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Frequency of Mycobacterium tuberculosis in Pneumonia Patients and Prevalence of Virulence Genes

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ABSTRACT

Tuberculosis is among the top 10 leading causes of death worldwide (TB). Tuberculosis is caused by the bacteria Mycobacterium tuberculosis. Acute pulmonary TB manifests as acute tuberculous pneumonia (TP). The development of drug-resistant TB, however, continues to pose a problem for TB treatment and efficient disease management. Different mutations in M. tuberculosis can lead to isolates that are rifampicin-resistant. The genes katG, inhA, and inhA's promoter are all involved in cell wall production, and most INH-R strains have mutations in these genes. The purpose of this research was to determine how often M. tuberculosis infection in patients suffering from pneumonia and also find the prevalence of Rifampicin and Isoniazid resistance isolates, and also to correlate the infection with the lipid profile of patients. This study included 200 subjects who are suffering from severe pneumonia. DNA have been extracted from all samples and then M. tuberculosis detection have been done by qRT-PCR. Lipid profile have also been estimated for all the included samples. Then detection of the antibiotic resistance have also done by qRT-PCR. The distribution of samples showed a significant difference among the different infection periods (chi-square= 44.19, P-Value= <0.001). The results showed that (7%) of the positive samples are resistance to Rifampicin. while resistance to Isoniazid are shown in 6.5% of the samples. The results of lipid profile showed a significant higher level in positive samples than negative samples regarding the serum level of total cholesterol (196.90 VS. 172.86, respectively), triglyceride (195.61 VS 152.33, respectively), and low density lipid (121.03 VS. 98.14, respectively).

Keywords: *isoniazid, rifampin, pyrazinamide, ethambutol, INH, rpoB*

INTRODUCTION

The extremely infectious disease tuberculosis (TB) is caused by the mycobacterium tuberculosis (Mtb), which primarily affects the lungs to form pulmonary TB and other body locations to induce extra pulmonary TB (Ravimohan et al. 2018). HIV/AIDS, to provide just one example, is the result of a number of factors. largest cause of mortality from a single infectious pathogen (WHO 2018). To lessen new Mtb infections and the progression to full-blown TB disease is critical because to the high rates of morbidity and death caused by this disease. (Singh et al. 2020). By assuring prompt and accurate TB diagnosis, proper infection control It is feasible to halt the development of drug-resistant TB in TB treatment facilities by reasonable and suitable pharmaceutical utilization for therapy and patient adherence to drug regimen. (Mase and Chorba 2019).

However, the rise drug-resistant tuberculosis is a major threat to public health worldwide and an obstacle to efficient disease control and TB treatment (Raviglione and Sulis 2016). The development of drug resistance in Mtb is facilitated by a number of mechanisms, including as compensatory evolution, epistasis, clonal interference, permeability of the cell membrane, efflux pumps, drug degradation and modification, target mimicry, and phenotypic drug tolerance (Rojas Echenique et al. 2019). That's why it's so important to investigate the genetic, molecular, and biochemical bases of resistance. order to develop novel treatment approaches and battle drug resistance (Mc Carlie, Boucher, and Bragg 2020).

The conventional TB therapy regimen currently consists of Isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) are the four first-line antibiotics that, when given to a patient in the correct dosages, eliminate the risk of transmission of tuberculosis.

Patients who do not get adequate treatment may develop drug-resistant MTB (acquired resistance), which may then transmit to others (primary resistance) (Micheni et al. 2021). Multidrug-resistant tuberculosis (MDR-TB), which is defined as resistance to both isoniazid (INH) and rifampin (RIF for treating TB), is extremely concerning since second-line antimycobacterial medications need to be used over an extended period of time, are costly, and have a number of adverse effects (Zetola et al. 2014).

Rifampicin, lipiarmycin, streptolydigin, and microcin J25 all target the rpoB gene (MccJ25). In M. There were 15 unique mutations in 8 conserved amino acid residues clustered in a 23-amino acid region in 64 of 66 rifampicin-resistant TB isolates. found (Zeng, Jia, and Tang 2021). Bacterial detection and identification are made possible by the robust, repeatable, and accurate rpoB gene sequencing. Additionally, it could make it possible to reclassify taxa and find previously undetected organisms in bacterial communities. According to Adékambi, Drancourt, and Raoult (2009), rpoB gene has phylogenetic resolution that is generally superior to 16S rRNA gene.

