

## Evaluation of Serum Interferon Regulatory Factors (IRF-1, IRF-2, and IRF-4) in Patients with Tularemia

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#### History

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### ABSTRACT


Tularemia caused by *Francisella tularensis*, is characterized by a complex interplay between the pathogen and the host's innate immune system. This study aimed to evaluate the diagnostic potential of serum interferon regulatory factors (IRFs) and their association with clinical outcomes in patients with tularemia. In this prospective case-control study, 35 adult patients diagnosed with tularemia at Cumhuriyet University Hospital (June–August 2024) and 30 age- and sex-matched healthy controls were enrolled. Serum levels of IRF-1, IRF-2, and IRF-4 were quantified using enzyme-linked immunosorbent assay (ELISA). Among the 35 patients (71.4% female; mean age 45.7±16.7 years), the oropharyngeal form was the predominant clinical presentation (80%). Serum levels of IRF-1, IRF-2, and IRF-4 were significantly higher in the patient group compared to controls ( $p < 0.001$ ). Receiver Operating Characteristic (ROC) analysis demonstrated that all three IRFs possessed high discriminative power between patients and healthy controls, with Area Under the Curve (AUC) values exceeding 0.80. Specifically, IRF-2 and IRF-4 exhibited sensitivities and specificities above 90% at optimal cutoff values. No statistically significant correlation was found between IRF levels and treatment failure or the requirement for lymph node dissection ( $p > 0.05$ ). Elevated serum levels of IRF-1, IRF-2, and IRF-4 reflect a robust systemic immune response against *F. tularensis*. These transcription factors, particularly IRF-2 and IRF-4, show promise as adjunct biomarkers for monitoring host immune activation during tularemia infection.

**Keywords:** *F. tularensis*, Innate Immunity, Interferon Regulatory Factors, Tularemia




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## 1. Introduction

Tularemia is a potent zoonotic disease caused by *Francisella tularensis* (*F. tularensis*), a highly infectious facultative intracellular bacterium [1]. Recognized globally as a potential bioterrorism agent, it is transmitted via direct contact with infected animals, contaminated water, soil, or arthropod vectors [2]. The clinical course of tularemia is notably heterogeneous; while many cases respond to standard antibiotic therapy, others progress to suppurative lymphadenitis requiring surgical intervention [3]. This variability suggests that the host's molecular immune landscape significantly dictates disease progression.

Interferons (IFNs) are pivotal cytokines in the antimicrobial arsenal, bridging innate and adaptive immunity [4]. The transcriptional regulation of IFN genes is orchestrated primarily by Interferon Regulatory Factors (IRFs). IRF-1, IRF-2, and IRF-4 are central to modulating the expression of IFNs and various pro-inflammatory mediators [5]. IRFs orchestrate the expression of IFNs and various antimicrobial proteins, thereby playing a central role in innate immune responses [6]. Additionally, many pathogens have evolved mechanisms to subvert IRF-mediated signaling pathways to evade host immunity [7].

Given this context, and considering the intracellular nature of *F. tularensis*, this study aimed to investigate the serum levels of IRF-1, IRF-2, and IRF-4 in patients with tularemia to explore their potential involvement in disease pathogenesis and host immune regulation.

While IRFs are traditionally recognized as intracellular transcription factors, recent evidence suggests that these proteins can be detected in the serum following extensive cell death, pyroptosis, or via release through extracellular vesicles during intense inflammatory states [8]. Given the intracellular nature of *F. tularensis* and its ability to induce host cell death [9], this study aimed to investigate the serum levels of IRF-1, IRF-2, and IRF-4.

