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

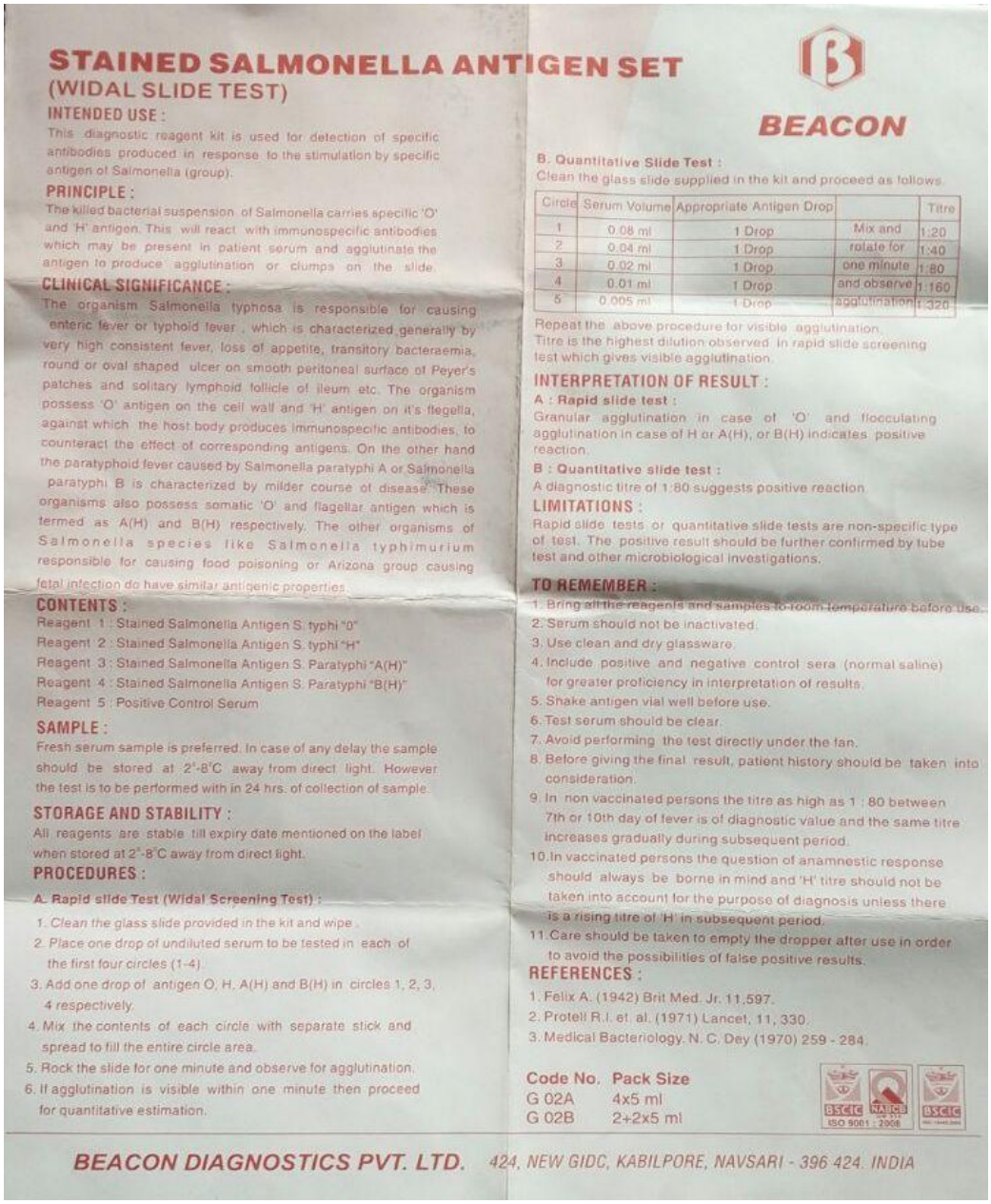
Cellular Immunology laboratory,

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.

