



DETERMINATION OF CASEOUS NECROSIS TO LYMPHOCYTE DENSITY IN HISTOPATHOLOGICAL RESPONSE OF HOST IMMUNE SYSTEM AGAINST MYCOBACTERIAL PATHOGENS

Faiza Wattoo^{1*}, Farhan Javed², Tabinda Roheen³, Khadija Saleem⁴

^{1*,3}Assistant Professor, University Medical and Dental College, University of Faisalabad

²Associate Professor, Madeena Teaching Hospital, Faisalabad

⁴Associate Professor, University Medical and Dental College, University of Faisalabad

***Corresponding Author:** Faiza Wattoo

*Assistant Professor, University Medical and Dental College, University of Faisalabad

Email: drfaizafarhan100@gmail.com

ABSTRACT

In mycobacterium infection, the characteristics of necrosis and inflammatory cells vary among host species and its hallmark in the diagnosis of a particular infectious disease remains unclear. Therefore, this study aimed to identify and characterize the morphological features of caseous necrosis with lymphocytes against mycobacterial pathogens in humans and animals. During this study, a total of 126 extrapulmonary tissue samples, and demographic data of individuals and animals suspected of mycobacterium infection in the Faisalabad district were collected and processed further at Al-Shifa Diagnostic and Research Laboratory, Faisalabad. In the results, the frequency analysis (%) showed that there were 21, 46, 38, and 21 *Mycobacterium* (MB) positive individuals in the 20-30, 31-40, 41-50, and 51-60 age groups. The maximum number of MB-positive individuals were in the 31-40 age group with a maximum number of females and males were more in the 41-50 age group. The presence of caseation necrosis in 110 (88%) individuals. The examination of the hematoxylin and eosin-stained slides revealed the presence of caseation in 22 (88%) and 3 (12%) showed no caseation. It is concluded that the host immune response is manifested with the development of caseation necrosis with lymphocyte density and is an applicable comprehensive approach to comparing histopathological markers in both animals and humans.

Keywords: Bacterial infection, Inflammatory response, Diagnostic tool, Frequency analysis, Lymphocytes

INTRODUCTION

Tuberculosis is a chronic progressive granulomatous infectious disease caused by a gram-positive, acid-fast bacillus classified under the genus *Mycobacterium* harboring over 170 species, the only genus in the family Mycobacteriaceae (Orgeur *et al.*, 2024). Tuberculosis an unremarkable ubiquity, has plagued humanity for centuries, and surpassed all the aetiologies of respiratory illnesses, claiming it to be the leading cause of death from infectious disease in adults worldwide (Kahase *et al.*, 2020). The 2020 global report published in October, ranked Asia as the third burden-carrying region with Pakistan having 5.7% of the total. This rising morbidity and mortality toll in Pakistan has affected our citizens' social and economic well-being as it ranks as a social barometer of welfare (Chakaya *et al.*, 2021).

The primary site of infection in humans is lung 95% and 5% extrapulmonary sites. The infection from aerosols results in the formation of tubercles in the lungs consisting of central caseation necrosis surrounded by epithelioid histiocytes, lymphocytes, and langhan type of giant cells surrounded by a rim of fibroblasts (Abou *et al.*, 2022). Patients show aggressive symptoms of cough, blood-tinged sputum, fever, fatigue, night sweats, anorexia and weight loss. During reactivation, the bacilli disseminate rapidly to regional lymph nodes, bones and joints, kidneys, brain and meninges, and genital tract (Muller *et al.*, 2013). In Pakistan pleural and lymphatic dissemination accounts for 50%, osteoarticular 9.3% in which the proportion of spinal tuberculosis is higher, central nervous system 6.0% of children and 4.2% of adults; abdominal tuberculosis 15%; Renal and genitourinary 08% presenting in a wide range of symptoms from simple cough and weight loss to intestinal obstruction in long-standing cases (Kumar and Kapoor, 2023).