## 2. Materials and Methods

### 2.1 Patient Selection

This study was designed as a prospective case-control investigation. A total of 35 patients aged 18 years and older, diagnosed with tularemia and followed at the Infectious Diseases and Clinical Microbiology clinics of Sivas Cumhuriyet University Hospital between June and August 2024, were enrolled in the patient group. The

control group consisted of 28 healthy individuals with no history of acute or chronic illness. Individuals under 18 years of age, pregnant women, and those in the lactation period were excluded from the study. For the patient group, only blood samples obtained at the time of initial diagnosis were included; lipemic, hemolyzed, insufficient, or pre-centrifugation delayed samples were excluded from analysis. The minimum sample size required to detect a medium to large effect size ( $d=0.75$ ) with a 5 % significance level ( $\alpha$ ), 80 % statistical power ( $1 - \beta$ ), and an allocation ratio of 0.8 ( $N1/N2$ ) was calculated to be 60 (33 patients and 27 control cases).

## 2.2 Diagnostic Criteria and Clinic Classification of Tularemia

The diagnosis and clinical classification of tularemia in this study were established according to the guidelines provided by the World Health Organization and the Ministry of Health Tularemia Field Guide (12,13). A case was considered tularemia-positive if at least one of the following criteria was met: isolation of *F. tularensis* in culture, a microagglutination test (MAT) titer of  $\geq 1/160$ , seroconversion or a fourfold or greater rise in MAT titers between paired serum samples taken at least two weeks apart, or a positive *F. tularensis* polymerase chain reaction (PCR) test in the presence of compatible clinical and epidemiological features [10,11]

Clinical forms of tularemia were categorized based on symptom presentation and physical examination. Patients presenting with fever, sore throat, and cervical lymphadenopathy, in combination with tonsillitis, pharyngitis, or oral mucosal ulcers, were classified as oropharyngeal tularemia. Oculoglandular tularemia was defined in individuals exhibiting conjunctivitis along with preauricular or cervical lymphadenopathy. Cases with isolated lymph node involvement were classified as glandular tularemia. When regional lymphadenopathy was accompanied by a skin ulcer at the site of inoculation, the ulceroglandular form was diagnosed. Pneumonic tularemia was identified in patients with lower respiratory tract symptoms and radiographic findings compatible with tularemia, in the absence of another identifiable etiology, and supported by serological confirmation. Finally, patients with non-localized systemic symptoms and laboratory evidence of tularemia, but without a focal clinical manifestation, were considered to have the typhoidal form [12].

## 2.3 ELISA Analysis of Serum IRFs

Serum concentrations of IRF-1 (E0429Hu), IRF-2 (E3333Hu), and IRF-4 (E3334Hu) were quantified using commercially available ELISA kits (Bioassay Technology Laboratory, China) according to the manufacturer's protocol. These kits utilize a double-antibody sandwich ELISA transition to ensure high specificity for the target human IRFs. Although IRFs are primarily intracellular proteins, their release into the systemic circulation during

acute infection due to cellular turnover or inflammatory signaling allows for their measurement in serum.

The detection ranges of the ELISA kits were 10-2000 ng/L for IRF-1, 20-3800 ng/L for IRF-2, and 5-1500 ng/L for IRF-4. The sensitivity of the assays was 4.82 ng/L for IRF-1, 9.54 ng/L for IRF-2, and 2.49 ng/L for IRF-4. The intra-assay coefficients of variation (CV) were documented as  $<8\%$ , while the inter-assay CVs were  $<10\%$ , ensuring high reproducibility. The manufacturer reported no significant cross-reactivity or interference with other cytokines or structural analogues. Samples exceeding the assay's detection range were reanalyzed following a 1:5 dilution.

## 2.4 Statistical Analysis

Descriptive statistics were computed to summarize the study variables, including mean, standard deviation, median, and quartiles for continuous variables and frequency and percentage for categorical variables. The Shapiro-Wilk test was employed to assess the normality of data distribution. Comparative analyses were conducted using the chi-square test for categorical variables. For continuous variables, group comparisons were performed with the independent samples t-test for normally distributed data or the Mann-Whitney U test for non-normally distributed data. Pairwise correlations between interleukin levels were evaluated using Spearman's rank-order correlation coefficient. Receiver operating characteristic (ROC) analyses were conducted to assess the performance of variables in predicting tularemia diagnosis. Due to the relatively small sample size, the ROC analyses were considered exploratory. While sensitivity and specificity values were calculated, these metrics represent preliminary diagnostic performance and warrant further internal and external validation in larger, independent cohorts. Statistical procedures were executed in IBM SPSS Statistics for Windows, Version 23.00 (IBM Corp., USA), and graphical representations were generated using GraphPad Prism, Version 8.3.0 (GraphPad Software, USA). A two-tailed p-value of  $<0.05$  was initially considered statistically significant. However, to account for multiple comparisons among the three IRF biomarkers and minimize the risk of type I error, a Bonferroni correction was applied, and a p-value of less than 0.0167 (0.05 divided by 3) was established as the threshold for significance in these primary comparisons.