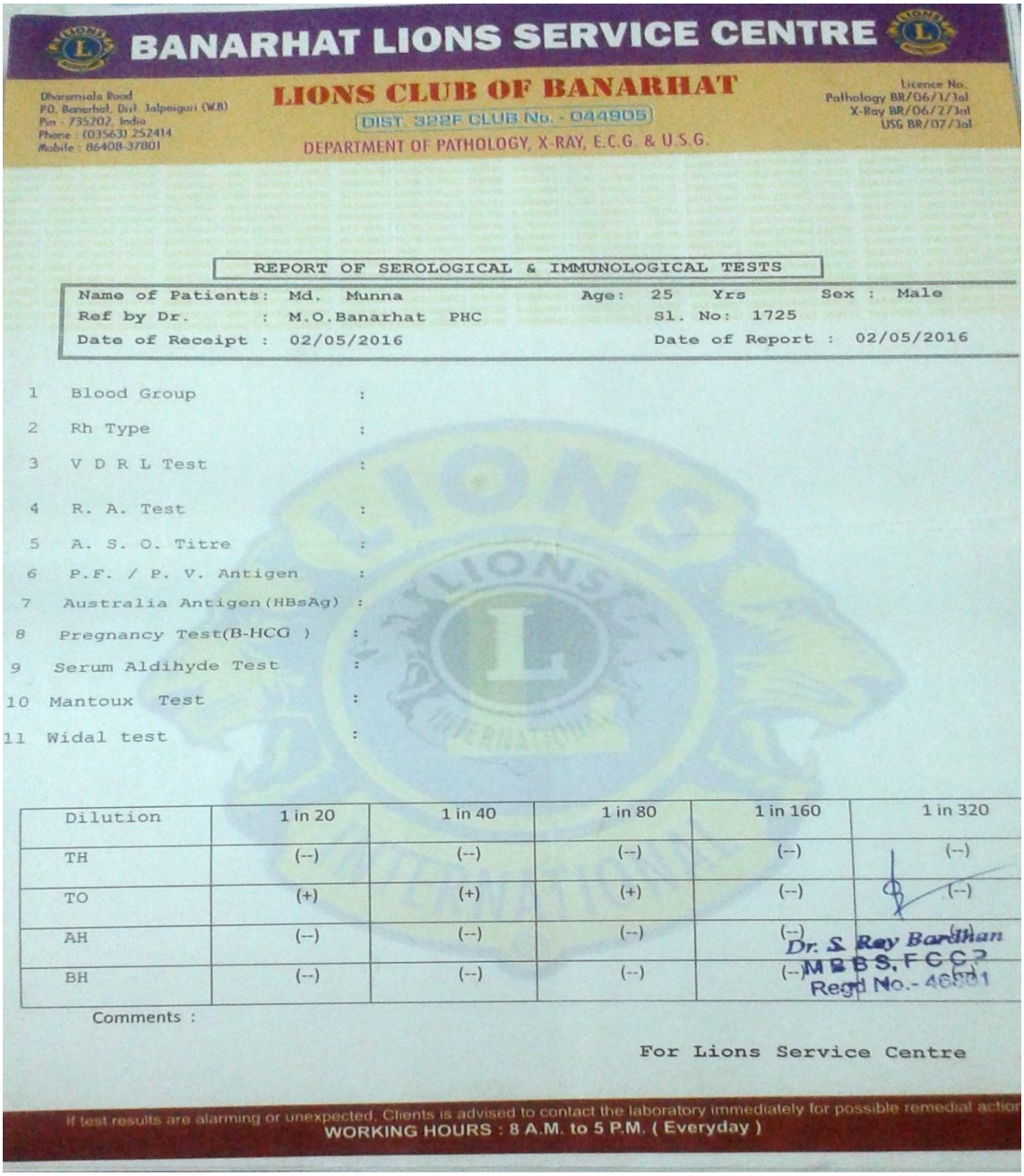
Code No.	Pack Size
G 02A	4x5 ml
G 02B	2+2x5 ml

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
P.O. Banarhat, Dist. Jalpaiguri (W.B)
Pin - 735202, India
Phone : (03563) 252414
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 :