Prodrug INH is converted into active INH by the catalase-peroxidase KatG. Metabolites eventually interact with nicotinamide adenine dinucleotide (NAD+), and bind to the NADH-dependent enzyme InhA, which is responsible for enoyl-ACP reductive reactions. InhA prevents the production of mycolic acid and the manufacture of cell walls in M. tuberculosis, which results in cell death (Manjunatha et al. 2015).

The majority of Mutations in the katG gene, the inhA gene and its promoter, or the oxyR-ahpC region, all of which are involved in cell wall synthesis, confer INH resistance (INH-R) to strains (Hsu et al. 2020).

Several papers have stated that strains with mutations in inhA or its promoter are less resistant to INH than those with katG deletion mutations. Additionally, INH resistance was related to the overexpression of INH inactivators or efflux pumps (Heidary et al. 2022).

Drug-resistant TB is a challenge in the treatment of TP. The effectiveness of the therapy will surely be improved by drug screening of infected individuals. Individualized therapy should also be taken into consideration reason being that TP is more common in certain groups such as kids, the elderly, and those living with HIV. If we are ever going to realize our deepest hopes, it is only fair that we look forward to the clinical introduction of newer, more protective vaccines. goal of eradicating TB (Wei et al. 2020).

METHODS

This study included 200 samples from patients visiting Baghdad Medical City/ Iraq. And suffering from dry cough, fever and chest pain. Those patients have been clinically diagnosed as tuberculosis. Sputum samples have been collected from the patients into screw-capped, disposable PP tubes of 50 mL capacity. The samples have been homogenized by being combined with an equivalent volume of 4% NaOH solution and then being stirred by tube rotator for 5-20 minutes within a biosafety cabinet. The samples were centrifuged at 2800-3000 g (3,000 rpm) for 15 minutes, and the supernatant was removed, leaving between 500 and 1000 l. A 1.5 cc tube was filled with sediment suspension. Thereafter, the samples were centrifuged at 12000 rpm for 5-10 minutes. DNA have been extracted by following the instruction provided by commercial Prep-Kit DNA/RNA (Sacace, REF K-2-9). A negative sample would be one that has no sample and instead has nuclease-free water added during the extraction process. Ten microliters of Internal Control were added to the sample/lysis combination at the start of the DNA isolation process. Total blood cholesterol was measured using a Biolabo laboratory kit; the method of measurement was based on the enzymatic hydrolysis. Glycerol and fatty acids were digested by enzymes to identify the triglycerides. By adding phosphotungstic acid, which includes magnesium chloride and has a pH of 6.2, the chylomicron fraction, very low density lipoprotein (VLDL), and low density lipoprotein (LDL) quantitatively precipitated. Using Friedwald's method, it is possible to quantitatively compute LDL cholesterol from total cholesterol, triglycerides, and the concentration of HDL cholesterol.

Detection of M. tuberculosis by qRT-PCR

The commercial kit TB15-50FRT has been used intended for identifying Mycobacterium tuberculosis. 10 ul of PCR-mix-1, 5 ul of PCR Buffer Flu, 0.5 ul of TaqF DNA Polymerase, and 0.5 ul of UDG should be combined in a sterile 0.2 ml tube. After that, a quick centrifuge has been used to create a vortex and mix the ingredients together. Finally, 10 l of purified DNA was introduced. In addition, 10 ul of DNA-buffer and 10 ul of C+ MTB were added, respectively, to tubes designated amplification Negative Control and Positive Control. Next, we inserted the tubes thermal-cycler. And then programmed as shown in table (1).

TABLE 1: qRT-PCR temperature cycling program for the detection of M. tuberculosis

Temp. C	Time	cycles	imaging
95	15 min	1	
95	15s	5	
65	30s		
72	15s		
95	15s	40	
65	30s		Fam/ Hex
72	15s		

Diagnosis of MDR isolates

For each sample 3 PCR tubes were prepared with 15 µl of the Reaction Master mix RIF N°1 or RIF N°2 or INH 5. In a new sterile 0.2ml tubes the following volumes were added; 10µl of PCR-mix-RIF N°1 or 10l of PCR-mix-2, 5l of PCR-mix-1, 0.5l of DNA polymerase, and 10l of PCR-mix-RIF N°2 or 10l of PCR-mix-INH (TaqF). short periods of vortexing and centrifuging. After that, we put 10 l of the purified DNA into each of the Master mix tubes. Three controls were made for every master mix. The tube labeled Amplification was loaded with 10 l of DNA-buffer. Add 10 l of C+ MTB-wt to the tube marked "Wild Type Control" for the Negative Control.; 10 µl of C+ MTB-mut added to the tube labeled Mutant Control; then all tubes have been placed in the thermal-cycler which has been programmed as shown in table (2).