Mycobacterial pathoadaptation is assisted by the modularity, flexibility, and interactivity of its cell wall components and virulence factors. After gaining access into the body, it lodges into the host i.e., primary infection; based on reprogramming the host defense by altering the capability of macrophages to clear or combat the bacilli, the formation of a well-defined granuloma in equilibrium with the host-defense, control of host cell metabolism and replication characterizing the so-called dormant state in which mycobacterium can stay for as long as the host defense downfalls to favor its flourish and replication (Pepperel, 2022).

The progression and disease resolution can generally be divided into four phases. The first stage lasts from 3 to 8 weeks, in which the aerosolized bacterium gets implanted into the alveoli and engages with the innate immune system within the alveolar spaces. Macrophages and dendritic cells ingest the bacteria and further recruit new immune cells thus activating the adaptive immune arm. Here the bacteria may get disseminated by lymphatic circulation, forming a Gohn or primary complex (Kalscheuer *et al.*, 2019).

The definitive diagnosis rests on histological and bacteriological evidence of tuberculosis in tissue specimens stained with H&E. Granuloma, the compact aggregate of immune cells is a hallmark structure of tuberculosis and can be seen with a microscope (Faiza *et al.*, 2022). This diffuse mixture of inflammatory cells including lymphocytes, plasma cells, circulating monocytes that convert into histiocytes, and multinucleated langhan giant cells with central caseation necrosis circled by the rim of fibroblasts represents the body's immune response to pathogen attack. Therefore, this study aimed to determine the relationship between the microscopic features of this central caseous necrosis and lymphocyte density in patients with extrapulmonary tuberculosis.

MATERIALS AND METHODS

Sample collection and processing: From July 2021 to December 2023, samples were collected, labeled properly, and transported to the Al-Shifa research and diagnostic center (BSL-III Lab), located at Jail Road, near Allied Hospital, Faisalabad (according to standard protocols/guidelines) for further processing, limited to the inclusion and exclusion criteria. In this study, a total of 206 suspected individuals were given purified protein derivative (PPD), and wheal formation was observed. A total of 126 individuals showed positive results with a desired wheal formation observed after 24, 48, and 72 hours. Sputum samples from these 126 individuals were taken and inoculated on Lowenstein Jensen (LJ) media and kept for 6 weeks. Demographic characteristics i.e., age, gender, vaccination status, educational status, and various social and environmental factors were recorded in a preformed questionnaire. Out of the total 126, only 25 individuals were positive for extrapulmonary tuberculosis. Out of the total 126, 77 (61.1%) were males and 49 (38.9%) were females. The patients were divided into four age groups i.e., 20-30 years, 31-40 years, 41-50 years, and 51-60 years. Assessment of the lymphocyte density was carried out by counting the number of lymphocytes in ten high power fields per slide, under high magnification (400X) around the area of central caseation necrosis. The number of lymphocytes was recorded, the mean and median values were calculated, and the overall mean value was taken as a cut-off point. To avoid miscalculation and inter-observer variation, the histopathological slide examination was carried out by the same person.

Statistical analysis: The obtained data was analyzed by using SPSS® to determine the frequency of demographic characteristics (variables).

RESULTS

Occurrence of *Mycobacterium* infection during various age groups: The frequency analysis (%) showed that there were 21, 46, 38, and 21 *Mycobacterium* (MB) positive individuals in 20-30, 31-40, 41-50, and 51-60 age groups. The maximum number of MB-positive individuals were in the 31-40 age group with a maximum number of females and males were more in the 41-50 age group (Table 2). These age groups were further observed for the occurrence of comorbidities including diabetes, hypertension, hepatitis, and cardiovascular diseases. The frequency analysis (%) showed that a total of 73 individuals were suffering from comorbidities whereas 53 individuals had no chronic conditions. In the 20-30 age group, a total of 11 (52.4%) individuals were suffering from comorbidities and 10 (47.6%) individuals had no chronic comorbidities. In the 31-40 age group, 31 (67.39%) individuals had comorbidities, and 15 (32.60%) individuals had no comorbidities. In the 41-50 age group, 17 (44.7%) individuals had comorbidities, and 21 (55.3%) individuals had no comorbidities. In the 51-60 age group, 14 (66.75%) individuals had comorbidities and 7 (33.3%) individuals had no comorbidities (Table 2).