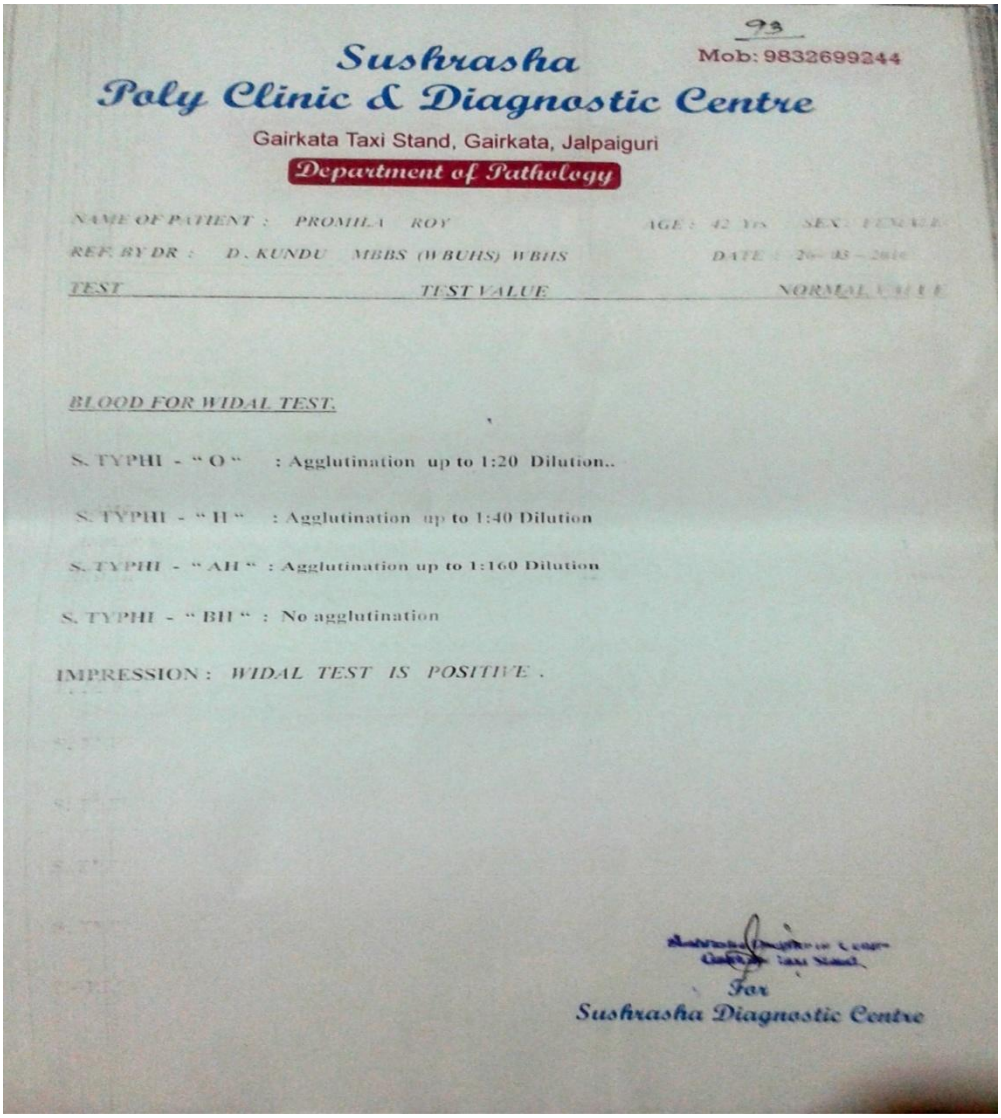
Dr. S. Roy Bardhan
MBBS, FCCP
Regd No.- 46881

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



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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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Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Raja Rammohunpur, Siliguri, West Bengal 734013, India–

***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.

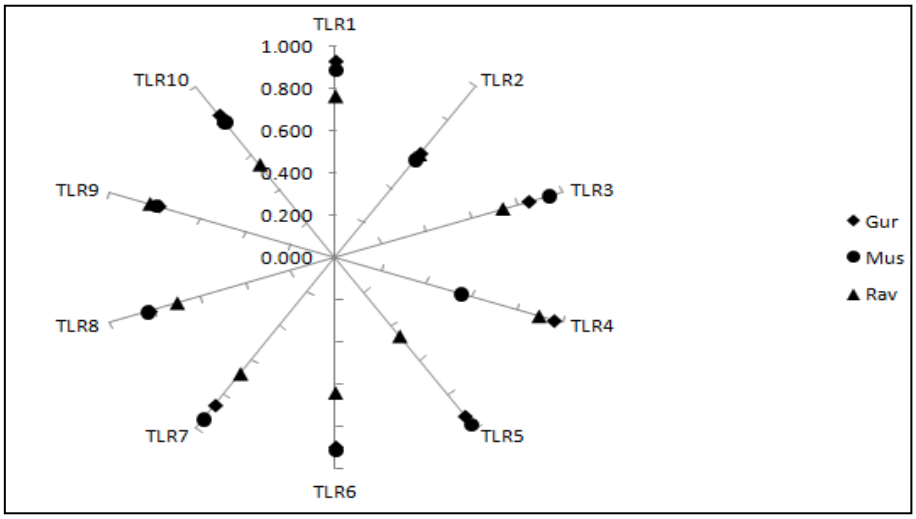
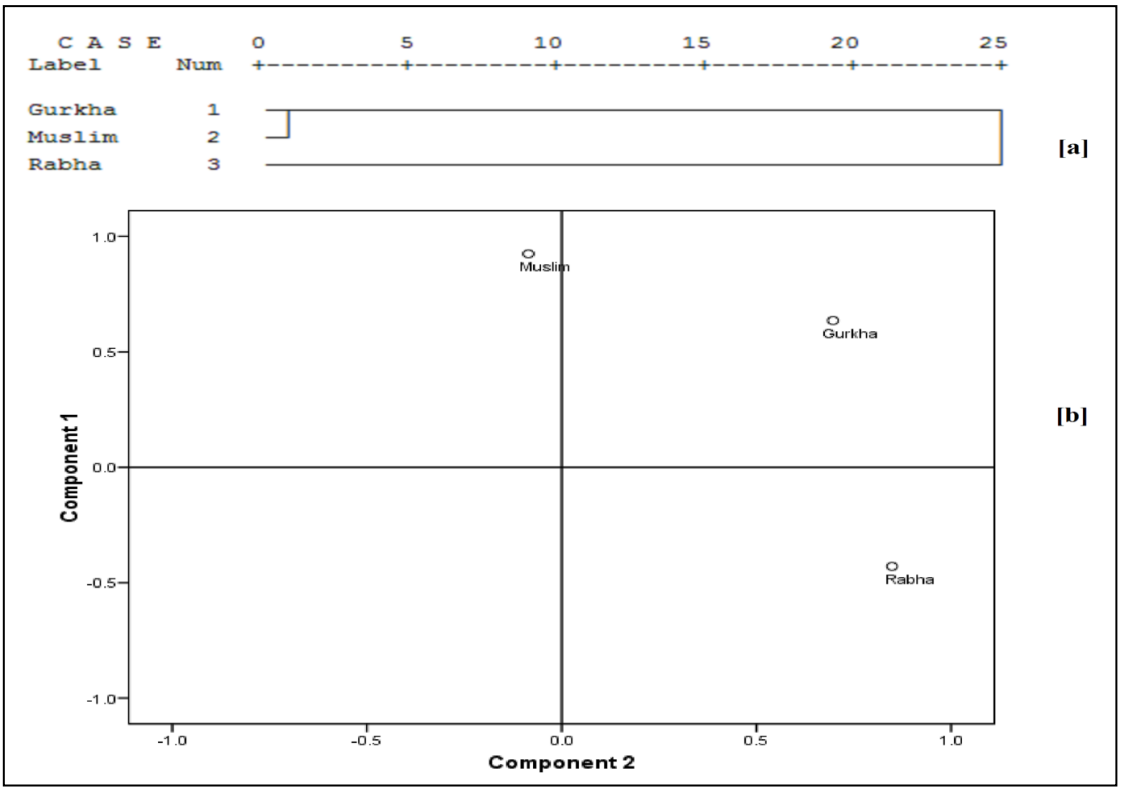


