically well characterized and lend themselves to genetic analysis in different populations in the world [5,26].

Typhoid is a major human enteric fever caused by bacterial infection in India. Although not common in urbanized countries, but the disease remains an important and persistent health problem in developing nations like India. Hospital-based surveys and reports from different parts of the country indicate that enteric fever is a major public health problem, with *Salmonella enterica* serovar t*yphi* (*S. typhi*) being the most common pathogenic agent [9]. Various risk factors such as sanitation problems, lack of safe drinking water supply and low socio-economic conditions amplify the rate of evolution of multidrug-resistant salmonellae with reduced sensitivity to different drugs have been reported in India [9,1].

The role of TLRs in typhoid fever patients has not been extensively studied in India, especially in the northern part of West Bengal where the health problems become the major issues among the tea garden workers. Some studies have documented the association of the TLRs with typhoid fever in India [12,27]. An association based study among the Malay population on TLR4 polymorphism confers a higher risk factor for typhoid infection [10]. According to Dunstan et al. (2005) premature stop codon of TLR5 polymorphism suggested no association with typhoid fever caused by *S. typhi*. TLR5 might not play an important role in TLR-stimulated innate immune responses during infection with *Salmonella enterica serovar typhi*. Initiation of these responses may rely on other TLRs that recognize different bacterial ligands [11].

In case of HIV+ patients, it was reported that polymorphism in TLR3 (Leu412Phe) has a protective role against the disease [28]. Two variants of TLR4 (Asp299Gly, Thr399Ile) which recognizes lipopolysaccharide (LPS) as their ligand are associated with increased infection risk in HIV+ patients [29]. According to Martinelli et al. (2007) pDCs, which normally secretes the IFN-gamma and activates the natural killer cell and also suppressed due to the presence of gp120 viral envelope of HIV virus? The viral envelope protein also inhibits the TLR9 mediated induction of proinflammatory cytokines in pDCs [30]. Thus the presence of the different types of polymorphic variants of TLR genes having susceptibility to HIV susceptibility or diseases depends on the ethnicity of different populations of the world [31].

The frequency and distribution patterns of ten TLR genes were analyzed and compared in case of typhoid fever and HIV+ patients of Siliguri and adjacent areas. It has been observed that the frequencies of some of the TLRs like TLR1, TLR4, TLR5 and TLR6 are very high when compared with healthy controls in case of typhoid patients, whereas the frequencies of TLR8 and TLR9 are the highest in HIV positive patients. These findings are in agreement with the previously reported work [30,32]. LPS and flagellin produced by the Salmonella elevates the frequency of TLR4 and TLR5 in macrophages and also in intestinal epithelium cells. In the contrary, the frequencies of TLR8 and TLR9 are higher in HIV-positive patients. In course of HIV viral infection, small single-stranded RNA/CpG oligonucleotides activate TLR8 and TLR9which are mainly expressed in monocytes and macrophages.

Recognition of different antigens like vi-capsule, flagellin, LPS and others, activate the signaling pathways for the production of different cytokines in the human. The interaction between TLRs and Pathogen-associated molecular patterns (PAMPs) produced from the bacterial and viral antigens increase the formation of inflammosome and other inflammatory products. It brings the neutrophil and macrophages and induces the production of pro-inflammatory cytokines like interleukin (IL)-6, IL-1b, tumor necrosis factor (TNF)-a, and interferon-gamma (IFN)-c [33]. In case of HIV infection Th1 cytokines like interleukin (IL)-2, and antiviral interferon IFN-gamma are generally decreased and the production of Th2 cytokines such as IL-4, IL-10, proinflammatory cytokines and TNF-α, are increased [34].

Chi-square analysis reveals the significant values for different TLRs. Significant associations have been found in cases of TLR8 and TLR10 among the patients and the control samples of typhoid fever. In case of viral infection, significant values are found among TLR2, 4, 8 and TLR9. Positive associations with the typhoid fever are found for TLR1 and TLR6. Door line association has been found among the patients in comparison to their relative risk and risk ratio for the *S. typhi* infected patients. It signifies the positive relationship of the disease among typhoid patients in respect to their TLRs. Increased level of TLR1, 4, 5 and TLR6 expression in the cells prove that antigen from *S. typhi* highly increased the frequency pattern of those TLRs in course of the disease progression. Sensitivity test for TLR1, TLR5, and TLR6 are very high in typhoid positive patients which signify the prevalence of the disease in the population. The predictive values of any diagnostic test are related to its disease prediction ability. The Positive predicted values (PPV) are found to be very low in comparison to the negative predicted values (NPV).

Positive and close association of TLR4, TLR8, and TLR9 with HIV are documented in the present study, which strongly supports the previously published reports. Sensitivity test for TLR8 and TLR9 are also very high, which suggests that the disease is detected in most of the patients. The PPV values are low when compared with NPV. It also inferred from the data that the expressions of some of the TLRs are also very high in patients in course of the viral replication.

6. Conclusion

TLR regulates the innate immune response and plays a crucial role in the initiation of adaptive immunity in human populations. Recent trends in the fields of genetics highly focused on the role or association of TLRs in case of bacterial and viral diseases. So, the overall frequency and the distribution pattern of TLRs have been focused in the present study in case of bacterial and viral infections. Positive associations are found for cell surface receptors such as TLR1, TLR2, TLR4, and TLR6, which influence the progression and positive risk for the disease in typhoid patients. But, in case of viral infection, endosomal TLRs play a crucial role in resisting the disease. The present study is one of the major first-hand reports on the association of TLRs with bacterial and viral infections caused due to *S. typhi* and for human immunodeficiency virus in North Bengal region of India.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmhg.2017.05.001.

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RESEARCH ARTICLE

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A rapid and efficient DNA extraction protocol from fresh and frozen human blood samples

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Background: Different methods available for extraction of human genomic DNA suffer from one or more drawbacks including low yield, compromised quality, cost, time consumption, use of toxic organic solvents, and many more. Herein, we aimed to develop a method to extract DNA from 500 μL of fresh or frozen human blood.

Methods: Five hundred microliters of fresh and frozen human blood samples were used for standardization of the extraction procedure. Absorbance at 260 and 280 nm, respectively, (A_{260}/A_{280}) were estimated to check the quality and quantity of the extracted DNA sample. Qualitative assessment of the extracted DNA was checked by Polymerase Chain reaction and double digestion of the DNA sample.

Results: Our protocol resulted in average yield of 22±2.97 μg and 20.5±3.97 μg from 500 μL of fresh and frozen blood, respectively, which were comparable to many reference protocols and kits.

Conclusion: Besides yielding bulk amount of DNA, our protocol is rapid, economical, and avoids toxic organic solvents such as Phenol. Due to unaffected quality, the DNA is suitable for downstream applications. The protocol may also be useful for pursuing basic molecular researches in laboratories having limited funds.

KEYWORDS
cost effective, DNA extraction, high yield, human blood

1 | INTRODUCTION

The increasing demand of genome based analyses in modern evolutionary and disease researches have also increased the need for bulk amount of pure genomic DNA[1,2] which should also be free from protein and RNA contaminants. It is indeed the primary requirement of various molecular biological techniques such as Polymerase Chain Reaction (PCR), restriction enzyme analysis, mutation detection, genotyping, and linkage analysis as well as determination of genetic abnormalities, epigenetic studies, and various diagnostic and preventive tests.[1–4] Moreover, it would become much more research friendly if the DNA extraction method becomes rapid and cost effective.

