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RESEARCH ARTICLE

Association of Tuberculosis with TLR-9 Gene polymorphism and C-Reactive Protein Levels in Blood of Humans and Animals

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ABSTRACT

Tuberculosis (TB) is an important bacterial zoonotic disease, causing mortality in humans and animals every year. Pakistan is ranked among top ten in the world based on the prevalence of tuberculosis in humans. Susceptibility to the disease has been linked with genetic variation in TLR-9 gene. Similarly, higher levels of C reactive protein have been reported in TB patients. This study was conducted to investigate the demography of tuberculosis in humans and to explore the association of TLR-9 genes and C-reactive protein levels in patients with tuberculosis in Faisalabad population. The study was also carried out on dairy animals. For TLR-9 gene polymorphism, DNA was extracted, amplified by PCR and fragmented by using restriction enzyme (*Bst* NI) and visualized after gel electrophoresis. To determine the C-reactive protein concentration, serum samples were sent to the commercial laboratory for ELISA. The demographic analysis showed that majority ($P<0.05$) of the patients had age between 18-50, height between 5.6-6.0 ft and weight between 50-60 kg. Furthermore, the majority were uneducated, un-employed or belonged to low-income status. Among tuberculosis patients, 86% were smokers, 32% had diabetes, 24% had cardiac disorders, 2% had HIV, 24% had a family history of TB and 20% had hepatitis. Furthermore, there was no family history of TB. The SNP analysis of TLR-9 gene (1237 C/T) showed no association with tuberculosis. However, the serum C-reactive protein levels were significantly ($P<0.05$) higher in TB positive cases as compared to controls, in both humans and animals.

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INTRODUCTION

Tuberculosis is a zoonotic and contagious disease caused by *Mycobacterium tuberculosis* in humans, which was identified by Robert Koch in 1982 and later was completely sequenced in 1998 (Cole *et al.*, 1998). The organism can be seen in sputum samples under the microscope by Acid-fast or fluorescent staining (auramine). The disease can be diagnosed by culture isolation, Mantoux/tuberculin test, ZN microscopy and PCR (Cudahy and Shenoi, 2016). According to the World Health Statistics 2018, 1.3 million deaths occurred due to TB in the year 2017. Ten million new cases have been reported in 2017 with 90% cases in adults. Globally, Pakistan is still among the top 30 countries with highest TB where 525,000 new cases and 27,000 MDR TB cases were notified in 2017 (WHO, 2018).

Toll-like receptors (TLRs), commonly known as pattern recognition receptors PRRs, are the important component of innate immunity against many pathogens including *M. tuberculosis*. As a first line of defense, they are expressed on the immune cells to identify a wide range of foreign patterns. This recognition leads to the transcription of proinflammatory cytokine genes which initiates the innate immunity and later there is an activation of the adaptive immunity through the production of cytokines (Horng *et al*., 2001). In humans, thirteen TLRs have been identified so far and they are either transmembrane, present on the cell surface or on the cytoplasmic subcellular membranes (Skevaki *et al*., 2015). In case of Mycobacterium, TLR-1, 2, 4, 6, 8 and 9 are involved in pattern recognition and interact with adaptor proteins MyD88 and TIRAP. This ultimately activates the macrophages and dendritic cells (O'Neill and Bowie, 2007). Susceptibility to TB and other diseases has been associated with mutation and polymorphism within TLR genes (Misch and Hawn, 2008). There have been reports about the association of TLR-9 gene (1237 C/T) polymorphism and the tuberculosis in humans, so we investigated the same in local human population, but we also included the animal population to see how they differ.

The level of serum C-reactive proteins (CRP) in the blood is associated with magnitude and type of systemic inflammations. The levels of C-reactive protein are different in intestinal disease, rheumatic disease, pneumonia and tuberculosis. In chronic diseases like asthma and diabetes mellitus, the very low concentration of CRP is a prognostic marker. In case of pulmonary tuberculosis, variation in the concentration of CRP is a sensitive indicator of the disease, so it can be used to assess the therapeutic response (Taikmura *et al*., 2006).

There is a need to explore the role of TLR-9 in local population suffering from TB and the levels of CRP. Therefore, the present study was carried out in both humans and dairy animals. The demography of human subjects suffering from tuberculosis was also studied.