Figure 2. (a) Euclidean distance based Hierarchial cluster analysis was performed using from the observed frequency data by using SPSS (Ver- 15) software. (b) Principle Component Analyses (PCA) based on observed frequencies of the 10 TLR genes in the three ethnic populations of North Bengal constructed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

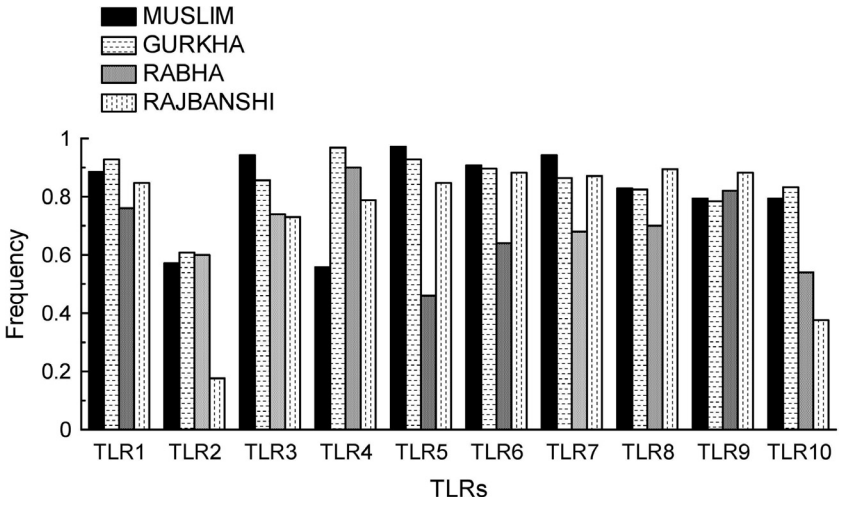

Figure 1 Frequency graph of ten TLR genes was constructed using Kyplot (ver-2.0) of the four populations in the North Bengal region.

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.

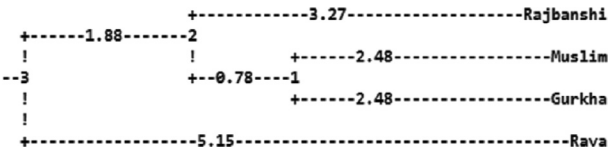

Figure 2 Neighbour joining tree was constructed using POPGENE (ver-1.32) and Phylip (ver-3.5) showing relationship among Rajbanshi and three other populations.