Occurrence of *Mycobacterium* infection in study population based on their educational status: The frequency analysis showed that a total of 49 (38.88%) individuals were illiterate followed by 23 (18.25%) individuals who could read and write only, 19 (12.70%) individuals had only junior grades, 15 (11.90%) had middle grades, 12 (9.53%) had senior grades and 8 (6.40%) were graduated (Table 3).

Occurrence of *Mycobacterium* infection in comparison to residence based on income per capita of study population: The frequency analysis showed that the study population was mostly from rural areas 66.7% (84/126) draining into Faisalabad as compared to urban areas 33.33% (42/126). The income per capita of the population was not enough in 44.4% (56/126), 31.0% (15/126) had taken loans from banks or other individuals of the society, 11.9% (16/126) had enough income per capita and 12.7% (16/126) had savings (Table 4).

Occurrence of *Mycobacterium* infection in comparison to family size regarding crowding index of study population: The frequency analysis showed that about 53.17% (67/126) of the individuals had more than five members in a family and 46.82% (59/126) had individuals under five. However, the crowding index of people living in a single home was significantly higher in which 53.17% (67/126) lived as more than four residents per living room 27.77% (35/126) of the individuals lived as 2-4 residents per room and 19.04% (24/126) lived as less than two residents in a single room. Overall, the total number of living rooms in 89.0% of the study population was two or less with the highest number of living rooms being three in only 4.0% of the population. The lowest household crowding index was 3.0 whereas the highest general crowding index was 12.0 (Table 5).

Distribution (%) of central caseation necrosis and lymphocyte density: The examination of the hematoxylin and eosin-stained slides revealed the presence of caseation in 22 (88%) and 3 (12%) showed no caseation (Table 6). However, the mean value of lymphocytes per 10 high-power fields was 128 to 41 in the 25 samples. As the cut-off value, 67 was taken as the median value in these 25 samples examined. The densities were calculated and grouped into high and low considering 67 as the cut-off value. Samples with a lymphocyte count of 67 and above were considered high density and those with a count of lower than 67 were considered low density. The distribution of high density of lymphocytes was considered in 19 (76%) samples and low density was considered in 6 (24%) samples (Table 7). The microscopic examination of the lymph node revealed multiple granulomas with central caseation necrosis surrounded by cuffs of lymphocytes and occasional multinucleated

giant cells (Fig. 1A; 10X; H&E). The granuloma with central caseation necrosis, lymphocytes, epithelioid histiocytes, and occasional langhan type of multinucleated giant cells surrounded by a rim of fibroblasts (Fig. 1B; 40X; H&E). Furthermore, the granuloma showed clusters of curved beaded rods of acid-fast bacilli scattered between necrotic debris and necrotic debris of caseation necrosis in a tissue section with moderately scattered acid-fast bacilli (Fig. 1D; 40X; Zn stain).

Table 1: Frequency and number of positive to negative samples of *Mycobacterium* pathogen during different sampling techniques

