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.

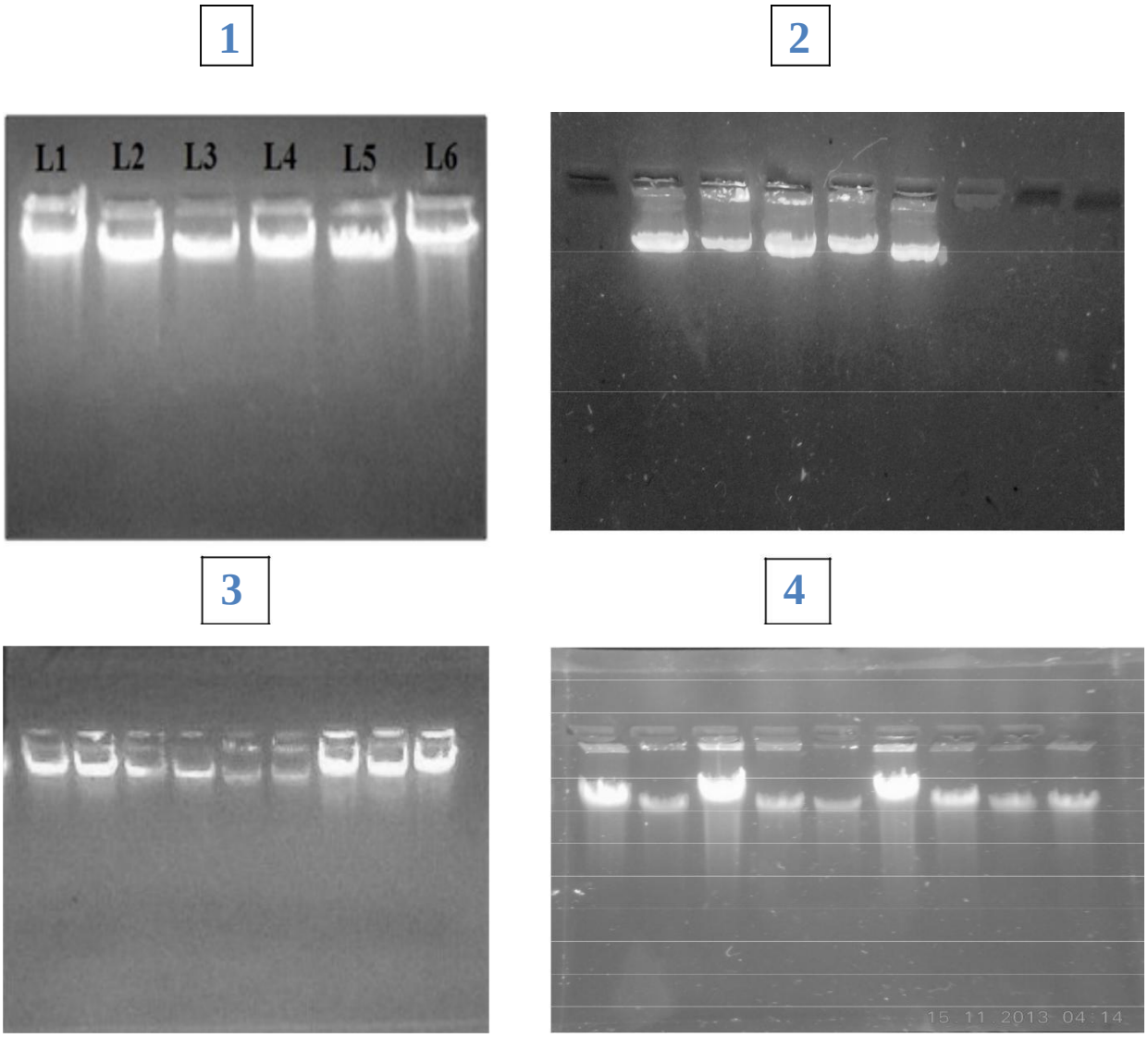

Fig 6: 1.2% agarose gel picture showing genomic DNA of different populations (1.Muslim, 2.Rabha, 3.Gurkha) and (4) HIV disease sample