TABLE 2: thermal-cycler cycling program for the detection of M. tuberculosis resistance genes

Temp. C	Time	Fluorescence detection	Cycle repeats
95	15 min		1
95	15 s		5
65	30 s		
72	15 s		
95	15 s		40
65	30 s	FAM, Hex, Rox, Cy5	
72	15 s		

Interpretation of results

The existence of the fluorescence curve crossing the threshold line provides the key to deciphering the data.

Rifampicin Resistance

a) The isolate considered not MTB Resistance to rifampicin if all the 3 conditions below are met:

1. Ct values are defined for channels FAM, JOE, Cy5 and there is no Ct value for channel ROX in mix PCR-mix-RIF N°1
2. Ct values are defined for channels FAM, JOE, ROX, and Cy5 in mix PCR-mix-RIF N ° 2
3. Ct value is defined for channel JOE in mix PCR-mix-INH

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b) Prevalence of Mycobacterium tuberculosis For at least one of the following conditions is met:

1. There is no Ct value defined for one or more channels in FAM, Joe, Cy5 and/or there is a Ct value defined for ROX channel in mix PCR-mix-RIF N°1

2. There is no Ct value defined for one or more channels in FAM, Joe, ROX, Cy5 in mix PCR-mix-RIF N°2

3. There is no Ct value defined for channel Joe in mix PCR-mix-INH.

Isoniazid Resistance

a) Absence of MTB Resistance to Isoniazid if there are Ct values defined for FAM and Cy5 channel in mix PCR-mix-INH.

b) Presence of MTB Resistance to Isoniazid if there is no Ct value defined for FAM channel (or for FAM and Cy5 channels) in mix PCR-mix-INH.

c) Presence of MTB Resistance to low level Isoniazid if there is no Ct value defined for Cy5 channel and there is a Ct value defined for FAM channel in mix PCR-mix-INH.

Statistical analysis

SPSS version 24.2 and Microsoft excel were utilized for the statistical analysis in this research. The probability, or P- value, has been determined to be 0.05. The numerical data have represented as mean ± Standard error (S.E.), t-student test has been used to compare two numerical data. ANOVA test used for numerical data more than two. Chi-square has used for two categorical parameters.

RESULTS

Distribution of the samples according to the demographic characteristics in tabular form (3). Sample percentages increased most rapidly between the ages of 31 and 40, with those between the ages of 21 and 30 following closely behind at 42%. (36.5%), 51-60 years (7%), 41-50 years (5%), the group of younger than 20 years (4%), the group of older than 71 years (3%), and the lower frequency had shown within the group age 61-70 years (2). According to blood group, O+ showed the higher frequency (35%), followed by both blood groups A+ and AB+ (17.5%), then B+ (9.%), AB- (7.5), B- (6.5%), A- (5%), O- (2%). According to gender distribution, male showed higher frequency than female (55% VS. 45%, respectively).

TABLE 3: samples distribution according to the demographic characteristics

Parameter		Frequency	Percent
Age group	<20	8	4.0
	21-30	84	42.0
	31-40	73	36.5
	41-50	10	5.0
	51-60	14	7.0
	61-70	5	2.5
	>71	6	3.0
Blood group	A+	35	17.5
	A-	10	5.0
	B+	18	9.0
	B-	13	6.5
	AB+	35	17.5
	AB-	15	7.5
	O+	70	35.0
	O-	4	2.0
gender	male	110	55.0
	female	90	45.0

M. tuberculosis was detected by Q-PCR and the results of the gene amplification are shown in the figure (1). The results showing increment curves which represent the amplifying process of the target region during each cycle. Each curve represent one sample.