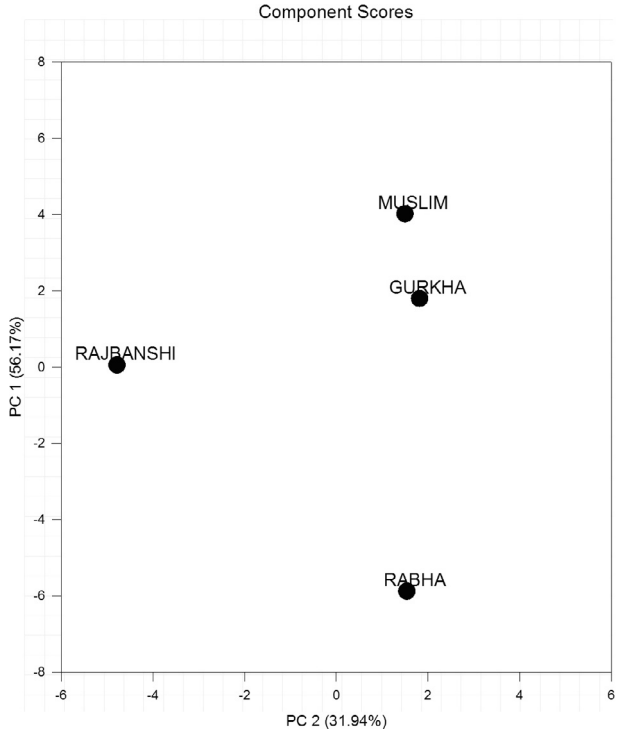

Figure 3 Principal component analyses (PCA) based on observed frequencies of the 10 TLR genes in the four ethnic populations of North Bengal constructed with Sigma plot (ver-13).

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).

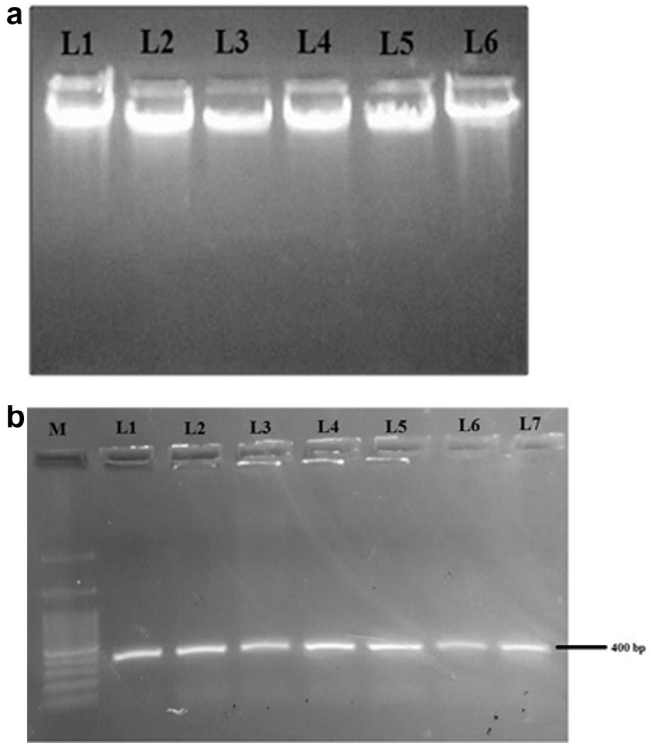

Fig. 1. a-Agarose gel electrophoresis showing results of DNA isolated from typhoid patient's blood samples (L1-L6). b-Agarose gel electrophoresis results showing PCR amplification of TLR5 in HIV+ samples (M-100 bp DNA ladder, L1-L7 positive samples).

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.