## 3. Results

A total of 35 adult patients with accessible laboratory results and antibiotic treatments were included in the study. Of the patients 25 (71.4%) were female, and the mean age was  $45.7 \pm 16.7$  years. The most common form of tularemia was oropharyngeal tularemia (80%,  $n=28$ ) and glandular tularemia (14.3%,  $n=5$ ). Lymph node drainage or excision was performed in 15 patients (42.9%). Treatment failure was observed in 12 patients (34.3%), but no patient died. The clinical data of the patients are summarized in Table 1.

**Table 1. Demographic and clinical data of the patients and controls**

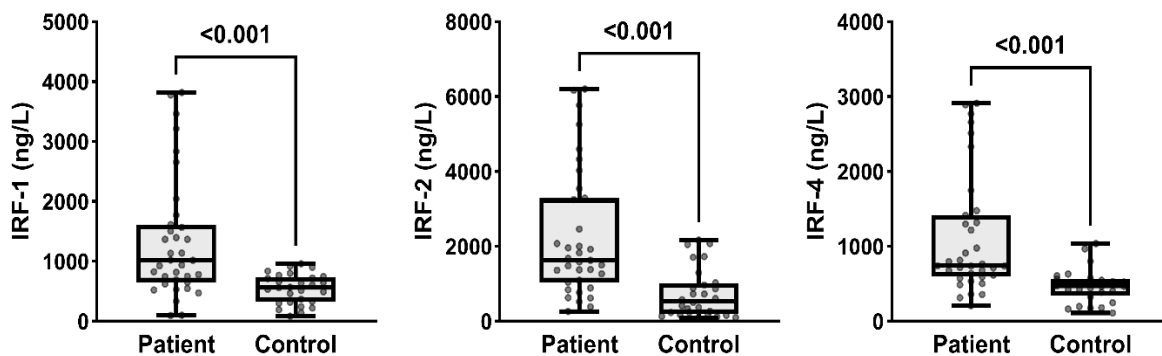
Variables	Values	
	Patients (n=35)	Controls (n=28)
Age (years)	45.7±16.7	37.3±12.3
Sex, female	25 (71.4%)	17 (%61)
MAT titer		
≤640	16 (45.7%)	
>1/1280	19 (54.3%)	
Tularemia form		
Oropharyngeal	28 (80%)	
Glandular	7 (20%)	
Presence of LAP	37 (92.5%)	
LAP region		
Cervical	36 (97.3%)	
Other	3 (8.1%)	
Antibiotic treatment		
Ciprofloxacin	26 (74.2%)	
Others	9 (25.7%)	
Duration of antibiotic treatment		
≤14 days	14 (40%)	
15-21 days	15 (42.9%)	
>21 days	6 (17.1%)	
Lymph node drainage performed	15 (42.9%)	

MAT: Microagglutination test

Categorical variables are presented as n (%), normally distributed numerical data as mean± SD

The levels of IRF-1, IRF-2, and IRF-4 were significantly higher in the tularemia patient group compared to the control group ( $p<0.001$ ) (Figure 1, Table 2). Moreover, IRF-1, IRF-2, and IRF-4 demonstrated high discriminative power between the patient and control groups, with area under the curve (AUC) values of 0.806, 0.826, and 0.821, respectively. Notably, IRF-1 exhibited a specificity exceeding 90% (Figure 2, Table 3). A moderate positive correlation was observed among all IRF levels (Figure 3). In our exploratory subgroup analyses, IRF-1 and IRF-4

levels were slightly higher in patients who did not require lymph node dissection compared to those who did; however, this difference was not statistically significant ( $p>0.05$ ) (Table 4). Similarly, no significant differences were observed in IRF levels between treatment responders and non-responders (Table 5). Given the small number of patients in these subgroups, these specific analyses should be considered preliminary and interpreted with caution due to limited statistical power.



