NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY

EVALUATION OF FOUR DIFFERENT METHODS FOR THE DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS* FROM CLINICAL SAMPLES IN A UNIVERSITY HOSPITAL

M.Sc. THESIS

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Supervisor

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Approval

We certify that we have read the thesis submitted by Gülten HASTÜRK titled **"Evaluation of Four Different Methods for the Diagnosis of *Mycobacterium tuberculosis* from Clinical Samples in a University Hospital**" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Educational Sciences.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Gülten HASTÜRK

22/04/2022

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Gülten Hastürk

Abstract

Evaluation of Four Different Methods for the Diagnosis of *Mycobacterium tuberculosis* from Clinical Samples in a University Hospital

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Objective: The aim of this study is to determine the prevalence of *Mycobacterium tuberculosis* by evaluating the samples of patients came to the Near East University Hospital, Microbiology laboratory between 2016 and 2020, which is the only mycobacteriology laboratory in North Cyprus. In addition, it is among our goals to discuss the necessity of the bacillus Calmette-Guerin (BCG) vaccine, which is not included in the routine vaccination calendar of North Cyprus. **Material and Method:** This study is designed both retrospectively and prospectively. For the retrospective study, test results of our group and demographic information of the patients were obtained from the information processing system of Near East University Hospital. For the prospective study, the culture method is accepted as the gold standard and an automated system BACTEC- MGIT 960 and Löwenstein Jensen media were used. Erlich Ziehl-Neelsen method, Polymerase Chain Reaction (PCR) and QuantiFERON-TB GOLD PLUS tests are also used. **Results:** SPSS Demo Ver 22.0 (SPSS Inc., Chicago, IL, USA) program was used for the statistical analysis. Total 288 (male: 162, 56.3%; female: 126, 43.8%) patient were analyzed. The number of patients who found as positive was 27 (9.4%). **Discussion:** It has been seen that TB positive patients increased significantly between 2016 and 2020, (p=0.000). Our results indicated that the higher TB positivity was found in other nations (18.8%) and the TB positivity among North Cyprus citizens has increased over the years. BCG vaccine is necessary or not ‘might be revisit by the Ministry of the Health according to our results.

Keywords: *Mycobacterium tuberculosis*, North Cyprus, prevalence, BCG vaccine

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List of Abbreviations

AIDS: Acquired Immuno Deficiency Syndrome

BCG: Bacillus Calmette-Guerin

DNA: Deoxyribonucleic acid

ELISA: Enzyme linked immunosorbent assay

HIV: Human Immunodeficiency Virus

PCR: Polymerase Chain Reaction

PPD: Purified Protein Derivative

TB: Tuberculosis

TR: Turkey

TRNC: Turkish Republic of North Cyprus

WHO: World Health Organization

UNICEF: United Nations International Children's Emergency Fund

MDR: Multi drug resistant

MTC: *Mycobacterium tuberculosis complex*

MGIT: Mycobacterium Growth Indicator Tube

BD: Becton Dickinson

LJ: Löwenstein Jensen

NaOH: Sodium hydroxide

NALC: N-acetyl-L-cysteine

RPM: Revolutions per minute

IGRA: Interferon gamma release assays

IFN-γ: Interferon gamma

SPSS: Statistical Package of the Social Sciences

CHAPTER I

Introduction

Tuberculosis (TB) is known as *Mycobacterium tuberculosis* (*M. tuberculosis*) bacteria-caused infectious disease, which affects both people and animals. TB is a health problem that causes morbidity and mortality all over the world. While pulmonary tuberculosis mainly affects the lungs of patients, it can also occur in the patient's cerebrospinal membrane, joints, skin, bones, intestines, kidneys, lymph nodes and other organs as extrapulmonary tuberculosis (Naveed, & et al., 2019). As aerosol droplets, tubercle bacilli can be inhaled by a person and directly reach to the lungs. In alveolar macrophages, *M. tuberculosis* starts to multiply inside and can be spread to other tissues and organs via the bloodstream and lymphatics (Delogu, & et al., 2013).

