Current Microbiology (2025) 82:154
https://doi.org/10.1007/s00284-025-04144-x

Association of TOLLIP mRNA Expression and TOLLIP rs5743899A/G, TOLLIP rs3750920C/T, and IL-10 -1082G/A Single-Nucleotide Polymorphisms with Susceptibility to Tuberculosis

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Received: 31 October 2024 / Accepted: 17 February 2025

Abstract

The early innate host immune response is crucial in the susceptibility to tuberculosis (TB). This study aimed to investigate the potential role of Toll-interacting protein (TOLLIP), a negative regulator of the human innate immune response, and interleukin-10 (IL-10), an anti-inflammatory cytokine, in susceptibility to TB. The peripheral blood mononuclear cells of 30 active pulmonary TB patients and 30 healthy volunteers were included. TOLLIP mRNA expression levels, single nucleotide polymorphisms (SNPs) of the TOLLIP gene, such as rs5743899A/G and rs3750920C/T, and IL-10 -1082G/A SNP were detected using real-time PCR, PCR-restriction fragment length polymorphism (PCR–RFLP) and amplification refractory mutation system-PCR (ARMS-PCR), respectively. TOLLIP mRNA expression levels were significantly higher in TB patients than in healthy controls (expression fold difference = 2.72, the delta-delta Ct method, also known as the 2-ΔΔCt method). The heterozygous (GA) genotype of the IL-10 -1080G/A polymorphism was significantly predominant among the patient group ($p = 0.02$, Hardy–Weinberg equilibrium). However, the Kruskal−Wallis analysis found no significant relationship between TOLLIP mRNA expression levels and SNPs. TOLLIP mRNA and IL-10 -1082G/A SNP were assessed as potential biomarkers to predict TB susceptibility. For more significant results, they should be examined with other negative regulators of innate immunity and other immune factors associated with susceptibility to TB. Our study is the first to investigate the association of TOLLIP polymorphisms and expression levels with TB susceptibility in Turkey.

Abbreviations

ARMS-PCR	Amplification refractory mutation system-PCR
cDNA	Complementary DNA
COPD	Chronic obstructive pulmonary disease
Ct	Delta delta threshold cycle
DM	Diabetes mellitus
IL-10	Interleukin-10

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Published online: 25 February 2025

LJ	Löwenstein-Jensen
MGIT	Mycobacterial growth indicator tube
MHC	Major histocompatibility complex
MTBC	Mycobacterium tuberculosis complex
NAP	p-nitro-alpha-acetylamino-beta-hydroxy-propriophenone
PCR	Polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
PPD	Tuberculin skin test
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SSP-PCR	Tuberculosis
TB	Charlson comorbidity index
TLR	Toll-like receptor
TOLLIP	Toll-interacting protein
WHO	World Health Organisation
ΔCt	Delta ct
ΔΔCt	Delta delta ct
*ΔCt	Average Ct of the TOLLIP gene - average Ct of the housekeeping gene (β2-microglobulin)
**ΔΔCt	ΔCt of the patient group - ΔCt of the healthy group

Background

Mycobacterium tuberculosis complex (MTBC) members, causative bacteria of tuberculosis (TB), infect about a quarter of the world's population and seriously threaten public health. According to the World Health Organisation (WHO) Report 2021, it is estimated that close to 10 million people were infected with active disease in 2020, and approximately 1.3 million people died from this contagious necrotising disease. Although there are effective prevention and treatment options, the immunopathogenesis of TB, which is still one of the deadliest diseases in the world, is still not understood [1].

Host immunogenetic risk factors are considered essential in susceptibility to TB and the pathophysiology of the disease, as in most chronic infections. As a result of few molecular studies on this subject, many polymorphisms in genes that negatively regulate the human innate immune response and gene expression levels of these effectors have been associated with the emergence of TB in different populations. In particular, Toll-interacting protein (TOLLIP) expression and single nucleotide polymorphisms (SNPs) such as rs5743899 and rs3750920 of the TOLLIP gene are associated with susceptibility to TB. TOLLIP is a negative regulator encoded on chromosome 11 in humans and can communicate with Toll-like receptors (TLRs) and convert the proinflammatory response to an anti-inflammatory response [2, 3]. Another crucial effector induced by mycobacteria for escape from the innate immune response is interleukin-10 (IL-10). This anti-inflammatory cytokine is a potent inhibitor of gamma interferon synthesis, regulating T-cell function, major histocompatibility complex (MHC) class-II expression, and antigen-specific proliferation, especially during the early inflammatory response [4]. Some researchers have associated the −1082G/A SNP of the IL-10 gene with TB susceptibility and clinical prognosis [5–8].