MATERIALS AND METHODS

An ethical approval was obtained from the ethical committee of University of Agriculture Faisalabad. Permission for sampling and working at the District TB Hospital, Faisalabad for human blood samples and at the Directorate of Livestock Research Centre, University of Agriculture Faisalabad Pakistan for animal samples, was also obtained.

Sample size: Samples from a total of 50 randomly selected humans positive for tuberculosis and an equal number of negative samples were collected, including males and females of all ages, from District TB Hospital Faisalabad and people at University of Agriculture Faisalabad Pakistan, respectively. The data from these humans about demography and other diseases they were suffering was also collected. The 50 samples collected from negative humans were from the University students and staff members who have no previous history of the disease and were clinically normal. Similarly, samples from 10 TB positive animals as diagnosed by the tuberculin test and a similar number of tuberculin negative animals were collected, including cattle and buffaloes of over two years of age at the Livestock Research Centre, University of Agriculture Faisalabad. Three ml peripheral blood from each case was collected in EDTA and Gel Clot tubes.

Diagnosis of tuberculosis: For the screening of tuberculosis in animals, single comparative intradermal skin test by using PPDs (avian and bovine) was conducted, while for the human's tuberculosis Zeihl Nielsen staining of sputum, PCR Based Genexpert Test and X-rays were utilized.

Demographic study: A comprehensive questionnaire, consisting of specific sections, was designed to study the demographic parameters of human TB positive subjects including age, height, body weight, level of education, occupation, income, smoking addiction, diabetes, cardiac disorders, HIV, family history of TB and hepatitis status.

TLR-9 gene polymorphism study: The genomic DNA from peripheral blood was obtained by using a kit method (BBI Life Sciences®) as per manufacturer's recommendations.

For PCR, 5 µL DNA template, 18 µL master mix, and 2 µL of each Primer (Table 1) were taken for each sample reaction (Selvaraj *et al.,* 2010). Thermocycler conditions given were: Initial Denaturation at 95ºC for 5 min; 35 cycles of denaturation (95ºC for 30 seconds) annealing (53ºC for 30 seconds), and elongation (72ºC for 30 seconds) with a final extension at 72ºC for 5 minutes. For amplicon digestion, 1 µL restriction enzyme (Table 1) was added for 16 hours at 37ºC in each sample, followed by enzyme deactivation at 90ºC for 1 hour. Fragments were analyzed on gel electrophoresis using 2% agarose gel having ethidium bromide @ 0.5µg/ml and seen under ultraviolet light.

C-Reactive Proteins (CRP): To determine the C-reactive protein concentration, serum samples were sent to the commercial laboratory which used ELISA method for determination of C-reactive proteins and results were received.

Statistical analysis: Statistical procedures, including the general linear model procedure for comparison of C-reactive protein levels in TB positive and negative cases were used and the means were compared with the Tukey's test, while X^2 of Hardy-Weinberg Equilibrium and Mantel-Haenszel Chi-Square was used to analyze demographic data on personal computer by using SAS statistical software (SAS-2007).

RESULTS

Demographic Study: The results of demographic parameters showed that among pulmonary tuberculosis patients, significantly ($P<0.05$) higher number of patients had age between 18-50 years, height between 5.0-6.0 ft and weight between 50-60 kg (Table 2). Similarly, significantly ($P<0.05$) larger number of people had primary level or no education, the majority were un-

employed and had monthly income of <10,000/month or no income (Table 2). Furthermore, 86% were smokers, 32% had diabetes, 24% had cardiac disorders, 2% had HIV, 24% had a family history and 20% had hepatitis. It was also revealed that majority of these patients belonged to rural areas and had few diabetics among them (P<0.0001) (Table 2).

TLR-9 Gene Polymorphism Study: The *Bst* NI digested PCR products, separated on agarose gel showed two bands of 27 bp and 108 bp sizes. P1-P5 were results of TB positive samples, while N1-N5 were those of healthy controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of toll-like receptor 9 (TLR9-1237 (C/T) single nucleotide polymorphism (SNP) in pulmonary tuberculosis had depicted the similar bands, i.e., similar genotypes and allelic frequencies among TB positive and negative humans and animals (Fig. 1). This single nucleotide polymorphism analysis showed no association between TLR-9 (1237 C/T) and tuberculosis.