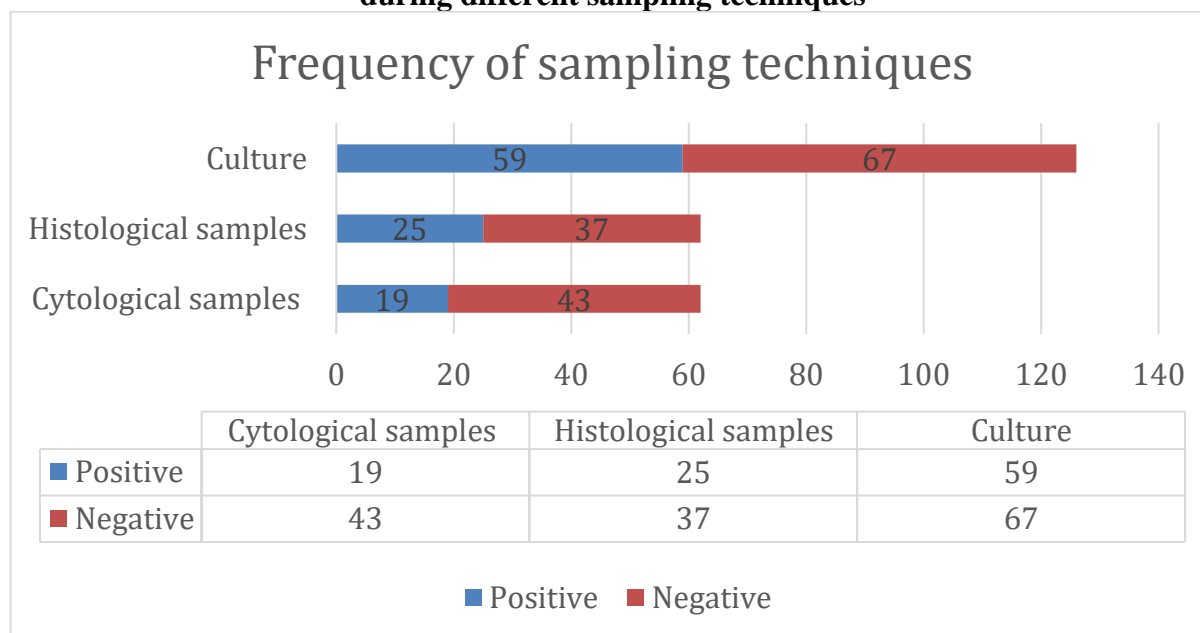


Table 2: Frequency analysis (%) of occurrence of *Mycobacterium* infection during various age groups

Age (years)	Comorbid		Total
	Yes	No	
20 – 30	11	10	21
31 – 40	31	15	46
41 – 50	17	21	38
51 – 60	14	7	21
Total	73 (57.9%)	53 (42.1%)	126

Table 3: Frequency analysis (%) of occurrence of *Mycobacterium* infection in males and females based on their educational status.

Educational Status	Gender		Total
	Male	Female	
Illiterate	29	20	49 (38.90%)
Read and write	14	9	23 (18.25%)
Junior	11	8	19 (12.70%)
Middle	7	8	15 (11.90%)
Senior	9	3	12 (9.53%)
Graduate	7	1	8 (6.40%)
Total	77	49	126

Table 4: Frequency Analysis (%) and occurrence of *Mycobacterium* infection in comparison to residence based on income per capita of the study population.

Income per capita	Residence		Total
	Urban	Rural	
Not enough	17	39	56
% within-residence	40.5	46.4	44.4%
% of total	13.5	31.0	
Not enough and loan	11	28	39
% within-residence	26.2	33.3	31.0%
% of total	8.7	22.2	
Enough	7	8	15
% within residence	16.7	9.5	11.9%
% of total	5.6	6.3	
Enough and saving	7	9	16
% within residence	16.7	10.7	12.7%
% of total	5.6	7.1	
Total	42	84	126
% of total	33.3	66.7	

Table 5: Frequency Analysis (%) and occurrence of *Mycobacterium* infection in comparison to family size regarding the crowding index of the study population.