Although the induction of IL-10 secretion by TOLLIP has been revealed [3], we have yet to encounter a study investigating the combined effect of these two factors, which limit the human innate immune response, on tuberculosis susceptibility in healthy and patient groups. Therefore, we aimed to investigate the association of the TOLLIP mRNA expression level and the SNPs of TOLLIP and IL-10 with susceptibility to tuberculosis by evaluating them together. Moreover, our study is the first to investigate the association of TOLLIP polymorphisms and expression levels with TB susceptibility in Turkey.

Methods

This research is a case−control study comparing TB patients with healthy controls and was carried out in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee for Non-Invasive Clinical Research of the Faculty of Medicine of Cukurova University.

Study Groups

Thirty patients whose sputum samples were sent to the laboratory of the Medical Microbiology Department of the Faculty of Medicine, Cukurova University from the Chest Diseases outpatient clinic of Cukurova University Balcali Hospital and diagnosed with pulmonary tuberculosis and 30 healthy volunteers who were determined not to have tuberculosis were included. Subjects with chronic diseases such as asthma, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), diabetes mellitus (DM), and cancer were excluded.

For TB diagnosis, the samples were analysed both phenotypically and genotypically. The presence of mycobacteria was investigated microscopically and by cultivation on Mycobacterial growth indicator tube (MGIT) media using the BACTEC™ MGIT™ 960 automated system (Becton–Dickinson, NJ, USA) and on Löwenstein-Jensen (LJ) media. To confirm the diagnosis of mycobacteria by the polymerase chain reaction (PCR) test, the primers TB11 (5'-ACCAACGATGGTGTGTCCAT-3') and TB12 (5'-CTTGTC GAACCGCATACCCT-3') targeting the 441 bp region of the genus-specific hsp65 gene were used [9]. DNA isolation was

performed mechanically using the Mickle instrument (The Mickle Lab. Engineering Co. Ltd., Surrey, UK). PCR was also applied to the culture-negative sputum samples to determine the healthy group. In the samples for which the diagnosis of mycobacteria was confirmed, identification of MTBC was performed using the p-nitro-alpha-acetylamino-beta-hydroxypropriophenone (NAP) and MGIT TBc Identification tests (Becton–Dickinson Diagnostic, MD, USA). The diagnosis was confirmed by PCR using the INS1 (5'-CGT GAGGGCATCGAGGTGGC-3') and INS2 (5'-GCGTAG GCGTCGGTGACAAA-3') primers targeting the 245 bp region of the IS6110 gene specific for MTBC members [10]. The *Mycobacterium tuberculosis* H37Rv strain, which we previously isolated in culture and identified at the species level by DNA sequence analysis, was used as a positive control in identifying mycobacteria and MTBC.

Investigation of Host Factors in Blood Samples

Eight milliliters of venous blood samples were collected from both TB patients and healthy individuals in anticoagulant-containing tubes. Following the manufacturer's instructions (Sigma−Aldrich, Missouri, USA), mononuclear cell isolation was carried out using the "Ficoll-Paque density gradient centrifugation method" from 6 ml of the blood sample, followed by RNA extraction using the "TRI-Reagent method". The DNA was directly extracted from the remaining 2 ml using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The extracted DNA and RNA samples were measured by a NanoDrop ND-1000 device (Thermo Fisher Scientific, MA, USA), and the ratio of absorbance at 260 and 280 nm was used to evaluate their purity. DNA samples with a ratio between 1.7 and 1.9 were used to investigate the rs5743899A/G and rs3750920C/T SNPs of the TOLLIP gene and the −1082G/A SNP of the IL-10 gene. From the RNA samples with a purity ratio between 2.0 and 2.2, complementary DNA (cDNA) was synthesised using the MultiScribe Reverse Transcriptase enzyme (Thermo Fisher Scientific, MA, USA) following the manufacturer's instructions to investigate the TOLLIP mRNA gene expression levels.