**Figure 1. Comparison of serum interferon regulatory factor levels between the tularemia and control groups**

Table 2. Comparison of serum interleukin levels between the tularemia and control groups

Variables	Control group (n=28)	Tularemia group (n=35)	p-value
IRF-1	560 (319-722)	1018 (653-1613)	<0.001
IRF-2	503 (193-1031)	1633 (1042-3291)	<0.001
IRF-4	475 (387-564)	741 (596-1416)	<0.001

Variables are given as median and quartiles (Q1-Q3) and compared using the Mann-Whitney U test. Significant p-values are shown in bold.

Statistical significance was determined using a Bonferroni-corrected p-value of <0.016 to account for multiple comparisons of the three IRF biomarkers.

Table 3. ROC analysis results showing the strength of the variables in separating the tularemia and control groups

Variables	Cut-off value	AUC	Sensitivity (%)	Specificity (%)	PPV (+)	NPV (-)
IRF-1 (ng/L)	>918	0.806	57.1 (39.4-73.7)	96.3 (81.0-99.9)	95.2 (74.1-99.3)	63.4 (54.0-71.9)
IRF-2 (ng/L)	>1031	0.826	77.1 (59.9-89.6)	77.8 (57.7-91.4)	81.8 (68.5-90.3)	72.4 (58.0-83.3)
IRF-4 (ng/L)	>632	0.821	71.4 (53.7-85.4)	88.9 (70.8-97.6)	89.3 (73.7-96.1)	70.6 (58.3-80.5)

Variables were compared using the Mann-Whitney U test.

All AUC values were statistically significant ( $p < 0.001$ ), which remains below the Bonferroni-corrected threshold of  $p < 0.016$ .

AUC: Area under curve, PPV: Positive predictive value, NPV: negative predictive value

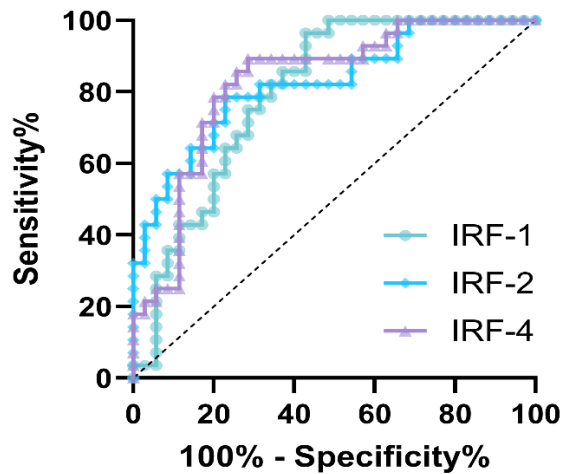


Figure 2. The discriminatory performance of predictive variables in stratifying tularemia and control groups: a ROC curve analysis