Family size	Crowding index			Total
	< 2	2 – 4	> 4	
1 – 4	11	20	28	59
≥ 5	13	15	39	67
Total	24 (19.04%)	35 (27.77%)	67 (53.17%)	126

Table 6: Frequency and distribution (%) of central caseation necrosis and lymphocyte density in human-origin sputum samples

Microscopic features		Frequency	Percentage (%)
Caseous necrosis	Positive	22	88
	Negative	3	12
Lymphocytes density	High	19	76
	Low	6	24

Table 7: Relationship of central caseation necrosis with lymphocyte density in human-origin sputum samples

Microscopic features		Lymphocytes density		<i>P-value</i>
		High	Low	
Central caseous necrosis	Positive	16 (84.21%)	3 (50%)	0.001
	Negative	3 (15.78%)	3 (50%)	
Total		19	6	

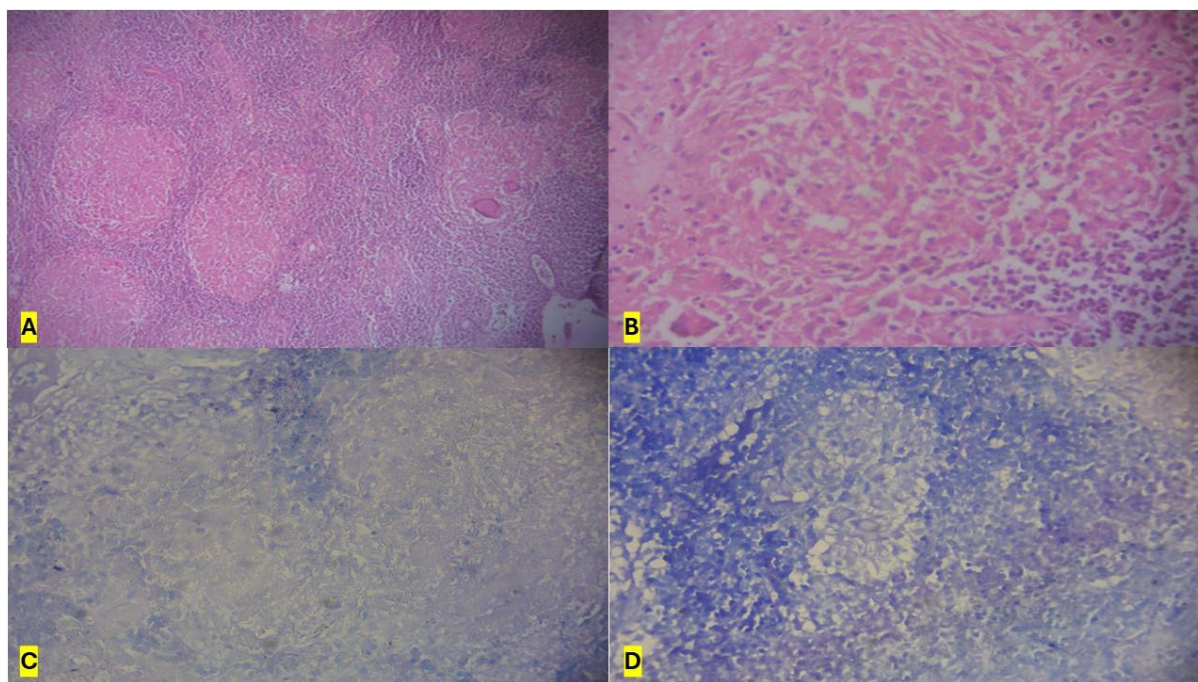


Fig. 1: Photomicrographs of extrapulmonary sites showing central caseation necrosis and lymphocytes scattered around with occasional Langhan type of multinucleated giant cells.

DISCUSSION

Tuberculosis control and eradication require high levels of leadership, investments, and uptake of rapid methods of diagnosis and treatment (Chauhan *et al.*, 2023). At the global level, the incidence declined by 6-26% between 2015 and 2020 as a result of refined control programs and recalibration of strategies toward ending tuberculosis. WHO Global Tuberculosis Report highlights the raised toll of diagnosis and treatment of 7.5 million lives since the year 2000. A single active case infects several other individuals due to a delay in diagnosis and poses a significant burden on the economy of a health system (Orgeur *et al.*, 2024). Hence management and early diagnosis are vital to curbing the TB burden, particularly in the high-burden countries in south-east Asia. Gender predilection in the results revealed 61.1% males and 38.9% females out of the total sampling population of 126 individuals (Salari *et al.*, 2023) showed similar findings i.e., 76.2% males and 23.8% females. However, another study showed that females were more prone to develop extra-pulmonary tuberculosis as compared to males with an overall prevalence of 20.9% with a rising trend from 22.6% in 2016 to 27.9% (Rolo *et al.*, 2023).