Calmette and Guerin developed the Bacillus Calmette-Guerin (BCG) vaccine in 1908 at the Institute Pasteur in Paris and in 1921, it has been used on a newborn baby as a first time (Yalçın & Hatipoğlu, 2005). BCG is still the only available vaccine for TB. BCG, a live attenuated strain of Mycobacterium bovis, is used to prevent TB and other mycobacterial infections. It is the most extensively used vaccine, and it is routinely included in a newborn's regular vaccination schedule (Okafor, & et al., 2022). BCG vaccine has been widely used in Turkey since 1951, which was included in the Turkish National Vaccination Calendar (Özmert, 2008).

Since BCG vaccination is not available in Northern Cyprus, the Tuberculin Skin Test (PPD-purified protein derivative) is used to identify positive individuals and in order to start the treatment of individuals as soon as possible. PPD test is regularly applied to first and fifth grade students in primary schools every year. Also there is a necessity to have a PPD test for all people who come from abroad to work or study and want to reside in North Cyprus.

CHAPTER II

General Information

2.1. History of Tuberculosis

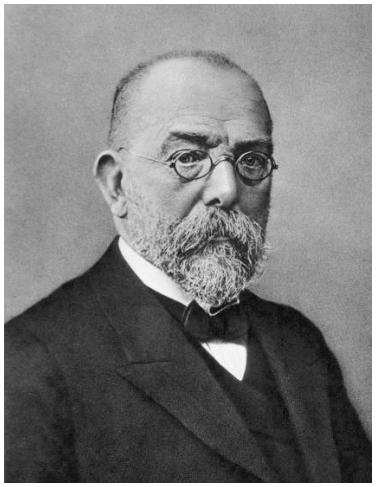
Researchers believed that the ancestors of *M. tuberculosis* appear to have lived in the world more than 2,6 million years ago. The first hominid tissue TB was found in a 500,000-year-old fossilized homo erectus skeletal tissue specimen of a bear isolated from a stone quarry located north of the village of Kocabas in Western Turkey (Agarwal, & et al., 2017). In Europe and Asia, much research has been done at various burial sites to confirm the existence of human TB and the first human TB found on a 9,000-year-old settlement of a Neolithic baby and woman found in the Eastern Mediterranean (Cambau & Drancourt, 2014). Zimmerman (1979) suggested that the evolution of human bovine tuberculosis occurred in the Nile Valley 5,000 years ago in the mummy of an Egyptian child with a documented case of TB. TB, known as Phthisis in ancient Greece, was described by Hippocrates as a fatal disease with its symptoms and characteristic tubercular lung lesions, especially for young adults. The Greek orator Isocrates was known as the first author who studied TB and assumed that TB is a contagious disease. Also in the Middle Ages, scrofula, a disease affecting the cervical lymph nodes, was described as a new clinical form of TB. In France and England, TB was known as "King's evil" disease. In 1363, the French surgeon Guy de Chauliac was the first to propose a curative intervention for the treatment of the "King's evil". In 1679, anatomical and pathological description of TB disease described by Francis Sylvius and in his well-known work Opera Medica, he discussed the development of tubercles, abscesses, cavities and empyema (Barberis, & et al., 2017). In the 1700s and early 1800s, TB prevalence reached the top as the largest cause of death in Western Europe and the United States. After 100 to 200 years, it completely spread to Asia, Africa, Eastern Europe, and South America. The epidemic then spread through Western Europe and almost all Western Europeans became infected with *M. tuberculosis* (Daniel & et al., 1994). In 1768, tuberculous meningitis discovered by

Robert Whytt and described in his remarkable treatise "On the Dropsy in the Brain" (Ruhräh, 1904). Tuberculous meningitis was a catastrophic disease until the discovery of chemotherapeutic agents and antibiotics in the twentieth century (Breathnach, 2014).