DNA can be extracted from many biological samples such as hair, blood, semen, saliva, skin cells, and many more. Among these, the one that has gained astounding importance in biological researches is blood. Blood has become an integral part of biochemistry, hematology and clinical studies and forensic investigations. It serves as an important source of genomic DNA because of the presence of nucleated white blood cells.

Many protocols have been published regarding DNA isolation from blood.[4–14] Some of these published protocols applied enzymes and organic solvents for yielding high quality DNA, devoid of PCR inhibitors, while others including salting out procedure targeted toward higher DNA yields.[15,16] Thus some protocols are expensive and time consuming[17] while others compromised with the DNA quality.[18–20]

Therefore, in order to fulfill the demand of a rapid and cost effective procedure for obtaining high quality genomic DNA, hereby we have aimed to develop a protocol free from costly enzymes and toxic organic solvents for extracting pure DNA from fresh and frozen human blood samples.

TABLE 1 Optical density (OD) ratios and DNA yield (μg) per 500 μL of blood extracted by the three protocols respectively. ST1 and ST2 denote Standard Protocol 1 and 2 respectively. Both OD ratios and DNA yield were expressed as mean±SD

	Experimental protocol		ST1		ST2	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
OD_{260}/OD_{280} ratio	1.88±0.07	1.86±0.06	1.96±0.03	1.90±0.05	1.72±0.11	1.7±0.09
Yield (μg) per 500 mL	22±2.97	20.5±3.97	19.5±3.95	17±2.12	25.2±3.32	24±4.28

2 | MATERIALS AND METHODS

2.1 | Blood samples

Blood samples were collected in EDTA-containing vacutainer tubes from 20 healthy individuals randomly chosen from the localities in and around the campus of University of North Bengal, Siliguri, West Bengal, India. The volunteers provided their prior informed consent in becoming a part of this study. All the donors were interviewed and a questionnaire concerning their health conditions was completed with the assistance of medical staff to ensure that none of the volunteers has any prevailing disease conditions. The investigation was approved by the Human Ethics Committee of the Department of Zoology, University of North Bengal, India and was performed in accordance with the with the Helsinki Declaration of 1975.[21] Fresh blood samples were used for DNA extraction after 1 hour from the time of collection and for frozen blood sample, the extraction was done generally after 15-21 days from the date of collection. In case of the frozen blood, the samples were refrigerated at −20°C for future use. Blood may sometimes act as potential biohazard and therefore suitable care was taken during handling of the blood samples.

2.2 | Reagents and solutions

RBC lysis Buffer (RLB): 0.155 mol/L NH_4Cl, 10 mmol/L $KHCO_3$ and 0.1 mol/L EDTA (Na_2) in 1000 mL of distilled H_2O. The pH was adjusted to 7.6.

Extraction Buffer: 1.5 mol/L Tris pH 7.6, 0.4 mol/L disodium salt of ethylenediaminetetra acetic acid (Na_2EDTA; Merck, Darmstadt, Germany), 2.5 mol/L NaCl, 2% Cetyl trimethyl ammonium bromide (CTAB; Merck, Germany) 850 mL H_2O. Adjust the pH to 8.0 and make the final volume to 1 L.

10% SDS (Sodium dodecyl sulfate).

β-Mercaptoethanol.

Chloroform: Isoamyl alcohol (24:1).

Isopropanol.

70% and 90% ethanol (Merck, Germany);

2.3 | DNA extraction procedure

The extraction procedure was standardized both for fresh and frozen blood samples.

Step 1. 500 μL of blood sample was transferred from the vacutainer to an eppendorf tube. In case of frozen blood, the sample was thawed at room temperature for 20-30 minutes before transferring the blood to the eppendorf tube.

Step 2. Plasma was aspirated out carefully by centrifuging the sample at 2664 RCF for 7 minutes at 4°C.

Step 3. 1 mL of RLB was added to the precipitate, mixed gently and was allowed to stand at room temperature for 1-2 minutes.

Step 4. The mixture was then centrifuged at 2664 RCF for 6 minutes at room temperature.

Step 5. The supernatant was discarded. This step may be repeated 1-2 times until a white colored pellet is obtained.

Step 6. 500 μL of prewarmed DNA extraction buffer was added to the pellet followed by 30 μL of 10% SDS and 2 μL of B-Mercaptoethanol respectively and mixed gently. The mixture was then incubated at 56-60°C for 1 hour.

Step 7. 500 μL of Chloroform: isoamylalcohol (24:1) was added to the mixture after incubation and shaken well. The mixture was then centrifuged at 10 656 RCF for 12 minutes at 4°C.

Step 8. The supernatant was pipetted out in another fresh sterilized centrifuge tube containing chilled ethanol. The tube was shaken for a while until fine white threads appeared in the solution. The sample tube may be kept at −20°C for 20 minutes instead of shaking.

Step 9. The sample was then centrifuged at 10 656 RCF for 12 minutes at 4°C.

Step 10. The supernatant was discarded without disturbing the pellet and 500 μL of 90% alcohol was added to it.

Step 11. The sample was then centrifuged at 10 656 RCF for 12 minutes at 4°C.

Step 12. Step 10 and 11 were repeated with 500 μL of 70% alcohol.

Step 13. The supernatant was discarded and the pellet was allowed to dry at 37°C.

Step 14. The pellet was then dissolved overnight in 100 μL of TE buffer.

Step 15. The DNA solution was then stored at −20°C for future use.

2.4 | DNA assessment

Ratio of the absorbance at 260 and 280 nm respectively (A_{260}/A_{280}) were estimated to check the quality and quantity of the extracted DNA sample (Table 1). The absorbance ratio was measured using UV spectrophotometer (Rayleigh UV-2100, Beifen-Ruili Analytical Instrument (Group) Co., Ltd., Beijing, China). DNA concentration was measured based on A_{260} values. Absorbance value of 1 at 260 nm equals to 50 μg/mL of pure dsDNA. This concentration was multiplied with the total eluted volume to provide the total yield of DNA.

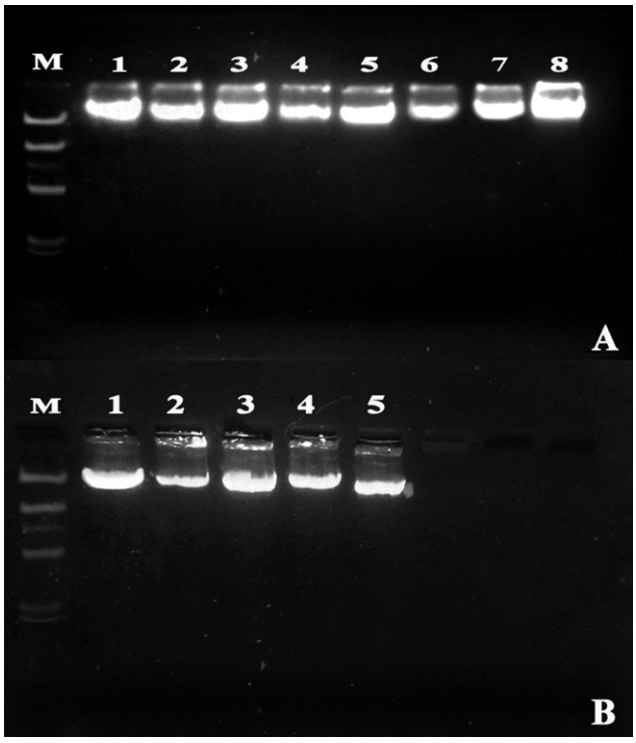

FIGURE 1 One percent agarose gel electrophoresis to demonstrate the extracted DNA. (A) Fresh Bloods and (B) Frozen Bloods. M stands for Marker (λ-DNA/Hind III Marker)

Furthermore, electrophoresis of 5 μL of each extracted DNA sample was done on 1% agarose gel in order to trace any degradation of the DNA sample during the extraction procedure (Figure 1).