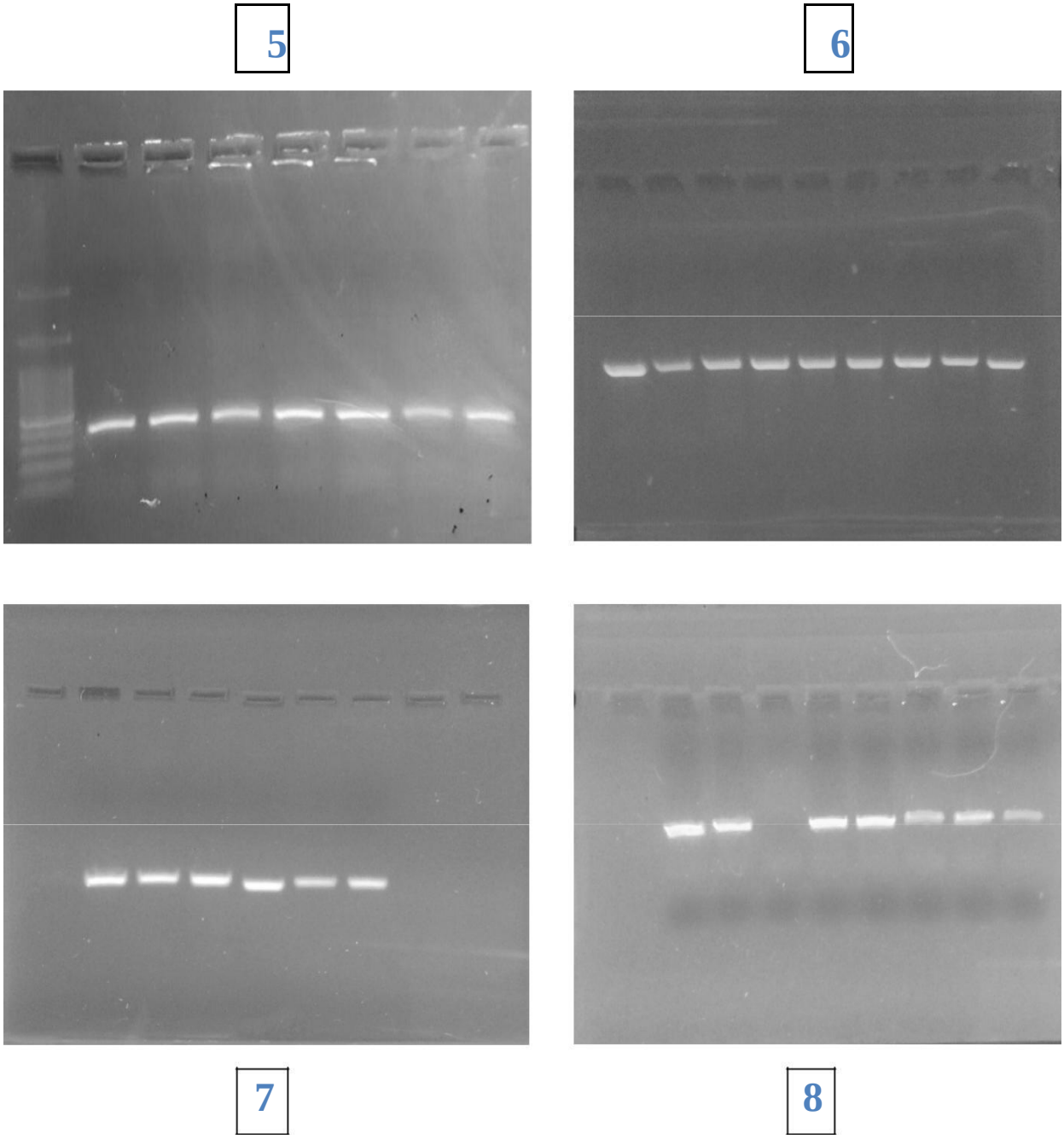

Fig 7: 1% agarose gel of TLR bands (after PCR) in case of (5) Rheumatoid arthritis, (6) Human Immunodeficiency virus, and (7) (8) Typhoid fever in different disease patients.