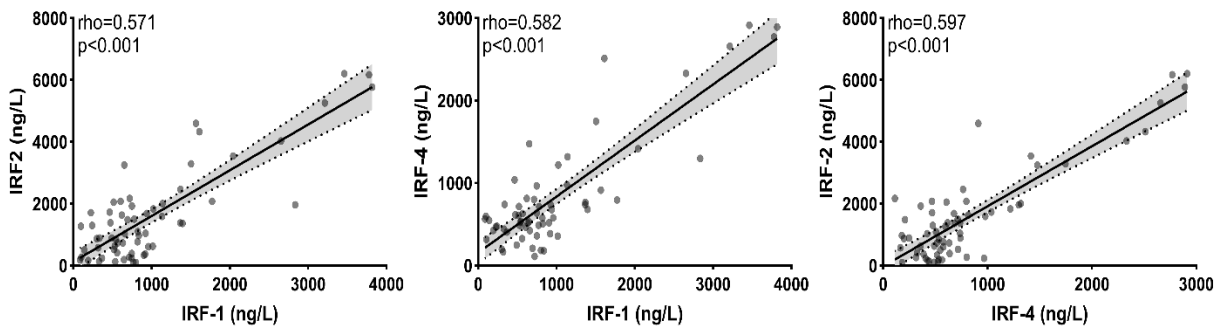


Figure 3. Spearman's correlation analysis of interferon regulatory factor

Table 4. Comparison of tularemia patients according to lymph node excision status

Variables	Lymph node excision (+) (n=15)	Lymph node excision (-) (n=20)	p-value
IRF-1	1023 (621-1397)	877 (674-2134)	0.882
IRF-2	1633 (630-2079)	1714 (1068-4181)	0.419
IRF-4	765 (617-1218)	739 (586-2040)	0.521

The variables were presented as median and quartiles (Q1-Q3) and compared using the Mann-Whitney U test.

Table 5. Comparison of patients with and without treatment response

Variables	Treatment response (+) (n=23)	Treatment response (-) (n=12)	p-value
IRF-1	874 (497-1264)	1141 (694-2043)	0.195
IRF-2	1442 (967-2561)	1927 (1094-3543)	0.526
IRF-4	727 (648-1483)	797 (506-1416)	0.905

<sup>a</sup>Compared with t test in independent groups. Other variables were determined by Mann-Whitney U test.

#### 4. Discussion

In this study, we investigated the serum levels of interferon regulatory factors (IRF-1, IRF-2, and IRF-4) in patients with tularemia to understand their potential roles in disease pathogenesis and treatment response. Our results revealed significantly elevated levels of IRF-1, IRF-2 and IRF-4 in tularemia patients compared to healthy controls. These findings suggest that IRF-1, IRF-2, and IRF-4 may play an essential role in the immune response to *F. tularensis*, a facultative intracellular pathogen. The moderate positive correlation observed among all IRFs suggests that these factors may be regulated through common or similar immunological pathways. This finding implies that evaluating the IRF family collectively may offer diagnostic value in the clinical context.

IRF-1 acts as a key regulator of innate and adaptive immune responses against infections [13]. Activated downstream of interferon signaling, IRF-1 initiates the transcription of cytokines and antimicrobial effectors, bridging pathogen recognition with effector responses [14,15]. In the specific context of *F. tularensis* infection, IRF-1 serves as a critical component of the host's last line of defense [16]. After entering macrophages, *Francisella* rapidly escapes the phagosome to replicate in the host cell cytosol. This escape triggers cytosolic DNA sensors, such as AIM2, which rely on IRF-mediated signaling specifically the induction of guanylate-binding proteins (GBPs) to initiate an effective response [17].

While IRF-1 deficiency has been linked to increased disease severity in various intracellular infections such as *M. tuberculosis* and *Brucella* [18,19], its role in tularemia is directly tied to restricting this cytosolic niche. IRF-1 is essential for inducing inducible nitric oxide synthase (iNOS) and other antimicrobial proteins that are vital for restricting the cytosolic replication of *F. tularensis* [20]. In line with these mechanisms, our study demonstrated significantly elevated IRF-1 levels in tularemia patients compared to healthy controls. This elevation likely reflects the host's intensive effort to

overcome the bacterium's sophisticated immune evasion strategies and its escape into the cytosol. However, IRF-1 levels did not differ significantly between patients who underwent lymph node dissection and those who did not, nor between treatment responders and non-responders.

The structural similarity between IRF-1 and IRF-2 allows for a competitive balance in the transcription of interferon-stimulated genes [20], a process that appears to be specifically activated during *F. tularensis* infection. Our findings of elevated IRF-2 levels in tularemia patients align with the host's need to control the potent Type I IFN response, which can be both protective and pathological during *Francisella* pathogenesis [20,21]. Given that *F. tularensis* is an intracellular pathogen that requires robust NK cell activity for clearance, the essential role of IRF-2 in NK cell differentiation [22] suggests its elevation is part of a broader recruitment of the innate immune system. However, similar to IRF-1, the non-significant association between IRF-2 levels and treatment outcomes suggests that IRF-2 expression is a fundamental part of the host's immune signature against *F. tularensis*, regardless of the clinical course. Consequently, IRF-2 appears to function as a systemic modulator of the anti-tularemia response rather than a biomarker of disease progression.