Polymerase Chain Reactions were carried out using the extracted DNA samples in order to check the proficiency of the extracted DNA in gene amplification studies and also to check whether any inhibitory component were present in the samples which may hinder the participation of the DNA in PCR reactions. A fragment of Growth Hormone (GH) gene was amplified using the extracted DNA sample for which the forward and reverse primers were 5′-CTT CCC AAC CAT TCC CTT A- 3′ and 5′-CGG ATT TCT GTT GTG TTT C-3′ respectively. Each 25 μL PCR reaction contained 2.5 μL 10× PCR buffer (Bangalore Genei, Bangalore, India), 0.2 mmol/L of deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP; (Bangalore Genei), 1.0 μmol/L each of forward and reverse primers (Imperial LifeSciences, Gurgaon, India), 50 ng of template DNA and 1 U of Taq polymerase (Bangalore Genei). The PCR reactions were carried out in MJ Mini Gradient Thermal Cycler (Bio-Rad PTC 1148, BioRad, CA, USA). The amplification program consisted of an initial denaturation step at 94°C for 3 minutes, followed by 30 cycles of 30 seconds at 95°C, 50 seconds at 58°C for primer annealing, and 60 seconds at 72°C for extension and finally terminating with a final extension of 10 minutes at 72°C. Electrophoresis of the PCR products were done on ethidium bromide (0.5 μg/mL) prestained 1% agarose gel after which a photograph of the agarose gel was taken over a UV-transilluminator (Spectroline TVD-1000R, Spectronics Corporation, Westbury, NY, USA; Figure 2A).

Qualitative assessment of the extracted DNA was also checked by double digesting the DNA with restriction enzymes: Eco RI and Hind III (Genei; Figure 2B). Briefly, each reaction mixture contained: 1.5 μg DNA,

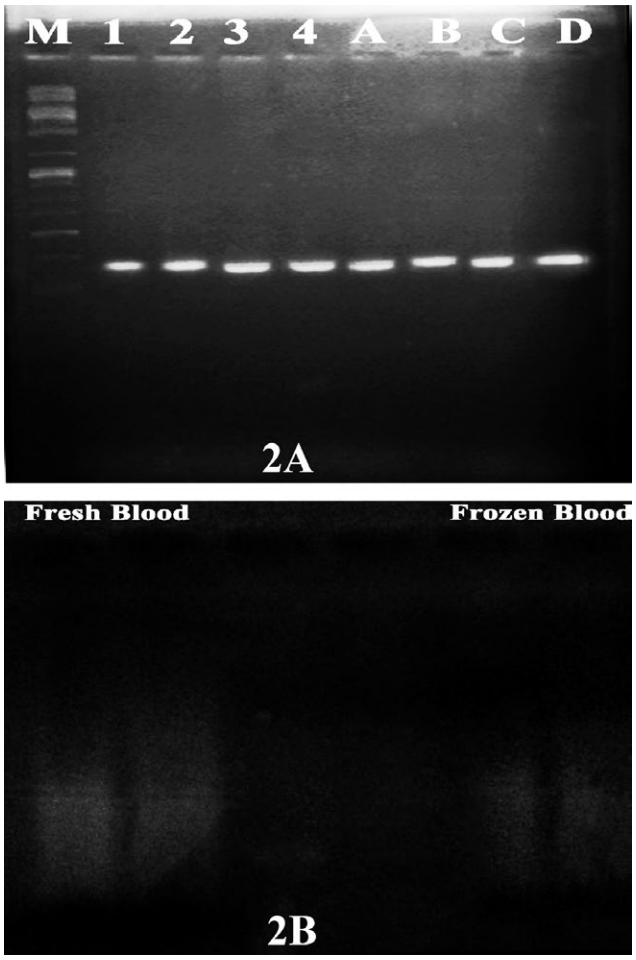

FIGURE 2 (A) One percent agarose gel electrophoresis showing the PCR amplification products of the GH gene from fresh and frozen blood samples. Lanes 1-4 corresponds to fresh blood from samples 1, 3, 7,10, respectively, while lanes A-D corresponds to frozen blood from the same samples. (B) Double digested fresh and frozen blood samples run on 1% agarose gel. Eco RI and Hind III were used for the digestion

2 μL of 10x assay buffer D (Genei) enzyme specific, 1 μL of restriction enzymes, EcoR1(10 U/μL) and Hind III (10 U/μL), 0.2 μL of 100x acetylated BSA and ddH_2O to make the final volume to 20 μL. After brief centrifugation, the mixture was incubated at 37°C for overnight. Gel electrophoresis of the digested product was carried out on 1% agarose gel, prestained with ethidium bromide and visualized on a UV transilluminator.

Our data were also compared with some of the previously published protocols[10,12,22–24] and commercially available kits which includes Wizard Genomic DNA purification kit (Promega, Madison, WI, USA), QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany), Archivepure™ DNA purification kit (5 Prime, Hilden, Germany) and GenElute™ Blood Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). In case of the reference protocols, average DNA yields, wherever mentioned, were considered for the analyses, else the maximum yield was considered if a range of value was provided (Figure 3). Bar graphs and scatter plots were constructed using MS Excel software (Redmond, WA, USA). The box plot was constructed using SPSS ver. 15.0 (Chicago, IL, USA).