Serum C-Reactive Proteins: Test for C-reactive proteins showed significantly (P<0.0001) higher values in serum of TB positive humans with a mean value of 41.57±29.63 as compared to controls with a mean value of 5.432±0.728 (Table 3). A similar pattern was seen in bovines where positive cases and controls had mean values of 25.85±12.82 and 5.625±0.518, respectively (Table 3).

DISCUSSION

Tuberculosis is a bacterial disease mainly caused by *M. tuberculosis* in humans and *M. bovis* in animals. It is a highly fatal disease, with every year, 10 million new cases occur worldwide.

Results of our study showed that majority of tuberculosis positive people had age between 18-30 years. Ahmed *et al.* (2016) also found higher susceptibility to tuberculosis in young people, with a high rate of depression as compared to children and old age people. According to Irum *et al*. (2017), tuberculosis was present @ 1/3rd in children with age ≤2 years. The majority of people suffering from TB had height between 5.6-6.0 ft, weight 51-60kg, had no education, no job and had an income between Rs. 1,000-10,000. In a previous study, the disease was seen significantly (P<0.05) associated with poor income status, married and jobless population as compared to other respective groups (Ali *et al*., 2016). Further it was revealed that majority of the people those had TB were from rural areas. Similarly, Khan *et al*., (2015) also reported that tuberculosis was significantly (P<0.05) more prevalent in rural population as compared to the urban. The reason for this can be the socioeconomic factors.

Both innate and adaptive immunities are involved against tuberculosis. In specific immunity, toll like receptors, a type of pattern recognition receptors, are involved. The TLR 9 gene is located on chromosome 3p21.3 with a total length of approximately 5 kb (Chen *et al*., 2015). Polymorphism of these receptor genes previously has been linked to disease susceptibility (Schurz *et al*., 2015; Stein *et al*., 2017). The results of the present study revealed similar patterns of TLR9 gene (TLR9-1237 C/T) single nucleotide polymorphisms in TB positive and negative humans and animals. Similarly, Selvaraj *et al*. (2010) had also not seen different allele and genotype frequencies of TLR9-1337 C/T gene in healthy and affected population of Southern India. Therefore, our results and those of Selvaraj *et al*. (2010) indicate that in the subcontinent the polymorphism in this gene is not associated with the susceptibility to TB. However,

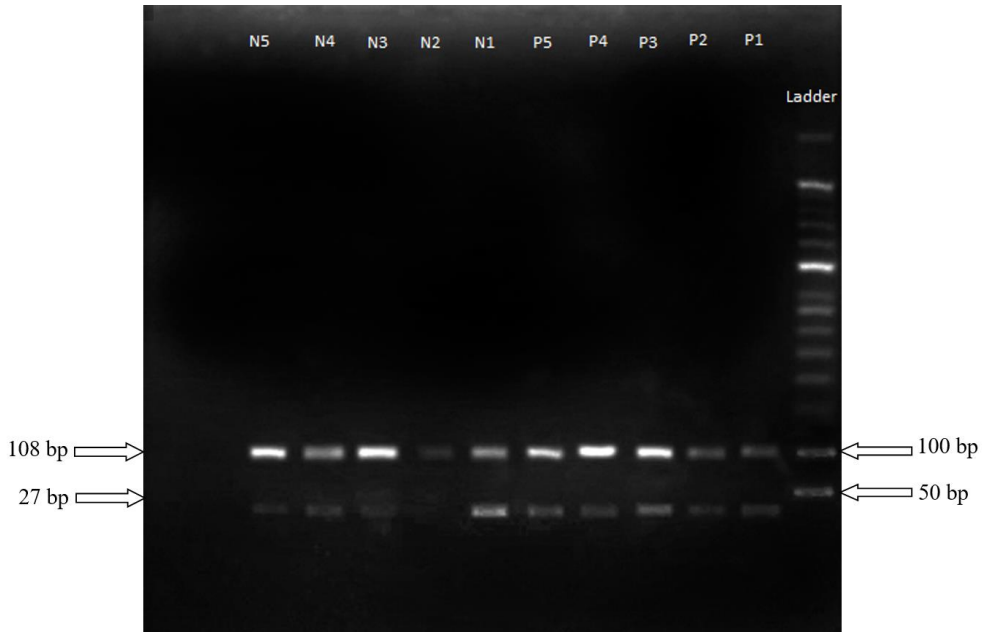

Fig. 1: Representative gel image showing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of toll-like receptor 9 (TLR9-1237 (C/T) single nucleotide polymorphisms (SNPs) in pulmonary tuberculosis, versus healthy controls.