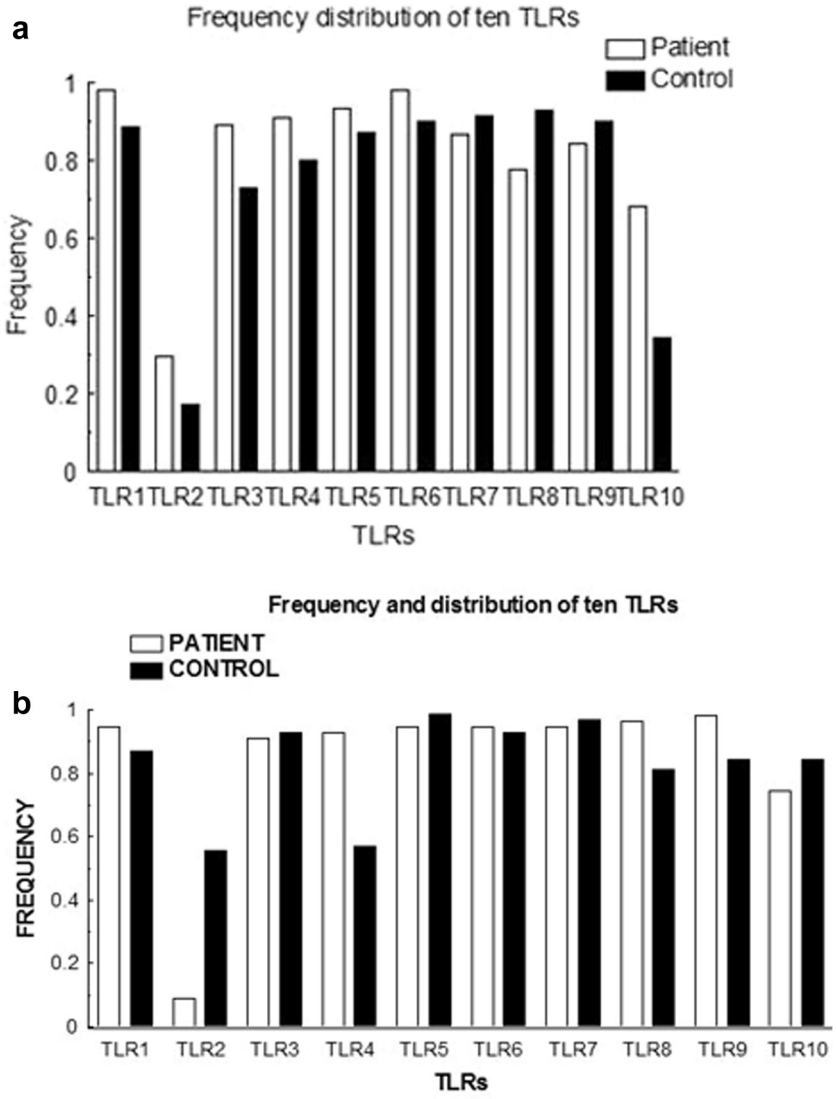

Fig. 2. a-Frequency graph of ten TLR genes was constructed using Kyplot (ver-2.0) of typhoid patients. b-Frequency graph of ten TLR genes was constructed using Kyplot (ver-2.0) of HIV positive patients.

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

WILEY

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.

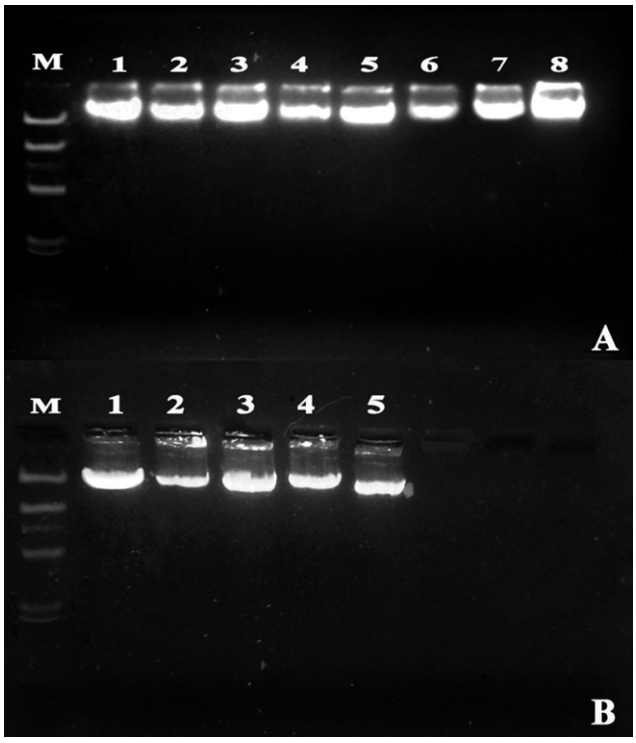

FIGURE 1 One percent agarose gel electrophoresis to demonstrate the extracted DNA. (A) Fresh Bloods and (B) Frozen Bloods. M stands for Marker (λ-DNA/Hind III Marker)

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,

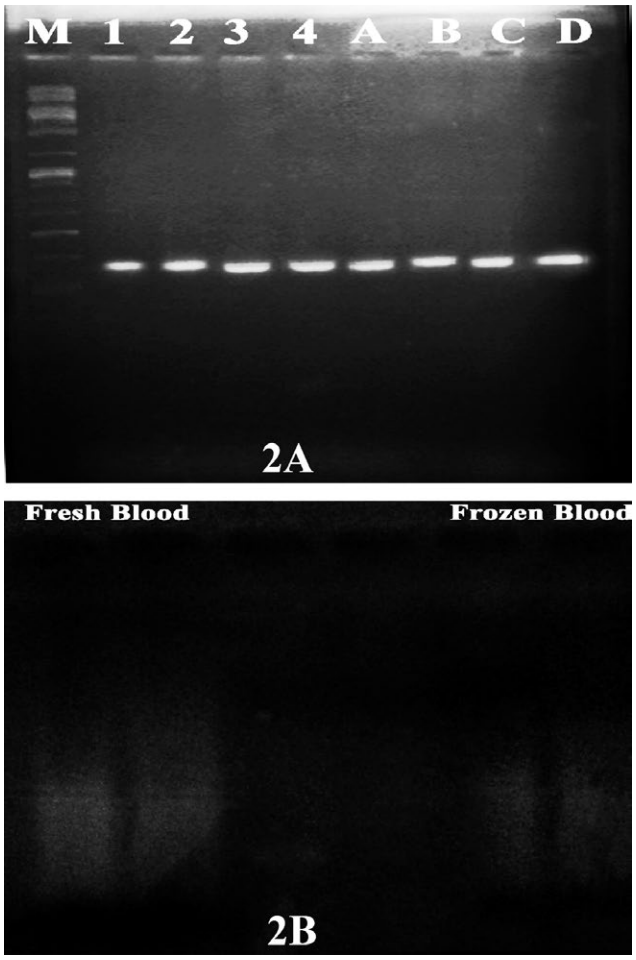

FIGURE 2 (A) One percent agarose gel electrophoresis showing the PCR amplification products of the GH gene from fresh and frozen blood samples. Lanes 1-4 corresponds to fresh blood from samples 1, 3, 7,10, respectively, while lanes A-D corresponds to frozen blood from the same samples. (B) Double digested 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).