Determination of the TOLLIP mRNA Expression Levels

As previously described [11], the TOLLIP mRNA expression levels were determined in cDNA samples synthesised from the RNA of mononuclear cells using the 7500 Fast Real-Time PCR System (Applied Biosystems™, TX, USA). The primer sequences targeting the 160 bp region of the TOLLIP gene were 5'-CTGATGCCAACAGTGTACCAG-3' (forwards) and 5'-ACATGTCCTGGATGGCTTTC-3' (reverse). The primer set (forwards: 5'-CTCGCGCTA CTCTCTCTTTCT-3' and reverse: 5'-TGCTCCACTTTT TCAATTCTCT-3') targeting a 185 bp region was used for β2-microglobulin, a "house-keeping" gene.

Detection of the rs5743899A/G and rs3750920C/T SNPs of the TOLLIP Gene

PCR-restriction fragment length polymorphism (PCR–RFLP) method was used to detect the rs5743899A/G and rs3750920C/T SNPs of the TOLLIP gene. For amplification, the primers targeting the 279 bp specific region of rs5743899 were 5'-GGCAATGGCATGGCCACCAGTGA-3' (forwards) and 5'-CCGATGCCCGCACACCTGTGTGAT-3' (reverse), while 5'-AGGCGTGCAGCTCACCGCGTAGGA -3' (forwards) and 5'-GAGAGCCTTCTCCATGGACGA CCGC-3' (reverse) were used to target the 169 bp region of rs3750920 [12].

After digestion of the rs5743899 region using the HhaI restriction enzyme, DNA bands of 125, 93 and 61 bp identified the “G” allele and bands of 218 and 61 bp identified the “A” allele. Similarly, after digestion of the rs3750920 region by the MspI restriction enzyme, bands of 117 and 52 bp identified the “T” allele and a 169 bp-size uncut single band identified the “C” allele.

Detection of the −1082G/A SNP in the Promoter Region of the IL-10 Gene

The −1082G/A polymorphism (rs1800896) in the promoter region of the IL-10 gene was detected by amplification refractory mutation system-PCR (ARMS-PCR). Two specific sense (forwards) primers and a common antisense (reverse) primer targeting the 258 bp region were used. The sense primers were 5′-CTA CTA AGG CTT CTT TGG GAG-3′ for the “G” allele and 5′-ACT ACT AAG GCT TCT TTG GGA A-3′ for the “A” allele, while the antisense primer (5′-CAG TGC CAA CTG AGA ATT TGG-3′) was joint for both alleles. As previously described, amplification was performed by preparing two separate PCR mixes for each sample to determine these alleles [13, 14].

Statistical Analysis

IBM SPSS Statistics for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA) was used for the data analysis. The categorical data were presented as numbers and percentages, while the numerical measurements were summarised as mean and standard deviation (median and range, where necessary). Analyses were performed at a 95% confidence interval, and the statistical significance level was set at 0.05. The association of the SNPs with susceptibility to TB (differences between healthy control

and patient groups) was examined using the Pearson chi-square test. The Hardy–Weinberg library in Hardy–Weinberg equilibrium R programming language version 3.5.1 was used to evaluate polymorphisms in terms of allele frequencies [15, 16].

To analyse the relationship of TOLLIP mRNA expression with susceptibility to TB, the expression levels of TB patients and healthy individuals were compared using the "delta delta threshold cycle (Ct)" method [17]. The first measurable amplification cycle in the quantitative real-time PCR method is defined as the "Ct" value. Delta Ct (ΔCt) values were determined by calculating the differences between the Ct averages of the TOLLIP gene and the reference housekeeping gene (β2-microglobulin) separately in both groups. The "delta delta Ct (ΔΔCt)" value was determined by calculating the difference between patient ΔCt and healthy control ΔCt. The TOLLIP gene expression fold difference between the two groups was calculated using the formula "2 − ΔΔCt". An expression fold difference of ≥ 1 was considered statistically significant.