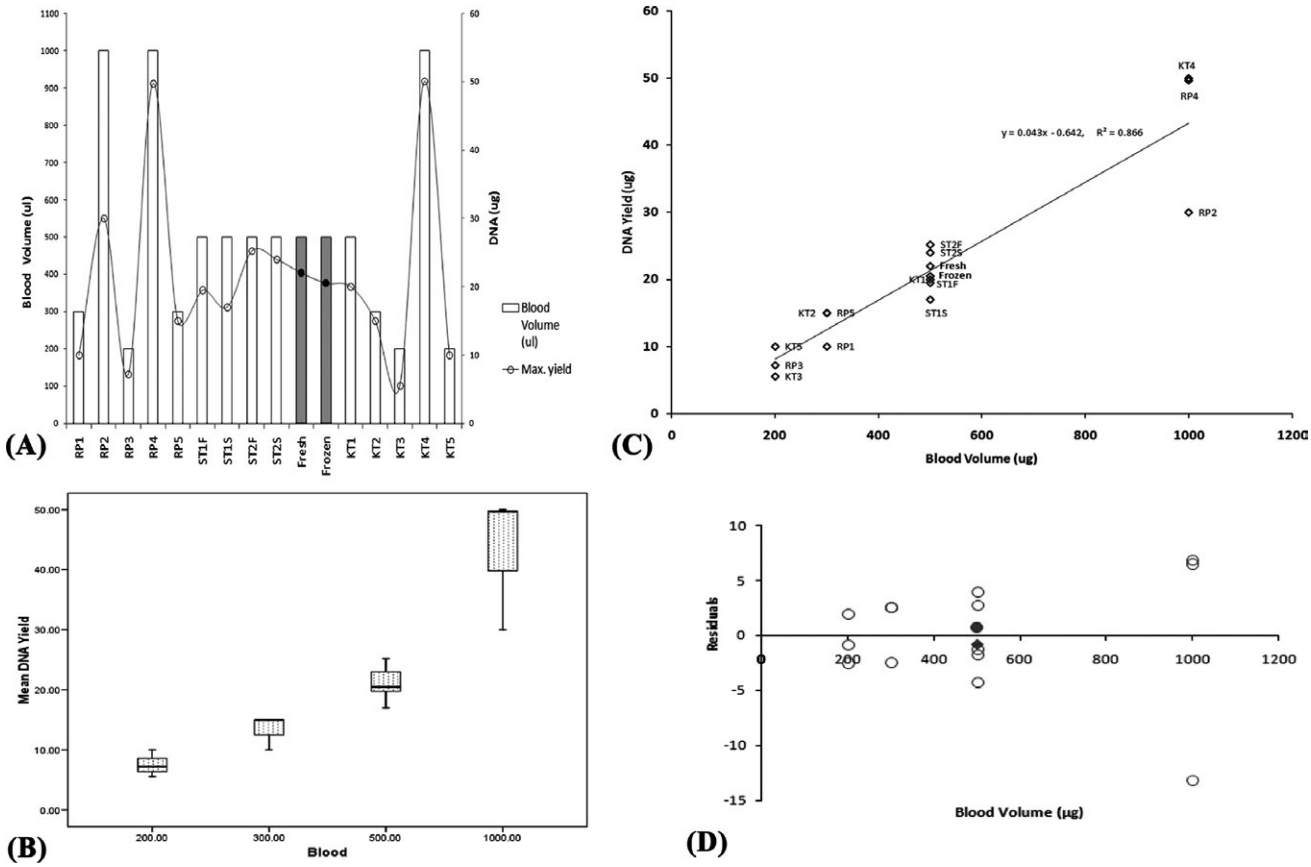

FIGURE 3 (A) Bar graph to compare the yield of DNA and the amount of blood volume required in our study protocols with other published protocols and kits. R1-5(Reference Protocols), KT1-5 (Reference kits), ST1-2(Standard Protocol) F or S (fresh or frozen). The results of the experimental protocol are mentioned "Fresh" or "Frozen" in bold. (B) Box plot to compare the average yield of DNA from different blood volumes as reported in our study and other published reports. (C) Scatterplot to compare DNA yield from our protocol with that of published reports. R1-5 (Reference Protocols), KT1-5 (Reference kits), ST1-2(Standard Protocol) F or S (fresh or frozen). The results of the experimental protocol are mentioned "Fresh" or "Frozen" in bold. (D) Residual Plot to show whether the linear regression model is appropriate for the dataset Residual values for DNA from fresh and frozen blood samples following our protocol are symbolized as (●) and (♦) respectively

2.5 | Standard protocols

Furthermore, two standard protocols were also used to extract DNA from the collected blood samples both in fresh and frozen conditions, namely the phenol-chloroform DNA extraction procedure by Sambrook et al.[25] and the high salt DNA extraction procedure by Miller et al.[14] respectively. Both the protocols were standardized for 500 μL of blood.

As per the phenol-chloroform method, after removal of the RBCs (by EDTA) nucleated cell pellets from both fresh and frozen blood samples were suspended in extraction buffer (10 mmol/L Tris HCl [pH 8.0], 0.1 mmol/L EDTA, 0.5% (w/v) SDS and 20 μg/mL pancreatic Ribonuclease A) along with Proteinase K (20 μg/mL). This step was followed by phenol-chloroform-isoamylalcohol (25:24:1) extraction and ethanol precipitation, finally dissolving the DNA in TE buffer.

On the other hand, in high salt precipitation method, after removal of the RBCs, the WBCs were treated with nuclei lysis buffer (10 mmol/L Tris-HCl, 400 mmol/L NaCl and 2 mmol/L Na2EDTA, pH 8.2) following overnight digestion at 37°C with 10% SDS and 20 mg/mL Proteinase K. This was followed by addition of 6 mol/L saturated NaCl. Protein pellets and DNA in the supernatant were procured after shaking the mixture vigorously followed by centrifugation. This was followed by ethanol precipitation and dissolving the DNA in TE buffer. The total DNA yield (μg) and the absorbance ratio (A_{260}/A_{280}) estimated from these two standard protocols were then compared with our experimental protocol.

3 | RESULTS

A_{260}/A_{280} absorbance ratio in case of both fresh and frozen human whole blood samples ranged consistently between 1.8 and 2.0 averaging at 1.88±0.07 and 1.86±0.06 for fresh and frozen blood respectively, which efficiently signified purity and successful deproteinization of the samples (Table 1). It has also suggested that the extracted DNA samples were also free from RNA contaminations. Following our protocol, the yield of genomic DNA per 500 μL of fresh blood ranged between 15 and 29 μg while that for 500 μL of frozen blood ranged between 14 and 28 μg respectively. Thus, the average yields for fresh and frozen blood samples were calculated to be

22±2.97 μg and 20.5±3.97 μg, respectively, which were comparable to the outcomes of both the standard protocols (Table 1).

DNA samples, run in 1% agarose gel, demonstrated bands of varying intensities, which were more or less comparable in case of fresh and frozen, blood (Figure 1). However, all the DNA bands were prominent and unified with very negligible smearing in the lanes. This suggests that no degradation has occurred in the extracted DNA inspite of the exposure to several chemical washes. Furthermore, agarose gel containing PCR products showed very prominent bands and therefore further demonstrating the quality and purity of the extracted DNA (Figure 2A). Double digestion by restriction enzymes (Figure 2B) showed that the extracted DNA samples were free from any inhibitory and interfering compounds.

It was observed that our standardized protocol yielded sufficient amount of high quality human genomic DNA, as was represented by the Bar graph and scatterplot (Figure 3A and C) when compared with other published protocols and commercially available kits. The mean DNA yields (μg) of different sample volumes were shown by box plot constructed in SPSS version 15.0 software (Figure 3B). The *R*-Squared value was estimated to be 0.866 which signified that our data fitted the line of the linear regression model with other reference data following the equation $y=0.043x-0.642$ (Figure 3C).

4 | DISCUSSION

Pure and intact genomic DNA is the first and foremost requirement for many modern applications in molecular biology. Therefore, the efficiency of a DNA extraction protocol will be affected by its robustness and ability to yield bulk amount of clean and unblemished genomic DNA. A number of DNA extraction protocols have already been published by several workers around the globe, which were verified to be reproducible and efficient in yielding sufficient amount of high quality DNA.[4–14] However, use of expensive enzymes and toxic solvents in these extraction procedures raised questions on their competence. Therefore, search for an inexpensive but efficient genomic DNA extraction methodology is still in progress. Herein, we have aimed to establish and standardize a simple, inexpensive yet useful procedure devoid of costly enzymes and toxic reagents for extraction of genomic DNA from whole blood samples.