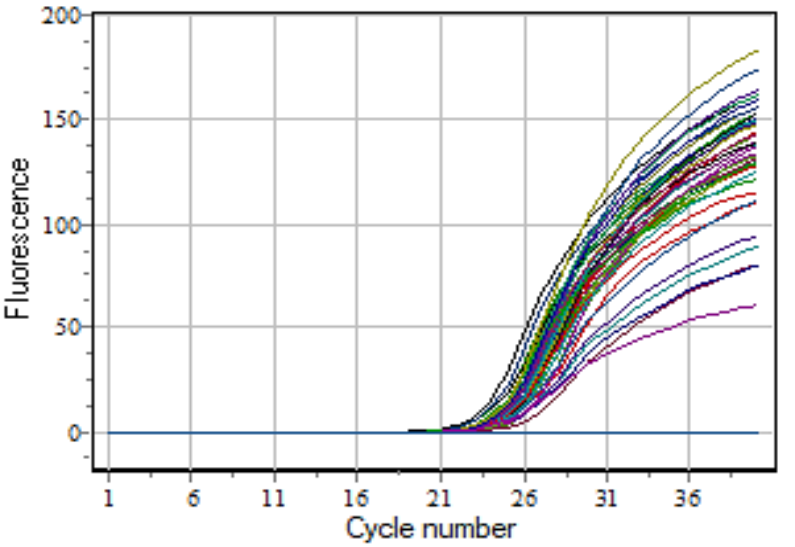

FIGURE 1: amplification curves of M. tuberculosis results of qRT-PCR

The results of the lipid profile are summarized n table (4). The results showed a significant higher level in positive samples than negative samples regarding the serum level of total cholesterol (196.90 VS. 172.86, respectively), triglyceride (195.61 VS 152.33, respectively), and low density lipid (121.03 VS. 98.14, respectively).

TABLE 4: comparison of lipid profile serum levels between positive samples with M. tuberculosis and negative samples

Mycobacterium tuberculosis		TC	TG	HDL	LDL	VLDL
positive	Mean	196.9000	195.6154	44.3846	121.0308	39.0077
	Std. Deviation	36.52093	37.10918	6.90626	35.77952	13.40451
Negative	Mean	172.8649	152.3316	49.8386	98.1456	32.1579
	Std. Deviation	37.40988	60.58150	10.33672	28.11547	16.42785
P-Value		0.04	0.016	0.075	0.014	0.167

The resulted positive/ negative number of samples have been distributed according to the demographic characteristics as shown in table (5). According to age group which showed a significant differences among the groups \, samples within The highest rate of positive (4%) was seen in those aged 21–30, followed by those aged 31–40 (3%), 41–50 (2%) and those aged 51+ (2%). <20 years (1%), and the lower frequency showed by age group 51-60 years (0.5%), none of the 5 samples within the age group 61-70 showed positive result. According to the blood group, the three blood groups A-, AB- and O- didn't show a positive results, higher frequency of positive samples are shown by the blood group O+ (5%) followed by the blood group A+ (2%), blood group AB+ (1.5%), then both blood groups B+ and B- showed the lower frequency (1%). According to gender showed same frequency in both gender (5.5%).

TABLE 5: positive/ negative samples distribution according to the demographic characteristics

Parameter			RT		Chi-square	P- Value
			positive	Negative		
age	<20	Count	2	6	13.83	0.05
		% of Total	1.0%	3.0%		
	21-30	Count	8	76		
		% of Total	4.0%	38.0%		
	31-40	Count	6	67		
		% of Total	3.0%	33.5%		
	41-50	Count	4	6		
		% of Total	2.0%	3.0%		
	51-60	Count	1	13		
		% of Total	0.5%	6.5%		
	61-70	Count	0	5		
		% of Total	0.0%	2.5%		
	>71	Count	1	5		
		% of Total	0.5%	2.5%		
blood	A+	Count	4	31	5.64	0.58
		% of Total	2.0%	15.5%		
	A-	Count	0	10		
		% of Total	0.0%	5.0%		
	B+	Count	2	16		
		% of Total	1.0%	8.0%		
	B-	Count	2	11		
		% of Total	1.0%	5.5%		
	AB+	Count	3	32		
		% of Total	1.5%	16.0%		
	AB-	Count	0	15		
		% of Total	0.0%	7.5%		
	O+	Count	11	59		
		% of Total	5.5%	29.5%		
	O-	Count	0	4		
		% of Total	0.0%	2.0%		
gender	male	Count	11	99	0.25	0.39
		% of Total	5.5%	49.5%		
	female	Count	11	79		
		% of Total	5.5%	39.5%		

Distribution of samples according to the infection that periods restrained by months are shown in figure (2). The distribution of samples showed a significant difference among the different infection periods (chi-square= 44.19, P-Value= <0.001). The higher number of positive samples are shown by the group of people that suffering from the symptoms for 20 months (6 samples), followed by those who were suffering for 21 months (4 samples), then 19 months (3 samples), then the infection periods 17 and 12 showed 2 samples, and the infection periods 15, 11, and 8 showed only one sample.