The combined effect of TOLLIP mRNA expression levels and SNPs on tuberculosis susceptibility was analysed within each of the healthy control and patient groups by nonparametric "Kruskal−Wallis test" using the IBM SPSS Statistics 22.0 program.

Results

We determined the SNPs of TOLLIP rs5743899A/G, TOLLIP rs3750920C/T and IL-10 −1082G/A and the TOLLIP mRNA expression levels and compared them between the healthy control and patient groups. Of the patients included in the study, 14 (46.7%) were female, and 16 (53.3%) were male, while these rates were equal (50.0%) in the healthy control group. The distribution of the population by age is shown in Table 1. In the immune-genetic factors we investigated, no significant difference was found between the healthy control and patient groups regarding age and gender.

The Pearson chi-square test found no significant difference between the healthy and patient groups in the genotype distribution of the SNPs we investigated ($P > 0.05$ in all comparisons). Nevertheless, as a result of the evaluation of polymorphisms by Hardy–Weinberg equilibrium in terms of allele frequencies, the heterozygous (GA) genotype of the IL-10 −1080G/A polymorphism was observed to be significantly predominant in the patient group ($P = 0.02$) (Table 2).

In addition, the statistical analysis by the "ΔΔCt" method detected the TOLLIP mRNA expression level to be significantly higher in tuberculosis patients than in healthy controls (expression fold difference = 2.72) (Table 3). By analysis using the Kruskal−Wallis method, no significant relationship was found between TOLLIP mRNA expression

Table 1 Distribution of the population by age

Group	Mean	Standart deviation	Median	Minimum	Maximum
Healthy control (n = 30)	33,3333	8,01435	32,5000	21,00	50,00
Patient (n = 30)	33,6333	9,50,312	33,5000	16,00	55,00
Overall (n = 60)	33,4833	8,71,681	33,0000	16,00	55,00

Table 2 Distribution of the SNPs to allelic genotypes

SNP	Genotype	Healthy controls (n = 30)			Patients (n = 30)		
		Detected value	Expected value	p value*	Detected value	Expected value	p value*
TOLLIP rs5743899A/G	AA	12	13	0.423	10	10	0.874
	AG	16	13		15	15	
	GG	2	3		5	5	
TOLLIP rs3750920C/T	CC	8	8	0.777	8	8	0.883
	CT	15	15		16	15	
	TT	7	7		6	7	
IL-10 −1082G/A	GG	7	8	0.897	3	7	**0.020**
	GA	16	15		22	15	
	AA	7	8		5	8	

In the TB patients, the TOLLIP mRNA expression levels were significantly higher, and the heterozygous genotype of the IL10-1082G/A SNP was predominant

*$P \leq 0.05$ is considered statistically significant (Hardy–Weinberg equilibrium test)

Table 3 TOLLIP mRNA expression analysis by the "delta delta *C*t ($2^{-\Delta\Delta Ct}$)" method

Group	Average *C*t value		Delta *C*t (Δ*C*t)* value	ΔΔ*C*t value	Expression fold difference ($2^{-\Delta\Delta Ct}$)
	TOLLIP gene	Housekeeping gene (β2-microglobulin)			
Healthy	35.732	25.914	9.818	− 1.445	**2.72**
Patient	34.761	26.388	8.373		

In the TB patients, the TOLLIP mRNA expression levels were significantly higher, and the heterozygous genotype of the IL10-1082G/A SNP was predominant

levels and SNPs of TOLLIP and IL-10 genes, neither in tuberculosis patients nor in healthy controls ($P > 0.05$ in all comparisons).

Discussion

Previous studies separately associated TOLLIP and IL-10, which both negatively regulate the innate immune response, with susceptibility to TB [5–8, 18–28] and additionally revealed that TOLLIP induces IL-10 secretion [3]. However, in some studies, these factors could not be associated with TB susceptibility [5, 18, 19, 29, 30], while those associated were reported to differ socially or geographically [5, 19]. We investigated the association of the TOLLIP mRNA expression level and SNPs such as TOLLIP rs5743899A/G (in the intron region), TOLLIP rs3750920C/T (in the exon region), and IL-10 −1082G/A with susceptibility to tuberculosis. As a result of our study, there was no statistically significant difference in the allelic distribution of TOLLIP rs5743899A/G and TOLLIP rs3750920C/T SNPs between the healthy and patient groups. On the other hand, TOLLIP mRNA expression was found to be significantly higher in patients. Whereas there was no significant difference between the allelic genotypes of the IL10-1082G/A SNP in the healthy group, the heterozygous (GA) genotype was observed to be significantly dominant in the patient group. However, by evaluating these three polymorphisms and TOLLIP mRNA expression levels together, they were not associated with TB susceptibility.