Clear understanding of the chemistry and function of different reagents and buffer helped researchers to construct alternative methodologies for genomic DNA extraction from different sample sources. While designing the protocol for DNA extraction, the compositions of the reagents were determined based on the chemical effect of each reagent on various cellular organelles. Heme protein present in RBC is a strong inhibitor of Taq DNA polymerase such that even 1 μL blood can completely inhibit the PCR in a 100 μL PCR reaction. Therefore, sufficient attention should be paid toward the effective removal of hemoglobin and other contaminating proteins present in the blood sample. The Red Cell Lysis Buffer contained NH4Cl, $KHCO_3$, and Tris buffer. NH_4Cl results in increased osmotic pressure inside the RBC until the cells burst from water influx. However, it has least effect on other cellular contents of the blood. It did not affect other cell types especially leucocytes due to the absence of Cl-/HCO_3- transmembrane anion exchanger in leucocytes which are present in case of RBC. $KHCO_3$ increases the rate of swelling of RBC and can serve as a buffer component. On the other hand, low concentration of Tris Buffer would fasten the erythrocyte lysis process without having considerable deleterious effect on WBCs. Moreover, it also helps to maintain the pH of the buffer at a steady state. In case of the DNA extraction buffer, Tris buffer was used in higher concentration. This was followed by the addition of EDTA in the extraction buffer, which binds divalent cations such as calcium and magnesium. These ions help to maintain the membrane integrity. Their binding with EDTA destabilizes the membrane. CTAB is a cationic surfactant, which helps to lyse the cell membrane. It may also help to precipitate and remove all the unnecessary junk materials such as membrane debris, denatured proteins, polysaccharides etc. However, due to its positive charge, CTAB may form complex with DNA and precipitate it. This is undesirable and therefore NaCl is added which provides Na^+ ions into the reaction. These Na^+ ions neutralizes the negative charges on phosphates of DNA by forming ionic bond which otherwise would cause the DNA molecules to repel each other. Moreover, when present at higher ionic strength, NaCl disturbs the formation of CTAB-DNA complex and helps to keep the DNA in solution. SDS used along with the extraction buffer acted as strong anionic detergent that can solubilize the proteins and lipids of the membranes. This will help the cell membranes and nuclear envelopes to break down and expose the chromosomes that contain the DNA. In addition to removing the membrane barriers, SDS may also be useful in releasing the DNA from histones and other DNA binding proteins by denaturing them. B-Mercaptoethanol was used along with the DNA extraction buffer in the digestion step because it is a very strong reducing agent. It breaks down disulfide bonds between the cysteine residues of protein molecules, resulting in denaturation of the proteins by linearizing them. These linearized proteins were entangled, messed up and finally removed during centrifugation along with CTAB. After this step, Chloroform: Isoamyl alcohol was added which help in binding and precipitation of protein and lipids of cell membrane This step resulted in the formation of an aqueous phase containing DNA and a non-aqueous phase containing lipids and proteins. At this stage, DNA molecules are surrounded by water molecules forming the shell of hydration. Therefore, isopropanol is added at this stage as it may act as a dehydrating agent and disrupts the hydration shell resulting in precipitation of the DNA, which can then be separated, from the remaining soluble components through centrifugation.

The average yield of DNA per 500 μL of fresh and frozen blood was found to be 22±2.97 and 20.5±3.97 μg. As per the recent estimates, the sizes of diploid human female and male genomes are 6.406×10^9 bp and 6.294×10^9 bp respectively. Following Doležel et al., 2003 the mean molecular weight of 1 base pair was estimated to be 1.023×10^9 pg. Based on these values, diploid human female and male nuclei in G1 phase of the cell cycle should contain approximately 6.55 and 6.436 pg of DNA respectively.[26] As total count of WBC in a normal adult human being ranged from 4.5-10 × 10^3/μL of

human blood, the total amount of DNA per μL of blood was calculated to be within the range of 29.48-65.5 ng approximately. Thus, it was seen that using our protocol, the average DNA yield was within the normal range. Furthermore, the protocol roughly consumes two and a half hours for successful completion.

An earlier protocol of DNA extraction using CTAB was published by Thomas et al.,[27] but it was found that the buffer compositions and the sequences of the steps differed considerably from our experimental protocol. When compared with other published protocols and commercially available kits, it was observed that our protocol yielded comparable amount of high quality human genomic DNA, as was represented by the scatterplot. We also tested the appropriateness of the model by examining residual plots (Figure 3D). It was observed that the points in the residual plot were more or less randomly dispersed around the horizontal axis and thereby suggesting that the linear regression model is appropriate for the data.

At last, it can be said that the present method is unique as it cuts down both time and expenditure of DNA extraction per human blood sample more robustly when compared to many other available protocols. It is simple and can be carried out successfully even by a nonprofessional. Furthermore, this protocol does not contain any toxic reagents and therefore provides safety while performing the extraction procedure. Lastly, although the protocol is simple and inexpensive, but the protocol did not compromises with the quality and integrity of the extracted DNA.

5 | CONCLUSION

The protocol mentioned in this study may prove to be efficient in yielding considerable amount of genomic DNA from both fresh and frozen human blood samples. Furthermore, the elimination of time consuming steps such as enzymatic incubation (for Proteinase K and RNAase) and avoiding the use of toxic organic solvents such as Phenol made the protocol time-saving and economical without affecting the quality of the DNA samples which could be reliable enough for applications in advanced molecular biological techniques. Moreover, it may prove to be useful for laboratories with limited funds to pursue basic molecular biological researches.

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Rapid Communication

Study of genetic diversity of KIR and TLR in the Rabhas, an endogamous primitive tribe of India

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ABSTRACT

The Rabha tribe is a little known small endogamous population belonging to Indo-mongoloid group of north-eastern India. We have analyzed 16 KIR and 5 TLR gene polymorphisms in the Rabha population of northern West Bengal, India for the first time. The observed frequencies of the KIR genes (except framework and pseudogene loci) ranged between 0.26 (KIR2DS3) and 0.96 (KIR2DL1). Comparisons based on KIR polymorphism have revealed that although the Rabhas are of Indian origin the presence of mongoloid component in their gene pool cannot be denied. The frequencies of the 5 TLR genes ranged between 0.90 (TLR4) and 0.46 (TLR5). TLR variations found in the Rabhas may play a synergistic role in fighting against the bacterial invasions. Our results may contribute to the understanding of (1) genetic background and extent of genetic admixture in the Rabhas, (2) population migration events and (3) KIR-disease-TLR interactions.

1. Introduction

Rabha is a very little known small endogamous scheduled tribe community of India [1–3] with a relatively conserved gene pool of their own. In West Bengal, they are mainly distributed in the forest villages of the Dooars region of Jalpaiguri and Coochbehar districts. Historically they are considered to be the primitive inhabitants of the region who remained isolated from other neighboring populations due to strict endogamy. According to H.H. Risley, the Rabhas belong to the Indo-mongoloid stock [4], having a unique genetic constitution. Thus, there is an urgency to undertake genetic diversity studies in this group.

Herein, we have used two very recent genetic marker groups namely Killer Cell Ig-like Receptor (KIR) and Toll-like Receptor (TLR) genes to unravel the genetic profile of the Rabhas. The KIR genes cluster together on chromosome 19q13.4. [5,6]. They directly interact with HLA Class I molecules and play significant role in modulation of immune responses. We have genotyped 16 KIR genes in the Rabha population and compared our data with that of other previously published reports from different parts of the world to trace the phylogenetic relationship of the Rabha population with that of other populations around the world.