A cross-sectional community survey conducted in East Africa yields the old age burden of the disease accounting for 23000 cases in age of more than 50 years in 5 years (Obeague and Onuoha, 2023). Deteriorating immunity with increasing age is one factor that contributes to a high burden of tuberculosis. Prevalence per 100,000 population showed an increasing trend with age in twelve surveys in Africa however, the absolute number of cases was significantly higher in individuals aged 35-44 years (Law *et al.*, 2020). These findings are consistent with the results showing a higher trend in ages 31 to 40 years irrespective of gender. Similarly, comorbidities predispose individuals to acquire infection rapidly as 57.9% of individuals in our study revealed a high occurrence rate of the disease as compared to 42.1% of the population who had no comorbidities. This observation is also seen in a study conducted in India that revealed a 38.9% increased incidence of tuberculosis in people with comorbidities (Gouda *et al.*, 2021).

The innate and adaptive immune cells collaborate to eradicate the bacteria or restrict its active replication within a granuloma. At this time, the tuberculin reactivity occurs. The second stage, which lasts for 3 months, is marked by the hematogenous spread to various parts of the lungs, and also to the distant organs including the brain, spinal cord, gastrointestinal system, genital organs, and skin called miliary tuberculosis (Nyarko *et al.*, 2021). The third stage lasts for 3 to 7 months but can be

delayed up to 2 years. Pleurisy or inflammation of the pleural surfaces is the hallmark of this stage characterized by the subpleural concentrations of the bacteria in the lung and the interactions of its components with the sensitized CD4 T lymphocytes that further release inflammatory cytokines (Kahase *et al.*, 2020). This stage fails to control the infection and is associated with high rates of cell death. The last resolution stage may take up to 3 years, in which the extrapulmonary lesions e.g., those in the bones and joints take place (Saini *et al.*, 2020).

A cross-sectional survey in Gambia, targeting 4309 individuals with 59.2% (2553) females and 60.7% (2614) males showed that almost 83% (3617) did not know about tuberculosis as most of these had no formal education (Bashorun *et al.*, 2020). Another study revealed that the baseline knowledge about the disease was only seen in 54% of the individuals out of the sample size of 236 individuals (Kalscheuer *et al.*, 2019). These results are to the findings in our study that revealed 38.90% of the individuals were illiterate and could not read or write, 18.25% could read and write, 12.7% attended junior school, 11.90% attended middle school, 9.53% attended senior school and only 6.40% were graduates. Likewise, 66.7% of the affected population was residing in rural areas as compared to 33.33% residing in urban areas. This finding is inconsistent with a study from India that revealed a 72.2% prevalence in rural areas as compared to 40.8% in urban areas (Singh and Ramamohan, 2020). However, another study conducted in Indonesia revealed a higher prevalence in urban areas than in rural areas per 100,000 population (Noviyan *et al.*, 2021). This difference exists as the living conditions and income per capita of Southeast Asian countries like India and Pakistan is significantly low as compared to Indonesia which is the world's fourth most populous and 10th largest economy in terms of purchasing power parity (Law *et al.*, 2020).

There has always been a direct and documented connection between income and health access as more privileged societies have better access to medical care and therefore people who face financial hardship have increased vulnerability to disease (Muller *et al.*, 2013). Similar results appeared in this study yielding 44.4% of the population with not have enough income, 31.0% of the population who had not enough income and had taken loans from others, 11.9% of the individuals with enough income per capita and only 12.7% had savings. The high burden of tuberculosis relates directly to living standards in terms of crowding, poor ventilation, and increased transmission. The risk of progression from exposure to disease is increased in overpopulated and crowded spaces further deteriorating the situation (Trajman, 2024). In this study, 53.17% population with a crowding index of 4 individuals per room had more prevalence as compared to 27.77% of people with a crowding index of 2-4 individuals per room and 19.04% of people with less than 2 individuals per room.