As a transcription factor primarily involved in adaptive immunity, IRF-4 governs the differentiation, polarization, and antibody-producing functions of T and B lymphocytes, which are essential for the long-term clearance of *F. tularensis* [23, 24]. Unlike IRF-1 and IRF-2, which regulate innate type I IFN responses, IRF-4 is indispensable for the development of Th17 and Th2 cell subsets [25,26] both of which are critical components of the host's protective response against *Francisella* infection [27]. Furthermore, IRF-4 acts as a master regulator of macrophage polarization, typically promoting an anti-inflammatory M2-like phenotype [28]. Since *F. tularensis* is an intracellular pathogen that survives by suppressing classical M1 macrophage activation, the expression of IRF-4 represents a key regulatory point: it may either serve the host by limiting immune-mediated tissue damage or be

exploited by the pathogen to facilitate its persistence within modulated macrophages. Experimental models of tularemia have demonstrated that IRF-4 deficiency leads to impaired T-cell responses and significantly increased susceptibility, underscoring its necessity for a balanced and effective immune defense [27].

Consistent with these regulatory roles, our findings of significantly elevated IRF-4 levels in tularemia patients suggest a robust mobilization of the adaptive immune system and a host-driven attempt to calibrate macrophage-mediated inflammation against *F. tularensis*. However, similar to our observations for IRF-1 and IRF-2, IRF-4 levels were not significantly associated with clinical outcomes, such as the need for lymph node dissection or treatment failure. It is important to emphasize that these subgroup analyses are likely underpowered due to the limited number of clinical events (n=12 for treatment failure and n=15 for surgery). Therefore, the absence of a significant association in this study should not be interpreted as definitive evidence that IRFs lack prognostic value; rather, it underscores the need for larger, multicenter studies to adequately assess their potential in predicting clinical outcomes. Consequently, IRF-4 appears to function as a critical immune modulator in the host's orchestrated response to tularemia, even if its current potential as a standalone diagnostic or prognostic biomarker remains limited.

Consistent with their elevated levels, our ROC analysis demonstrated high discriminative power for IRF-1, IRF-2, and IRF-4, with AUC values exceeding 0.80. However, these findings should be interpreted with caution due to the pilot nature of the study and the limited sample size, which may lead to overestimation of diagnostic accuracy. To confirm the stability of these biomarkers, future studies employing internal validation techniques (e.g., bootstrapping or cross-validation) and external validation in larger, multi-center populations are essential.

Despite the high discriminative power observed in our cohort, it must be emphasized that IRFs are not pathogen-specific markers. As key components of the interferon signaling pathway, elevated levels of IRF-1, IRF-2, and IRF-4 are likely present in various other intracellular bacterial, viral, or chronic inflammatory conditions. Therefore, these factors cannot replace gold-standard diagnostic tools such as PCR or MAT for *F. tularensis*. Instead, their clinical value may lie in providing real-time insights into the magnitude of the host's innate immune response and the extent of systemic inflammation.

In this context, it is also important to distinguish between general systemic inflammation and the specific IRF-mediated response. While common markers such as C-reactive protein (CRP) and leukocyte counts reflect a broad and non-specific acute-phase reaction, IRFs are essential components of the Type-I and Type-II interferon pathways. These pathways are specifically triggered by the presence of intracellular bacteria like *F. tularensis*. Therefore, the significant elevation of IRF-1, IRF-2, and IRF-4 observed in our study likely reflects a targeted molecular response to the pathogen's intracellular

survival mechanisms, rather than a non-specific byproduct of general inflammation. Assessing these transcription factors collectively may offer more granular insight into the host's cellular defense landscape compared to traditional inflammatory indices.