The TLR genes, located in different chromosomes, act in synergism to combat pathogens that infect the body. Recently there is a trend to establish the immunological connection between KIR and TLRs [7], following which we have also genotyped 5 TLR genes (TLR1–TLR5) in the Rabha population. Sivori et al. [8] mentioned in one of their publications that KIR3DL2 directly bind unmethylated microbial cysteine guanine dinucleotide (CpG) DNA and deliver it to endosome-resident TLR9 resulting in its activation and signaling [8]. Following this, Held [7] commented that immune cells such as NK and T-cell subsets co-expressing specific KIR and TLR9 may play a prominent role in directly recognizing and responding to pathogens or to host-cell death [7]. Another study by Artavanis-Tsakonas et al. [9] has shown a significant correlation between the carriers of a particular KIR3DL2 allele and the induction of an efficient NK-cell response to *Plasmodium falciparum* infected erythrocytes which lack HLA molecules [9]. These evidences may signify the importance of the KIR–TLR connection(s) [7]. Therefore in this study, we have analyzed TLR genes along with KIR genes in the Rabha population.

2. Materials and methods

2.1. Sample collection, DNA extraction and KIR specific PCR-SSP typing

Blood samples were collected from 50 medically examined healthy Rabha individuals from the Tufanganj Block (26°19′N/89°40

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′E) of Coochbehar District of West Bengal who provided their consent in written form after knowing the purpose of the study. Individuals with common ancestry for earlier 3 generations were excluded from the analyses. The investigation was approved by the Human Ethics Committee of the Department of Zoology, University of North Bengal, India.

Genomic DNAs were extracted by phenol–chloroform method [10]. The purity of the DNA samples was checked by UV Spectrophotometry. The extracted DNAs were then amplified using SSP-PCR reactions for detecting 16 KIR genes (KIR2DL1-5, KIR2DS1-5, 3DL1-3, 3DS1, 2DP1 and 3DP1). The KIR primer sequences were similar to that of Guha et al. [11] which were generously provided by R. Rajalingam on enquiry. The PCR reactions were performed according to Guha et al. [11]. We have also genotyped 5 TLR genes (TLR1–TLR5) in the Rabha population. The primer sequences generated by Primer-Blast [12] are shown in Table 2. The PCR reactions and amplification cycles were modified as per requirement.

2.2. KIR genotyping and statistical analyses

The frequency data of KIR genes and genotypes in populations to be compared to the Bengalis were extracted from the following publications and from the 'http://www.allelefrequencies.net' database [13] as follows: Indian Rajbanshis [11], South Indian Paravar, Kanikar and Mollukurumba [14], Finnish, French Caucasian, Senegal African, Guadeloupe Caribbean, and Reunion, a population from Indian Ocean origin [15], North Indian [16], Cook Island, Samoan, Tokelau, Tongan [17], Mestizo, Huichol, Purepecha, Tarahumara [18], Amazonian Amerindian [19], Wichis and Chiriguanos [20], Northern Irish [21], Basque population [22], Eastern Mainland Chinese [23], Chinese Han [24], Korean [25], Japanese [26], Warao, Bari, Yucpa [27], Vietnamese and Australian Aborigine [28], American Caucasian, Hispanic, African American [29], Thai, British Caucasian, Palestinian [30], Australian Caucasian [31], New York Caucasian [32], Greeks [33], Afro-Caribbean, Trinidad Asian, Pakistani [34], Chinese, Malay and Indian in Singapore [35], Indian Parsis and Maharashtrian [36], Tibetans [37] and Iranian Arabs and Persians [38].

Based on gene content, two distinct KIR haplotype groups can be designated, namely A and B. Certain assumptions were made while assigning genes to a specific haplotype which are as follows: (i) the framework genes (KIR3DL3, 2DL4, 3DL2 and 3DP1) are present in all the haplotypes; (ii) KIR3DL1 and 3DS1 are likely equivalent to alleles and (iii) either KIR2DL2 or KIR2DL3 are present in a haplotype but not both. Assignment of haplotypes was performed as described by Marsh et al. [39] with group B haplotypes defined by the presence of one or more of the following genes: KIR2DL5, KIR2DL2, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, and KIR3DS1. Conversely, the absence of all these genes define group A haplotypes [39]. Therefore, if any of the B haplotype genes are present, the genotype is considered as Bx. If none of these is present then the genotype is considered as AA.

The numbers were assigned to the KIR genotypes as provided in the Allele Frequencies website (http://www.allelefrequencies.net) [13]. The KIR genes and genotypic frequencies of the reference populations were also extracted from the 'http://www.allelefrequencies.net' database [13] and Guha et al. [11].

2.3. Statistical analyses

The observed frequency (OF) for each KIR gene was estimated as the ratio of the number of individuals carrying the gene to the total number of individuals in the sample population. KIR Locus Frequencies (KLFs) were calculated by using the formula: $KLF = 1 - \sqrt{(1-f)}$, where f is the OF of a particular KIR gene in a population. The standard $\chi 2$ test was executed using Kyplot 2.0 beta 15 to compare the differences of the Rabha population KIR gene content with that of some other neighboring populations. Nei genetic distances were calculated from 1000 bootstrap replicates of the KIR frequency data from the Rabhas and few other Asian populations and consensus NJ tree was constructed using

Table 1
KIR gene and genotypic frequencies in the Rabha population. Black boxes (■) indicate presence and white boxes (□) absence of KIR genes. Genotype IDs are considered according to www.allelefrequencies.net. The Observed Phenotypic Frequency (OF) for each KIR gene was calculated as the ratio of the number of individuals in the population carrying the gene to the total number of individuals in the sample population. KIR Locus Frequencies (KLF) were estimated from the OFs of the KIR genes.

Geno. ID	3DL1	2DL1	2DL3	2DS4	2DL2	2DL5	3DS1	2DS1	2DS2	2DS3	2DS5	2DL4	3DL2	3DL3	2DP1	3DP1	No.	Freq.
1	■	■	■	■	□	□	□	□	□	□	□	■	■	■	■	■	17	34.00
2	■	■	■	■	□	■	■	■	□	□	■	■	■	■	■	■	4	8.00
3	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	2	4.00
4	■	■	■	■	■	□	□	□	■	□	□	■	■	■	■	■	4	8.00
6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	3	6.00
8	■	■	■	■	□	■	■	■	□	■	□	■	■	■	■	■	2	4.00
15	■	■	■	■	□	□	□	■	□	□	□	■	■	■	■	■	2	4.00
22	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	1	2.00
68	□	■	■	□	■	■	■	■	■	□	■	■	■	■	■	■	2	4.00
70	□	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	4	8.00
101	□	□	□	□	■	■	■	■	■	■	□	■	■	■	■	■	1	2.00
161	■	■	■	□	■	■	■	■	□	□	□	■	■	■	■	■	1	2.00
192	■	■	■	■	■	□	□	■	■	□	□	■	■	■	■	■	2	4.00
277	■	■	□	■	□	□	□	□	■	□	□	■	■	■	■	■	1	2.00
300	■	■	■	□	■	■	□	□	■	■	■	■	■	■	■	■	1	2.00
486	■	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	1	2.00
530	■	■	□	□	■	■	□	□	■	■	■	■	■	■	■	■	1	2.00
N	□	■	■	□	■	□	□	□	□	□	□	■	■	■	■	■	1	2.00
Number	42	48	46	38	23	22	20	23	22	13	19	50	50	50	49	50	50	100.00
OF	0.84	0.96	0.92	0.76	0.46	0.44	0.40	0.46	0.44	0.26	0.38	1.00	1.00	1.00	0.98	1.00		
KLF	0.60	0.80	0.72	0.51	0.27	0.25	0.23	0.27	0.25	0.14	0.21	1.00	1.00	1.00	0.86	1.00		