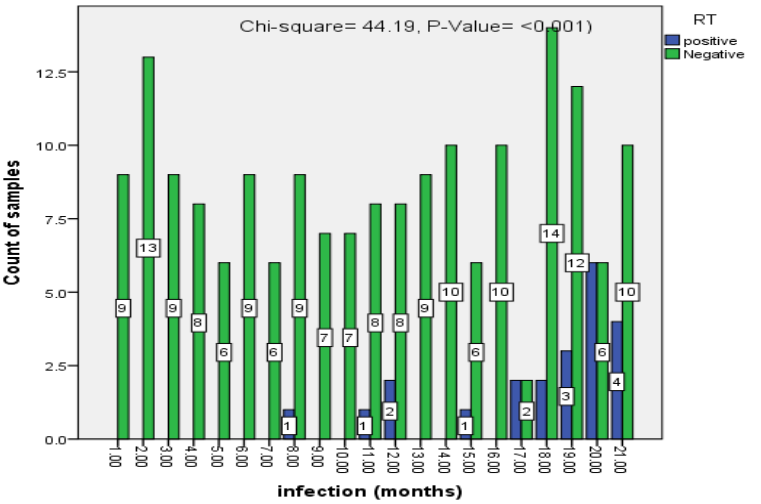

FIGURE 2: distribution of positive/ negative samples according to the infection periods

For diagnosis of antibiotic resistance the positive samples have been tested by qRT-PCR using SACACE kit for resistance detection. Fluorescence is detected at 65 °C in Fluorescence channels of various colors, including FAM/Green, Yellow/HEX, ROX/Orange, and Cy5/Red. The MTB MDR Resistance Real-TM has an analytical sensitivity of not less than 1x103 GE/ml. the results of amplification are shown n figure (3).

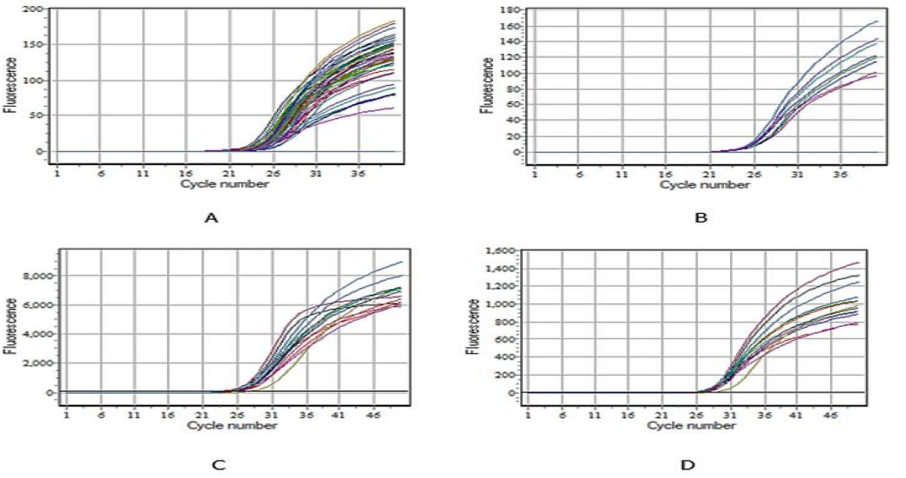

FIGURE 4: amplification curves of qRT-PCR. A; FAM/Green, B; Yellow/HEX, C; ROX/Orange, and D; Cy5/Red

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The results of isolates resistance to Rifampicin and Isoniazid in tabular form (6). The findings revealed that seven percent the positive samples are resistance to Rifampicin and (4%) were sensitive.

while resistance to Isoniazid are shown in 6.5% of the samples and 4.5% were sensitive.