The hallmark of tuberculosis is the establishment of clusters of immune cell-enriched aggregates called granulomas (Sawyer *et al.*, 2023). Based on histopathological examination of the hematoxylin and eosin-stained slides of the cases of extrapulmonary tuberculosis, it was found that caseation necrosis was found in 22 (88.0%) cases and 3 (12%) showed no caseation. The formation of central caseation necrosis determines the cellular hypersensitivity to invading (Orgeur *et al.*, 2024). Mycobacterial antigen is a special type of coagulated necrosis that is characterized by an unstructured granular red staining substance. The inflammatory response is amplified by the accumulation of cells of immune response giving rise to granulomas (Rojas *et al.*, 2023). The inner layer of granulomas contains macrophages and CD4+ T cells and the outer layer comprises CD8+ T cells (Singh and Ramamohan, 2024). The cells in the middle of granuloma necrotize eventually while the outer part becomes fibrotic which also signifies that the disease progression is towards chronicity. The tuberculous granuloma and central caseation are a classic example of delayed hypersensitivity response (Shivekar *et al.*, 2020). Lymphocytes are the main component of this hypersensitivity response. However, the decrease in the number of these cells reflects a dysfunction in the host's immune response. The reactivity of the immune cells towards an invading pathogen depends on the type of the pathogen, its activity, and the resulting damage (Pepperell, 2022).

Failure of appearance of granulomas in 03 cases depicts decreased host response and immunity. Moreover, high lymphocyte density in 19 (76%) cases highlights the essential role of inflammatory cells in evading the pathogen (Li *et al.*, 2023). Further elaboration of the characteristics of granulomas

revealed high lymphocyte density (84.21%) in low-density cases where there was no caseous necrosis. This proves the findings of Winchell *et al.*, 2023 who pointed out that depletion of CD8+ T cells allowed increased establishment of Mtb in lungs and dissemination into the body. Granulomas are designed to contain and eliminate the pathogen while the surrounding inflammatory cells prepare to control and fight the infection (Singh, 2024). However, mycobacterial antigens can proliferate due to immune response evasion mechanisms facilitating dissemination into the blood and extrapulmonary organs. The different ratios depict the intensity of the immune response and describe the occurrence of a defective immune response. The limitations of this study include a smaller sample size of the histopathological specimens and failure to use specific/special or immunostaining techniques to further isolate the inflammatory cells into different classes.

Conclusions: The host immune response manifested as caseation necrosis has a significant relationship with lymphocyte density in cases of extrapulmonary tuberculosis. The identification of factors contributing to acquiring tuberculosis and predictors for treatment enables strategized planning and collective intervention to be targeted at the high-risk areas in Pakistan to strengthen TB care and control. Monitoring of the risk factors facilitates our understanding of the distribution of strains of mycobacterium in different species and helps target the interventions to control and eradicate the deadly strains causing significant morbidity and mortality.

Ethical statement: This study was approved by the Institutional Biosafety Committee (IBC) of the University of Agriculture, Faisalabad (1021/ORIC, dated: July 2021) and the Human Research Ethic Review Committee of Madina Teaching Hospital, The University of Faisalabad (TUF/2021/92, dated: August 2021).

Statement of Novelty:

This study provides a comparative analysis of the histopathological response, specifically the relationship between caseous necrosis and lymphocyte density, in both animal and human immune systems against Mycobacterial pathogens. By examining these responses side-by-side, we aim to uncover unique and shared immunopathological mechanisms across species, offering insights into potential cross-species therapeutic strategies and contributing to a deeper understanding of Mycobacterial pathogenesis. This research is novel in its comprehensive approach to comparing histopathological markers in both animals and humans, which has not been extensively explored in previous studies.

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