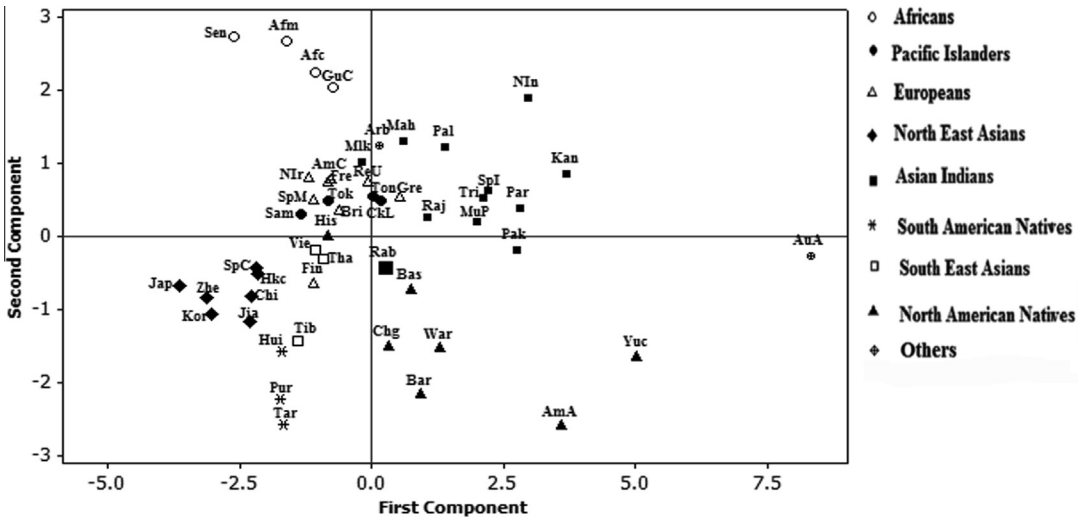

Fig. 1. Principle Component Analysis (PCA) of carrier frequencies of nine variable KIR genes (2DL1-3, 2DS1-4, 3DL1, and 3DS1) in the Rabhas and other world populations developed by MINITAB v16. The PCA graph shows a global relationship between Rabhas and other previously reported world populations. (Jap, Japanese; Chi, Eastern Mainland Chinese; Zhe, Zhejiang Chinese; Kor, Korean; SpC, Singapore Chinese; Jia, Jiansu Chinese; Hkc; Hongkong Chinese; Hui, Huichol; Pur, Purepecha; Tar, Tarahumara; Tib, Tibetans; Vie, Vietnamese; Tha, Thailand; Fin, Finnish; Chg, Chiriguanos; War, Warao; Bar, Bari; AmA, Amazonian Amerindian; Yuc, Yucpa; ReU, Reunion; Gre, Greek; CkI, Cook Island; Sam, Samoan; Tok, Tokelau; Ton, Tongan; Mez, Mestizo; Bri, British Caucasian; NIr, Northern Irish; Fre, French Caucasian; AmC, American Caucasian; SpM, Singapore Malay; His, Hispanic; MlK, Mollukurumba; Sen, Senegal African; GuC, Guadeloupe Caribbean; AfA, African American; AfC, Afro-Caribbean; Arb, Arabian; Pal, Palestinian; Raj, Rajbanshis; **Rab**, **Rabhas**; Mah, Maharashtrian; Tri, Trinidad Asian; Pak, Pakistani; NIn, North Indian; Par, Paravar; Kan, Kanikar; MuP, Indian Mumbai Parsi; Bas, Basque population; SpI, Singapore Indians and AuA, Australian Aborigine). The Rabha population has been marked in bold (■).

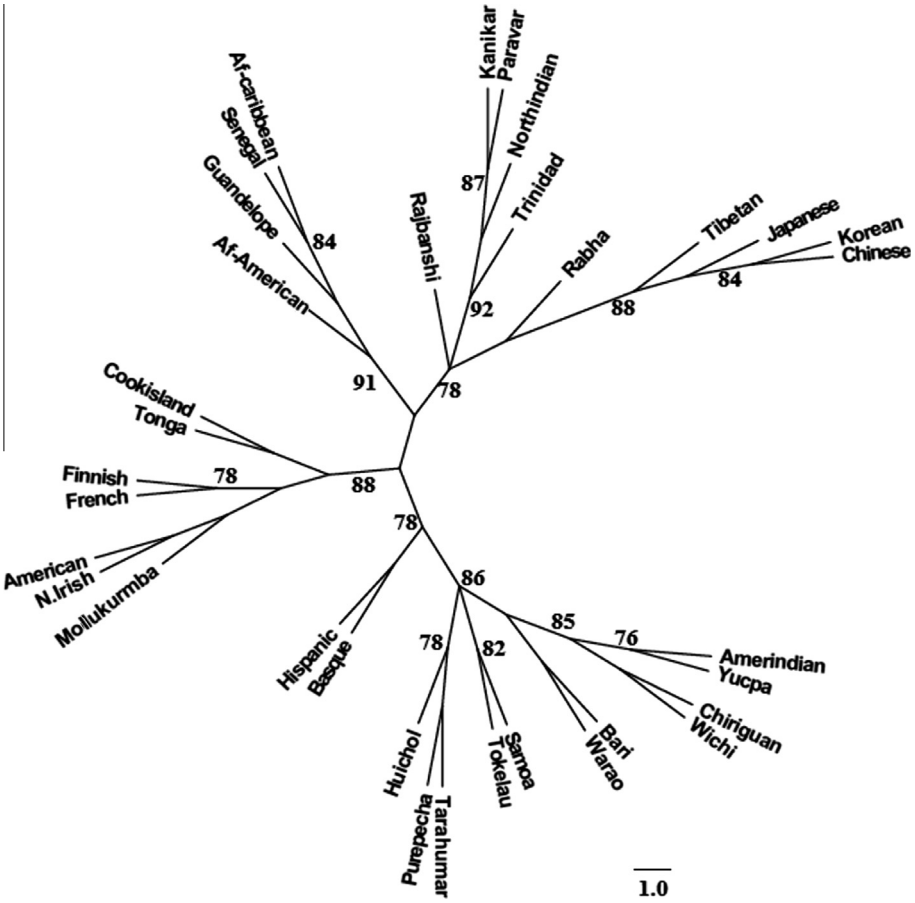

Fig. 2. Phylogenetic dendrogram based on REML analysis of the KIR genotypic profiles in the Rabhas and other previously studied World populations using PHYLIP package. Clusters relating to the Asian Indians, North East Asians, Africans, Europeans, Mexicans and American Natives can be distinguished separately. Populations having incomplete genotypes were excluded from the analyses. Bootstrap values were calculated for 100 replicates and the values are provided only to the branches supported by $\geqslant$75%.