In a multicenter study conducted in 2012, Shah et al. associated the rs5743899 and rs3750920 polymorphisms of the TOLLIP gene with TB susceptibility. They also detected a relationship between decreased TOLLIP expression and an increased risk of active TB. The researchers reported that the minor G allele of rs5743899 decreased TOLLIP expression, while the minor T allele of rs3750920 increased it [3]. In a study investigating the association of polymorphisms in TLR2, TLR4, and TOLLIP genes with susceptibility to tuberculosis in pulmonary TB patients, persons with latent TB infection, and healthy individuals among the Chinese population, TOLLIP rs5743899 was determined to be a risk factor, whereas TOLLIP rs3750920 had no relationship [18]. In another study conducted in China, the TOLLIP rs3750920 polymorphism was associated with susceptibility to TB in the Tibetan population, while the TOLLIP rs5743899 polymorphism was not associated with TB in any of the Chinese Han and Tibetan populations [19]. Different results from these studies may be due to different sample numbers and ethnic origins. Araujo et al. showed that these two TOLLIP polymorphisms separately increase the risk of the development of cutaneous leishmaniasis, which is caused by *Leishmania guyanensis*, an intracellular microorganism such as *M. tuberculosis* [12]. In our study, these TOLLIP polymorphisms were not associated with susceptibility to TB, while the increase in TOLLIP mRNA expression level in TB patients was statistically significant. When evaluating our results with the study conducted in 2012 by Shah et al. [3], both an increase and a decrease in TOLLIP mRNA expression appear to increase susceptibility to TB. In addition, considering that TOLLIP induces IL-10 secretion [3], it may indirectly cause TB bacillus survival.

In another study conducted by Shah et al. in 2017, infant monocyte cells were used to investigate the rs5743854 polymorphism, different from the rs5743899 and rs3750920 we used in our research, and the TOLLIP mRNA expression level. The researchers determined that the rs5743854 polymorphism reduced TOLLIP expression. They associated the minor GG genotype with TOLLIP deficiency, decreased BCG efficacy and increased risk of developing adult and latent TB [24]. The fact that decreased TOLLIP expression can increase susceptibility to TB even in adults becomes even more meaningful when considering the infant group vaccinated with BCG. In infants, decreased TOLLIP expression results in an increased inflammatory response due to reduced negative regulation. Therefore, the decrease in the number of live BCG bacilli following vaccination reduces vaccine efficacy and increases susceptibility to TB. On the other hand, it is thought that increased TOLLIP expression in adults raises negative immune regulation so that TB bacilli may escape the innate immune response. As mentioned above, TOLLIP expression was significantly higher in our study's patient group. The mean age of our study population was 33, and there were no infants in either group. In this condition, the balanced expression of TOLLIP, which is involved in the negative regulation of the human

innate immune response, appears to be more protective. Our data also support this opinion. Thus, we thought evaluating adult and infant groups together is crucial for brightening TB immunopathogenesis.

IL-10, an anti-inflammatory cytokine that regulates the T-cell response at the early stage of infection, has also been shown to play an important role in TB susceptibility [20, 31]. However, we found no significant direct relationship between the −1082G/A polymorphism of the IL-10 gene and susceptibility to TB. In Turkey, this SNP was previously associated with TB in two studies [21, 28] but not in another study [29]. In a study from India, Meenakshi et al. showed that this polymorphism is associated with susceptibility to TB [8]. As a result of a meta-analysis, Areeshi et al. reported that this polymorphism was not associated with patients with pulmonary TB in the Asian and African populations but with TB susceptibility in the Caucasian population [5]. In another meta-analysis including 18,553 participants from different races, 8625 of whom were TB cases, and 9928 of whom were healthy controls, the relationship between the −1082/−819/−592 polymorphisms of the IL-10 gene and susceptibility to TB was examined. The researchers concluded that the −1082 polymorphism might be associated with susceptibility to tuberculosis in the Caucasian race [6]. Both meta-analyses pointed out the effect of the IL10-1082 polymorphism on the Caucasian race.