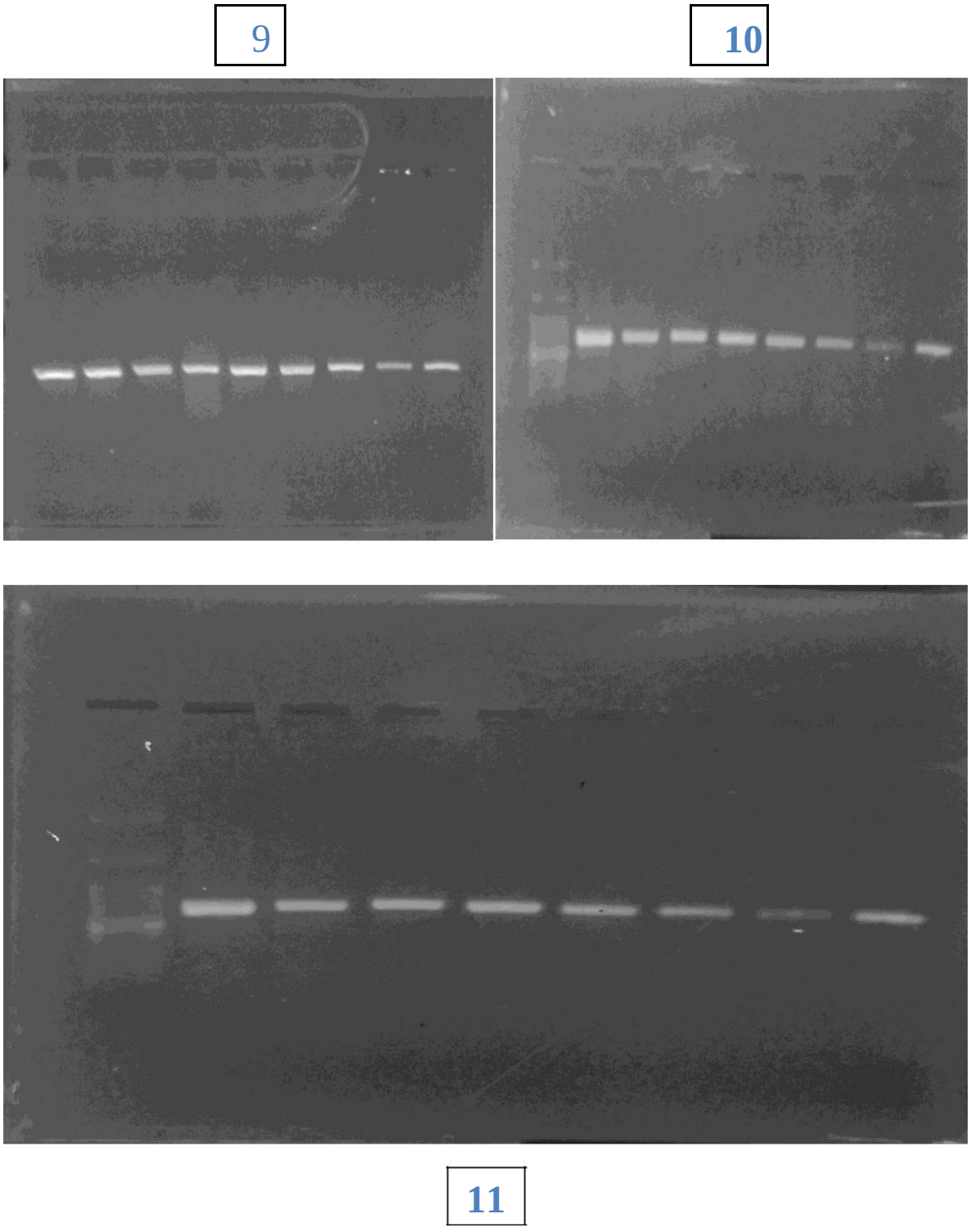

Fig 8: 1% Agarose gel picture of TLR4 genes (after PCR) in different populations of North Bengal region of India

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.

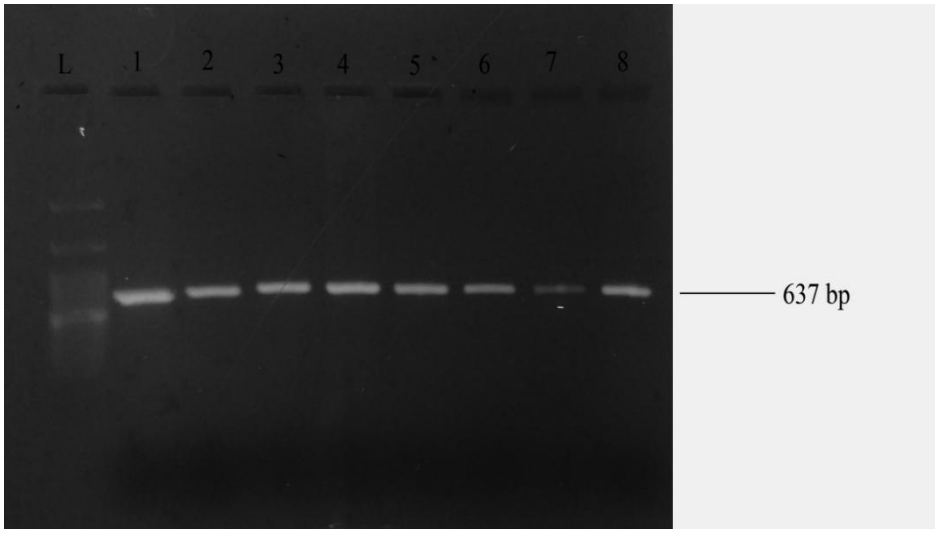

Figure 9: 1% agarose gel electrophoresis (after PCR) to show the presence of TLR8 gene in 8 Rajbanshi Individuals. The marker used in Lane 1 is Lambda DNA-Hind III double digested ΦX174 DNA.

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