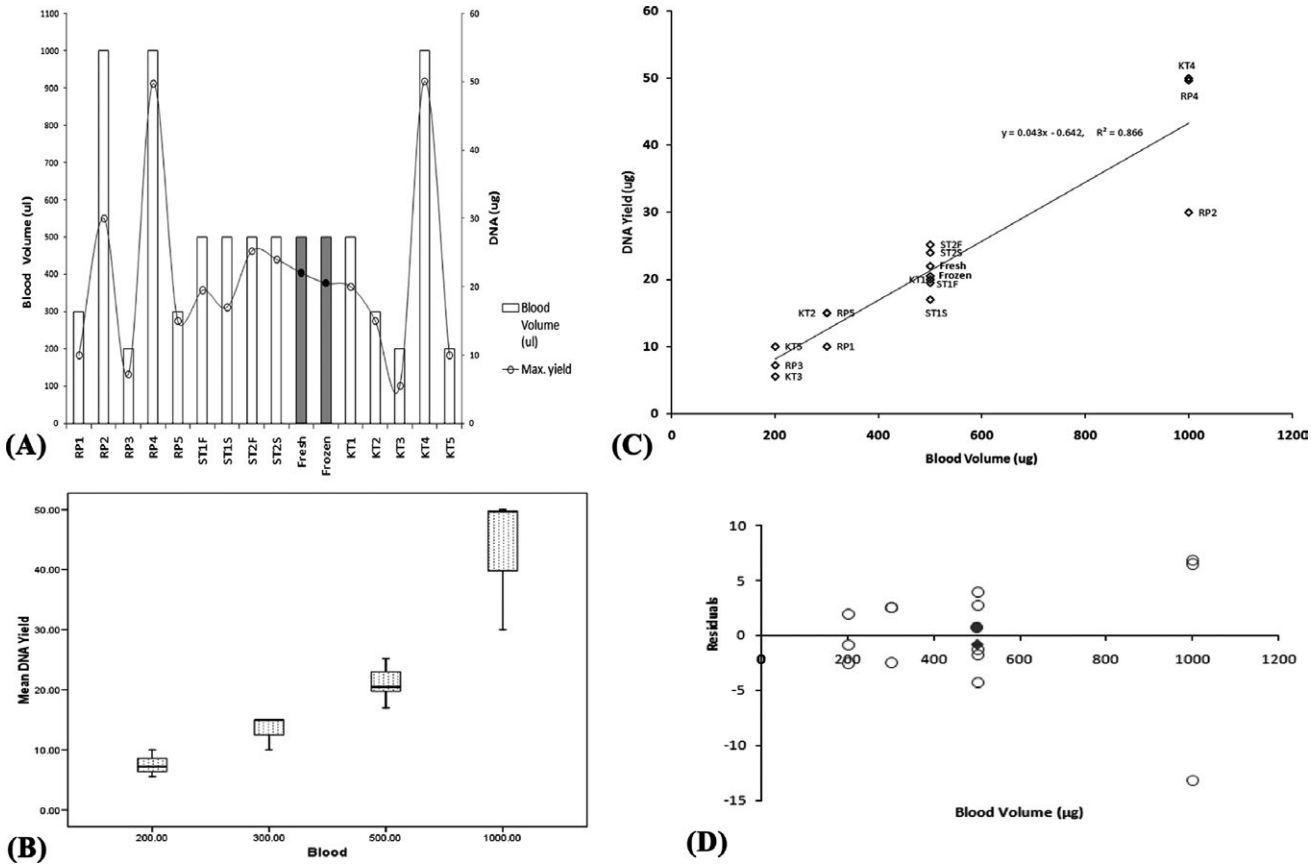

FIGURE 3 (A) Bar graph to compare the yield of DNA and the amount of blood volume required in our study protocols with other published protocols and kits. R1-5(Reference Protocols), KT1-5 (Reference kits), ST1-2(Standard Protocol) F or S (fresh or frozen). The results of the experimental protocol are 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		