Table 2
Frequencies of the TLR genes and the genotypes in the Rabha population. Black boxes (■) indicate presence and white boxes (□) absence of TLR genes. The Observed Phenotypic Frequency (OF) for each TLR gene was calculated as the ratio of the number of individuals in the population carrying the gene to the total number of individuals in the sample population. TLR Locus Frequencies (TLRF) were estimated from the OFs of the TLR genes.

Gene	Primers		OF	TLRF	Genotypes																
	Forward (5'-3')	Reverse (5'-3')			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
TLR1	TCAACCAGGAATTGGAATAC	AGTTCCAGATTTGCTACAGT	0.76	0.51	■	■	■	□	■	■	□	□	■	■	□	□	□	□	□	■	■	■
TLR2	GGATGGTTGTGCTTTTAAGTACTG	AAGATCCCAACTAGACAAAGACTG	0.6	0.37	■	□	■	□	□	■	■	■	□	■	□	□	■	■	■	□	□	■
TLR3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	0.74	0.49	■	■	■	■	■	□	□	■	□	■	□	■	□	□	■	□	□	□
TLR4	TTCTTCTAACTTCCTCTCCTGTG	TTAGCTGTTCGGCTCTACTATGG	0.9	0.68	■	■	■	■	■	■	■	■	■	□	■	□	□	■	□	□	■	■
TLR5	CATTGTATGCACTGTCACTC	CCACCACCATGATGAGAGCA	0.46	0.27	■	□	□	□	■	■	■	□	■	□	□	■	□	□	■	□	□	□
			Number		11	8	6	3	3	3	2	2	2	2	1	1	1	1	1	1	1	1
			Frequency		0.22	0.16	0.12	0.06	0.06	0.06	0.04	0.04	0.04	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02

PHYLIP software version 3.69 [40]. The Principal Components Analyses (PCA) was computed using MINITAB software version 16.0 based on KIR gene frequencies of the Rabha population and other reference populations. Restricted Maximum Likelihood (REML) analysis was computed using PHYLIP version 16.0 based on the KIR genotypic frequencies and the phylogenetic tree was developed using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk).

3. Results

The OFs of KIR genes in the Rabhas (except the framework and pseudogene loci) ranged between 0.26 (KIR2DS3) and 0.96 (KIR2DL1) (Table 1). The A haplotype associated KIR genes were much frequent compared to the B haplotype associated KIR genes in the studied population. Certain significant observations can be revealed from the above findings. Based on χ^2 analyses (Supplementary Table 1), it was found that the frequency of KIR2DL5 in the Rabhas differed significantly from all the reference populations of the Indian subcontinent, except from the Mollukurumbas and the Rajbanshis [11]. Another striking observation from the χ^2 analyses was that the Rabha population showed no significant difference for any of the KIR loci with that of the Rajbanshi population. Moreover, it was also observed that except for KIR2DS3 locus, the Rabhas did not differ significantly from the Tibetan population.

Principal Component analyses (PCA), based on OFs of the KIR genes, revealed that the Rabhas have occupied a slightly distant position from the Indian cluster in the score plot and have moved towards the lower left half of the score plot which have been mostly occupied by the North East and South East Asians (Fig. 1). Furthermore, the Neighbor Joining (NJ) dendrogram (Supplementary Fig. 1) based on Nei genetic distances (Supplementary Table 2) (computed using PHYLIP statistical package) also supported the result of the PCA score plot. The NJ tree showed that the Rabha population has branched away from the Indian cluster as an outlier group occupying a position somewhat intermediate between the Indian cluster and that of the other Asian populations i.e. the North East and South East Asian clusters.

Altogether 18 KIR genotypes were reported in this study (Table 1). One significant observation was that 17 out of 50 Rabha individuals have genotype ID 1 (34%) which has been found to occur predominantly among the North East Asian populations. Interestingly, it was also found that genotype IDs 161 and 300 that were found in the Rabhas were only reported in the North Indians till date. Genotype ID 486, found in the Rabha population was reported earlier only in the Chinese Shaanxi Han population [41]. It was evident from Restricted Maximum Likelihood (REML) based phylogenetic tree, computed from the genotypic frequencies (Fig. 2), that the Rabhas have branched out from the Tibetan–Japanese–Korean–Chinese clade and have occupied a position in between the Indian and the North East Asian Cluster.

The TLR frequencies were shown in Table 2. Interestingly, it was found that TLR4 has the highest frequency (0.90) among the studied TLR genes while on the other hand TLR5 has a considerably low frequency (0.46). Genotype ID 1 consisting of TLR1-5 was the most frequent among the genotypes.

4. Discussion

Historically, the Koch Rabhas and the Rajbanshis are considered to be very close relatives and they were thought to share the same ancestry [42]. This fact is evident from the $\chi 2$ analyses whereby no significant differences were observed for any of the KIR genes between the Rabhas and the Rajbanshis. Moreover, genetic distance measures also showed the least distance of the Rabhas from the Rajbanshis in comparison to other Indian populations. An earlier study by Guha et al. [11] suggested that the Indo-European speaking Rajbanshis from the northern part of Bengal has Tibeto-Burman influence in spite of having an Indian origin. Thus, the genetic proximity of the Rabhas with the Rajbanshis clearly indicates the inclination of the Rabhas toward mongoloid ethnicity.

Furthermore, both PCA score plot and NJ tree, based on KIR gene frequencies, showed the deviation of the Rabha population from the Indian cluster and also suggested their proximity to the mongoloid ethnicity. This view was further strengthened by the REML analysis where the Rabhas shared the same clade with the Tibetan population. Thus the results of the above analyses helped us to ascertain the fact that the mongoloid lineage has strongly influenced the gene pool of the Rabha population. The findings of our study were well supported by Y-chromosome haplogroup diversity study which have also extended similar views, where the Rabhas have clustered with the North East Asians [43]. Another study on Y-chromosome haplogroup diversity by Su et al. [44] also suggested the prehistoric migration of people belonging to Tibeto-Burman lineage to the Himalayas. Another earlier study by Chakraborty et al. [45] have also shown the presence of mongoloid element in the Rabha gene pool.

The TLR profile of a population in conjunction with the surrounding environment plays a complex role in disease pathogenesis [46]. Numerous published reports are available regarding the role of TLR in disease association studies [47–49]. Moreover, the presence of TLR genes can be documented from genetic pools of each and every population of the world. Thus, this marker may be considered to clarify genetic diversity and relatedness among

different populations [50–52]. During our preliminary investigations, we found that chronic gastritis and associated stomach problems are very common in the study population. Such profile of the TLR4 and TLR5 genes may indicate an upregulation of IFN-γ production by NK cells and may also indicate the prevalence of *Helicobacter pylori* negative gastritis [53,54], thereby signifying the possible association of TLR profile with the prevailing chronic gastritic condition in the Rabha population. However, this preliminary observation has to be substantiated experimentally.

Thus our investigation is a pioneering population-based analysis of KIR and TLR genes in the little known Rabha tribe of West Bengal, India which indicated the influence of mongoloid element in Rabha gene pool which was well maintained due to their strict endogamous character. It would be judicious to know the distribution pattern of these two gene families in populations before exploring their relations and roles in immune responses. Therefore, it may not be an overstatement to say that this study may lay the foundation to define KIR-TLR connections because fundamental studies always engineer the base of advanced researches.

Conflict of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2015.09.024.

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