This study has several limitations that should be acknowledged. First, the sample size was relatively small, which may limit the generalizability of the findings and reduce the statistical power to detect subtle associations, particularly in subgroup analyses such as treatment failure or surgical requirements. Second, the study was conducted at a single center over a limited time frame, which may restrict its external validity. Third, IRF levels were measured at a single time point during the acute phase of illness; longitudinal measurements would have provided greater insight into the dynamic changes of these biomarkers and their prognostic value throughout the disease course and recovery. Fourth, our study focused exclusively on protein levels in the serum without performing mRNA expression analysis or cellular validation in peripheral blood mononuclear cells (PBMCs). Consequently, we cannot definitively determine whether the elevated serum IRF levels originate from active cellular secretion or are a passive byproduct of cell death, such as pyroptosis or necrosis.

Additionally, the subgroup analyses regarding treatment failure and surgical interventions were significantly underpowered due to the small number of patients in these categories, limiting our ability to draw reliable conclusions regarding the prognostic value of IRFs.

Furthermore, the functional roles of these transcription factors in the pathogenesis of tularemia remain speculative, as no mechanistic or experimental studies were conducted to clarify their specific involvement. Another significant limitation is the absence of a non-tularemia infectious control group (e.g., other bacterial or viral infections), which prevents us from confirming the disease-specificity of IRFs as biomarkers. Finally, the lack of an independent validation cohort limits the immediate clinical applicability of the reported diagnostic performance metrics.

This study has several limitations that should be acknowledged. First, the sample size was relatively small, which may limit the generalizability of the findings and reduce the statistical power to detect subtle associations, particularly in subgroup analyses such as treatment failure or surgical requirements. In fact, these subgroup comparisons were likely underpowered due to the limited number of events (n=12 for treatment failure and n=15 for surgery), which underscores the need for larger, multicenter studies to adequately assess the prognostic value of IRFs.

Second, the study was conducted at a single center over a limited time frame, which may restrict its external validity. Third, IRF levels were measured at a single time point during the acute phase of illness; longitudinal measurements would have provided greater insight into the dynamic changes of these biomarkers and their

prognostic value throughout the disease course and recovery. Fourth, important inflammatory covariates such as CRP, leukocyte count, or other routine laboratory markers were not incorporated into the statistical models. Consequently, we could not perform a multivariate analysis to determine whether IRF elevations are independent diagnostic markers or simply reflect general systemic inflammation.

Fifth, our study focused exclusively on protein levels in the serum without performing mRNA expression analysis or cellular validation in peripheral blood mononuclear cells. Consequently, we cannot definitively determine whether the elevated serum IRF levels originate from active cellular secretion or are a passive byproduct of cell death, such as pyroptosis or necrosis. Furthermore, the functional roles of these transcription factors in the pathogenesis of tularemia remain speculative, as no mechanistic or experimental studies were conducted to clarify their specific involvement. Another significant limitation is the absence of a non-tularemia infectious control group (e.g., other bacterial or viral infections), which prevents us from confirming the disease-specificity of IRFs as biomarkers. Finally, the lack of an independent validation cohort limits the immediate clinical applicability of the reported diagnostic performance metrics.

In conclusion, our results show that IRF-1, IRF-2, and IRF-4 levels are elevated in tularemia and may reflect host immune activation. Although the ROC analysis suggests a high diagnostic potential for these factors, it is important to note the exploratory nature of these findings. Given the small sample size, this diagnostic performance metrics require validation in larger cohorts to ensure their reliability and clinical applicability.

### Conflict of Interest

There are no conflicts of interest in this work.

### Ethical Approval Statement

This study was approved by the Research Non-Interventional Clinical Research Ethics Committee of Sivas Cumhuriyet University with the decision number 2024/05-63 on 16.05.2024. All procedures performed in this study involving human participants adhered to the ethical standards of the institutional and national research committees and the 1964 Helsinki declaration and its later amendments. Written informed consent was obtained from all participants prior to their inclusion in the study.

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