CHAPTER 5

DISCUSSION

5. Discussion

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

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

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

5.1.1 Rajbanshi

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

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

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

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

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

5.1.2 Rabha

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

5.1.3 Gurkha

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

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

5.1.4 Muslim

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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