Table 1: Primers and Enzymes

TLR polymorphism	Sequences of the primers 5' to 3'	Restriction enzymes	Reference
TLR-9 1237C/T)	F: CTGCTTGCAGTTGACTGTGT R: ATGGGAGCAGAGACATAATGGA	*Bst* NI	Selvaraj *et al*., 2010

Table 2: The data on demographic variables and other diseases along with TB is presented in the table

Parameters of study	Tuberculosis positive (N)	Tuberculosis positive (Percentage)	95% CI
Age groups (year)			
18-30	15	30	17.9-44.6
31-40	11	22	11.5-36.0
41-50	12	24	13.1-38.2
51-60	7	14	5.8-26.7
61-70	4	8	2.2-19.2
71-80	1	2	0.1-10.6
Height groups (ft)			
4.0-4.5	0	0	0.0-5.8
4.6-5.0	10	20	10.0-33.7
5.1-5.5	17	34	21.2-48.8
5.6-6.0	23	46	31.8-60.7
6.1-6.5	0	0	0.0-5.8
Weight group (kg)			
30-40	5	10	3.3-21.8
41-50	14	28	16.2-42.5
51-60	19	38	24.7-52.8
61-70	8	16	7.2-29.1
71-80	4	8	2.2-19.2
81-90	0	0	0.0-5.8
Education level			
No	15	30	17.9-44.6
Primary	14	28	16.2-42.5
Middle	7	14	5.8-26.7
Matric	9	18	8.6-31.4
Intermediate	3	6	1.3-16.5
Undergraduate	2	4	0.5-13.7
Postgraduate	0	0	0.0-5.8
Occupation			
No	14	28	16.2-42.5
Student	6	12	4.5-24.3
Unskilled	13	26	14.6-40.3
Semi-skilled	6	12	4.5-24.3
Skilled	6	12	4.5-24.3
Professionals	5	10	3.3-21.8
Income groups (1000)			
0-0	18	36	22.9-50.8
1-10	21	42	28.2-56.8
10-20	9	18	8.6-31.4
20-30	2	4	0.5-13.7
30-40	0	0	0.0-5.8
Smoking addiction			
Yes	43	86	73.3-94.2
No	7	14	5.8-26.7
Diabetes			
Yes	16	32	19.5-46.7
No	34	68	53.3-80.5
Cardiac disorders			
Yes	12	24	13.1-38.2
No	38	76	61.8-86.9
HIV/AIDS			
Yes	1	2	0.1-10.6
No	49	98	89.4-99.99
Family history			
Yes	12	24	13.1-38.2
No	38	76	61.8-86.9
Hepatitis			
Yes	10	20	10.0-33.7
No	40	80	66.3-90.0

Table 3: Mean CRP levels in serum of pulmonary tuberculosis positive and healthy humans and bovines

Test	Mean±SD	P Value
Humans		
CRP positive	41.57±29.63	P<0.0001
CRP negative	5.432±0.728	
Bovines		
CRP positive	25.85±12.82	P<0.0001
CRP negative	5.625±0.518	