Although we could not directly associate the genotypes of the IL10-1082G/A SNP with susceptibility to TB, the heterozygous GA genotype of this polymorphism was significantly predominant in our study's patient group. In a comprehensive study from Colombia, the AA genotype of this polymorphism was associated with pleural TB using sequence-specific primary polymerase chain reaction (SSP-PCR) [22]. A study from Brasil reported that in Xavante, a Brasilian indigenous group with high rates of anergy to the tuberculin skin test (PPD), the risk of anergy was found to be higher (1.5-fold) in the homozygous GG genotype of the IL10-1082 SNP than in those carrying the mutant A allele [23].

Furthermore, various studies have suggested conflicting insights into the effects of IL-10 on TB control after vaccination with BCG. Pitt et al. showed that antagonists of IL-10 may play a positive role in preventive vaccination against *M. tuberculosis* infection by regulating BCG-mediated memory development in mice [32]. In contrast, Ferreira et al. showed that IL-10 overexpression did not attenuate the control of M. tuberculosis infection in mice between 6 and 12 weeks of age after BCG vaccination [27]. These studies thus suggest that the IL10-1082G/A SNP may be a crucial mediator for determining the vaccine's efficacy in addition to its possible role in susceptibility to TB. To support this thought, there is a need for comprehensive studies that will also involve the age factor, as we mentioned above on TOLLIP.

One of the limitations of our study was that we did not investigate plasma or mRNA expression levels of IL-10, so we could not compare these cytokine levels with the polymorphisms. We also didn't examine the protein expression levels of TOLLIP between TB patients and healthy volunteers. Another limitation was the small sample size. In addition, it would be more rational and instructive to consider the age factor in further investigations of TB immunopathology.

Conclusions

We obtained statistically significant results despite limitations. In the TB patients, the TOLLIP mRNA expression levels were significantly higher, and the heterozygous genotype of the IL10-1082G/A SNP was predominant. However, no associations with TB susceptibility were detected when TOLLIP mRNA expression levels were analysed along with SNPs such as TOLLIP rs5743899A/G, TOLLIP rs3750920C/T, and IL-10 −1082G/A. Hence, detecting the TOLLIP mRNA expression level alone appears insufficient to predict TB susceptibility. Therefore, considering that local and worldwide studies in this field are lacking and that a single marker is mainly used, our research is original. Moreover, our study is the first to investigate the association of TOLLIP polymorphisms and expression levels with TB susceptibility in Turkey. In this respect, it will shed light on subsequent immunogenetic studies. More significant results will be achieved by evaluating the gene expression levels of TOLLIP and other negative regulators of innate immunity together with tuberculosis-associated polymorphisms and proinflammatory and anti-inflammatory cytokine levels in comprehensive studies involving adults and infants. We concluded that, in this manner, these factors can be used as biomarkers for TB susceptibility and may contribute to vaccine development studies.

Acknowledgements Not applicable.

Author Contributions T.N. and E.E. contributed to the conceptualisation and design of this study. T.N. managed the project. T.N., E.E., A.U., I.H., B.K., and F.K. contributed by collecting patient samples and clinical data. T.N., E.E., and A.U. contributed to the data curation, formal analysis, investigation, methodology, and writing of the original draft. H.B. and Y.S. participated in statistical analysis. All authors contributed to data interpretation, reviewing and editing the manuscript and approved the final submission.

Funding This study was supported by the scientific research projects coordination unit of Cukurova University, Adana, Turkey (Project No: TDK-2016–5493).

Data Availability All the data is provided within the manuscript.

Materials Availability All the data is provided within the manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical Approval The study was approved by the Ethics Committee for Non-Invasive Clinical Research of the Faculty of Medicine of Cukurova University (Date: 06.11.2015 Decision No:31). We contacted the patients and explained our study's aim and the protocol for collecting sputum and blood specimens. After acquiring the permission of the patients, we began our work.

Consent for Publication Not applicable.

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