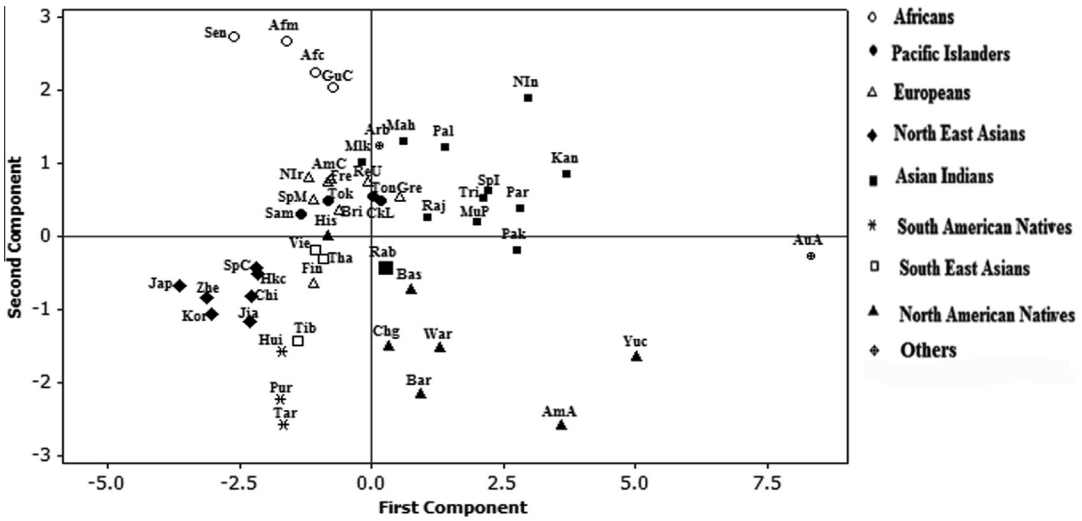

Fig. 1. Principle Component Analysis (PCA) of carrier frequencies of nine variable KIR genes (2DL1-3, 2DS1-4, 3DL1, and 3DS1) in the Rabhas and other world populations developed by MINITAB v16. The PCA graph shows a global relationship between Rabhas and other previously reported world populations. (Jap, 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 (■).

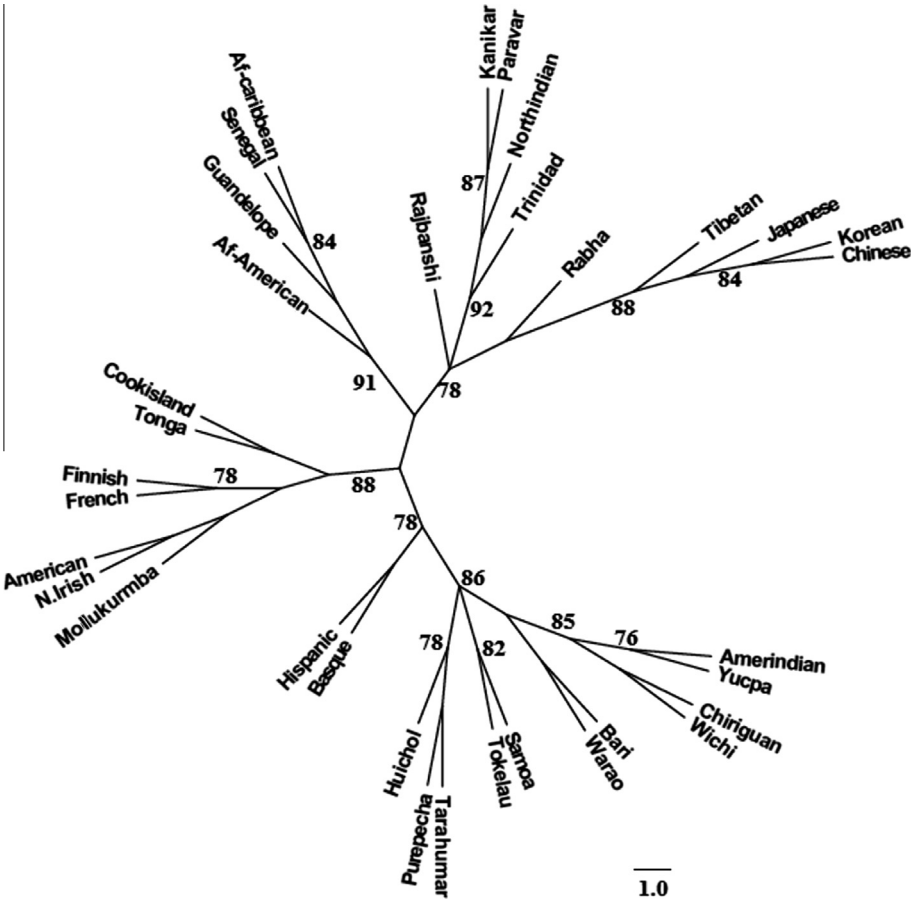

Fig. 2. Phylogenetic dendrogram based on REML analysis of the KIR genotypic profiles in the Rabhas and other previously studied World populations using PHYLIP package. Clusters relating to the Asian Indians, North East Asians, Africans, Europeans, Mexicans and American Natives can be distinguished separately. 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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