variation among populations for disease susceptibility to the specific polymorphism has also been reported that C genotype was associated with reduced TLR9 transcription activity as compared to the T genotype (Tao *et al*., 2007). Single nucleotide polymorphism in rs1146617 and rs412909 alleles of TLR-9 has been found significantly (P<0.05) associated with higher risk of pulmonary tuberculosis in Chinese Tibetan but not in Han population (Wang *et al*., 2018). The suppressed post-translational IL-8 response to innate immune ligands due to defective TLR-9 signaling has also been observed in patients with tuberculosis (Ramakrishna *et al*., 2017). Similarly, a significant association of TLR-9 with tuberculosis has been seen in Egypt (Omran *et al*., 2016). There are mixed and inconclusive results SNP in certain TLR genes across the world. Few studies carried out by using meta-analysis indicated an association of SNP in various TLR genes (Chen *et al*., 2015). They indicated that rs352139 is significantly (P<0.005) associated with TB risk (AA vs. AG, OR 0.77). Similarly, they further reported with reference to ethnicity that the Indonesians with AA genotype had a decreased susceptibility, while Mexicans with GG allele had an increased risk of tuberculosis (Chen *et al*., 2015). A similar study with meta-analysis on TLR polymorphism and association with susceptibility to tuberculosis indicated that TLR1 rs4833095, TLR1 rs5743557, TLR1 rs5743596, TLR2 rs3804099, TLR2 rs5743704, TLR2 rs5743708, TLR6 rs5743810 and TLR8 rs3764879 polymorphisms were significantly (P<0.05) associated with susceptibility to TB in the overall human population (Zhou *et al*., 2019). Therefore, it was reported that the TLR polymorphisms may be used to identify the individuals at high risk of developing tuberculosis. However, such studies are rare in animals and the results of the SNP analysis were the same in both animals and humans as observed during the present study.

Serum C-reactive protein is found to be associated with a systemic inflammatory process in the body (Sturmer *et al*., 2004). Its concentration can be used as a diagnostic marker for inflammation or infectious diseases. It has been reported that the C-reactive protein can regulate the immune system during the early stage of infection. It plays a role in destroying the infectious agents, minimizing the tissue damage, and facilitating the tissue repair and regeneration (Horadagoda *et al*., 1999). It was hypothesized that the C-reactive protein levels in serum of tuberculosis positive patients will be high. For this reason, we determined the CRP level in tuberculosis positive humans and animals and found significantly (P<0.05) increased levels in TB positive cases. Drain *et al*. (2014) also found increased levels of CRP in patients with tuberculosis in South Africa. The efficiency of this marker in tuberculosis has been compared with Genexpert which showed similar results. Compared to culturing, CRP based diagnosis had shown 71% specificity and 88% sensitivity in patients with tuberculosis and having co-infection with HIV (Yoon *et al*., 2017). A study indicated that the change in CRP may have utility in early evaluation of response to anti-tuberculosis therapy and to identify patients at increased risk of adverse outcomes (Wilson *et al*., 2018).

In our study, patients with pulmonary tuberculosis were having active disease and most of the patients had a

treatment duration of less than a month, which is also reflected by the higher levels of CRP in these patients. It has been reported that the levels of CRP start deceasing after two months of treatment on anti-tuberculous drugs (Kedia *et al*., 2018). Studies in cattle on C-reactive protein levels indicated that these increases with milk production, peaking during high lactation (2 to 4 months of pregnancy), and decreased when lactation ceases (Lee *et al*., 2003). In addition, Lee *et al*. (2003) found higher levels of CRP during naturally occurring infections, such as mastitis and other inflammatory diseases. Therefore, it has been reported that the levels of CRP can confirm the presence of an inflammation. To best of our knowledge, no studies in animals with reference to tuberculosis and levels of CRP have been conducted so far, especially in Pakistan. Thus, the present study has indicated the usefulness of this test in animals suffering from tuberculosis as well. However, this test must be interpreted very carefully in animals as the levels of CRP can be higher during peak lactation and in many other physiological and pathological situations/conditions.

Limitations of the study were the budgetary constraints to include other associated factors, including variation in cytokine profile and genetic polymorphism of other TLRs. The sample size for animal study was also small, in the future, a similar study on a large population of animals may be included to rule out the association.

Conclusions: It can be concluded that tuberculosis is more prevalent in un-educated, low income/ jobless poor people in rural areas of Faisalabad, Pakistan. The SNP in the TLR-9 (1237 C/T) was not found associated with the development of tuberculosis in humans and bovine population of Faisalabad Pakistan. Serum C-reactive protein can be used as a diagnostic marker for tuberculosis and treatment response against this disease, both in animals and humans.

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Authors Contribution: MTJ and MQHF collected the samples and involved in sample testing and/or lab activity. MTJ conceived the idea of research, drafted the skeleton, preparation of manuscript; SUKB, NT, SZ, MHA and AT drafted the details of the manuscript helped in data editing, data analysis and construction of tables; IJ, AK, RH and AUR did proof reading, added references and checked reference styling etc. all authors read and approved the final manuscript.

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