TABLE 6: results of samples resistance to b Rifampicin and Isoniazid

parameter			RT		Chi-square	P-value
			positive	Negative		
Rifampicin	positive	Count	14	0	122	<0.001
		% of Total	7.0%	0.0%		
	Negative	Count	8	178		
		% of Total	4.0%	89.0%		
Isoniazid	positive	Count	13	0	112	<0.001
		% of Total	6.5%	0.0%		
	Negative	Count	9	178		
		% of Total	4.5%	89.0%		

DISCUSSION

The results of this study showed a significant higher frequency of incidence in younger age subjects rather than elderly this results disagreed with a previous study that showed In younger patients, M. pneumoniae is the most common pathogen, the incidence of CAP is low but still significant (17.1% of cases in those aged 40 yrs), more than half of cases involve patients without comorbidity, most cases present with mild to moderate severity, but hospitalization rates are high (55.7%), and most patients are hospitalized. These are the most striking distinctions between the young and the old. (Klapdor et al. 2012).

This study's findings revealed a greater occurrence of patients were recorded by O+ blood group and followed by AB+ this results partially agreed with a previous subpopulation analysis study done by (Chen et al. 2021). The blood group AB posed the greatest threat from TB. Epidemiological research is unable to Unlike other Gram-positive bacteria, Mycobacterium tuberculosis possesses a periplasmic layer and a polysaccharide called lipo-arabino-mannan, which includes arabinogalactan and other compounds, which makes persons with blood type AB more susceptible to developing TB.

Arabinogalactan is a polysaccharide that consists of both arabinose and galactose. Conversely, erythrocytes, body fluids, and mucosal secretions all express a wide range of glycolipid and glycoprotein structures on their surface. determine ABO antigenicity (Stoop et al., 2013).

Therefore, there may be some parallels between M. tuberculosis polysaccharide and ABO antigens. Those with blood type AB lack both anti-A and anti-B antibodies, making it possible that they lack the necessary defenses against the polysaccharide produced by M. tuberculosis. Researchers observed that people with blood type AB were more likely to get tuberculosis (TB), especially in sub-Saharan Africa and India. African and African-Indian M. tuberculosis lineages predominate in these areas; their lipo-arabino-mannan structures may be similar to those of human blood types A and B. (Wirth et al., 2008).

As this study's findings shown, 63% of the positive samples were Rifampicin resistant this results is higher than what mentioned in previous study that showed all TB confirmed patients had a 9.9% (186/1876) rifampicin resistance rate.

Rifampicin resistance was present in 7.6 and 27.4% of newly diagnosed and previously treated cases of tuberculosis, respectively (Arega, Menbere, and Getachew 2019).

The most prevalent reason for isoniazid resistance is mutations in the katG gene. Despite the fact that there are more than 300 distinct katG variants known, On average, 64% of isoniazid-resistant clinical isolates include a katG 315 mutation, and these mutations occur most often at codon 315. globally. Serine to threonine, a specific amino acid alteration, responsible for 95% of all katG 315 mutations (Lempens et al. 2018). In this study, resistance to Isoniazid were shown in 59% of the positive samples. This is the higher frequency that find compared to other previous study. In previous study, 365 sputum cultures for M. tuberculosis were found to be positive. Resistance to rifampicin was observed in 3.5% (1.1 to 5.7%) and resistance to isoniazid in 19.8% (95% CI 14.7 to 24.9%). (Callum et al. 2022). There is a comparable proportion of isoniazid resistance (16.6%) and rifampicin resistance (2.0%) in southern Vietnam, according to earlier studies (Huong et al. 2006).

The results of lipid profile showed increased level of total cholesterol associated with M. tuberculosis infection. this results is agreed with a previous study that found a higher serum level of cholesterol in patients with TB than control and In people who did not take a statin, total cholesterol levels were significantly inversely related to tuberculosis risk, but patients using statins showed no such association (Jo et al. 2021). Previous research has shown that M. tuberculosis is capable of degrading cholesterol and obtaining carbon and energy from it. (Jo et al. 2021).

CONCLUSION

From the results of this study we can conclude that diagnosis with M. tuberculosis is more likely when suffering from the symptoms for a long period. Both Rifampicin-resistant and Isoniazid- resistant TB is prevalent in Iraqi adults.

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