Between 1779 and 1782, Sir Percivall Pott described extra-pulmonary phthisic tubercles as "Pott's disease" which is recognized with complaints of back pain, symptoms of fever, weight loss, chills, malaise and fatigue (Sternbach, 1996). French physician Rene Theophile Hyacinthe Laennec, defined pulmonary and extrapulmonary TB and clarified the majority of the physical symptoms of the disease in 1819. Johann Lukas Schönlein coined the word “tuberculosis” to characterize the disease in 1834 and Hermann Brehemer used the term tuberculosis of the lungs to refer to phthisis in 1853 (Perciaccante, & et al., 2018). Theodor Albrecht Edwin Klebs was the first scientist to try to isolate the TB bacillus by culturing TB material on egg whites and storing it in sterile flasks in 1867. (Barberis, & et al., 2017).

Figure 1.

Robert Koch, 1843-1910



Robert Koch was working with different stains to detect the visibility of the TB bacillus, known as the conventional stain resistant bacteria. Koch has detected

few tiny rods by using Ehrlich's methylene blue stain. Koch then uncovered more bacteria by adding a brown counterstain which is for coloring and contrasting the parts that are not stained with another dye. Koch succeeded to discover the tubercle bacillus with using this staining technique on March 24, 1882. (Blevins & Bronze, 2010).

Paul Ehrlich experimented the pure culture of tubercle bacilli with various stains which he obtained from Robert Koch. Koch's sample staining method took 24 hours, whereas Ehrlich tried to stain the samples with aniline water, fuchsin and gentian-violet by reduced staining time of 15-30 minutes. Also, in order to decolorize the surrounding tissues, he applied 30% nitric acid and alcohol for a few seconds. The red tubercle bacilli were seen more clearly than Koch's method when Ehrlich applied counterstaining. Furthermore, he placed the stained microscopic slides to the top of the stove found in his laboratory to dry. The fire had been out for few hours from the small iron stove while he was not there and in the next morning, he accidentally noticed the benefit of heating the slides when he saw the bacilli in clumps even more clearly by examining his prepared slides. Ehrlich published the details of his staining method in May 1882. Later on, the studies continued on staining methods and Franz Ziehl introduced the use of carbolic instead of aniline, while Friedrich Neelsen advocated the use of sulfuric instead of nitric acid. Thus, the "Ziehl-Neelsen" stain and the "acid alcohol fast bacillus" were discovered (Sakula, 1982).

For the treatment of TB, Robert Koch announced the success in "tuberculin" therapy, however he found that it was only useful as a diagnostic tool, not as an effective vaccine for TB. After that, in 1907, Viennese and Clemens Freiherr von Pirquet discovered Pirquet test, which was the cutaneous scratch tests of tuberculin to diagnose children with latent TB. In 1908, Charles Mantoux introduced a serological skin test, PPD, replaced with the Pirquet test, which helps to define if a person is infected with *M. tuberculosis* by an immune response and produced any skin reaction. In 1905, Koch was awarded the Nobel Prize for his achievements and

for his works on TB, and after five years later, he died of heart disease in Baden-Baden (Frith, 2014).

Between 1908 and 1921, a TB vaccine with an attenuated form of the TB bacillus was invented by Albert Calmette and Camille Guérin. The preventive effect of vaccine bacilli Calmette-Guérin known as "BCG", was partial at best, even it was uncontaminated. An effective treatment has not found for TB until 1943, however, laboratory of the soil biologist Selman Waksman developed streptomycin, the first of a series of antibiotics, which has been proved to be effective against TB (Barnes, 2000).

In 1943, a Swedish physician, Jorgen Lehmann, developed para-aminosalicylic acid and a German bacteriologist, Gerhard Domagk, developed thiosemicarbazone in 1945, which both are found to have very actual effect on TB. Since then, other anti-tuberculosis antibiotics such as rifampicin, isoniazid, ethambutol, and pyrazinamide have been developed. Recently, ciprofloxacin and viomycin are also added to be used in drug resistant infections (Frith, 2014).

2.2. Epidemiology of TB

2.2.1. *Global Epidemiology of TB*

One of the most frequent diseases in the world is TB. *M. tuberculosis* is thought to infect over 2 billion individuals and approximately 10 million people have TB each year, with 1,6 million dying as a result (Anon 2021). People with Human Immunodeficiency Virus (HIV) infection, immigrants/refugees, homeless, prisoners and drug or heavy alcohol users are always at greater risk to be infected with *M. tuberculosis* (Sulis, & et al., 2014).

In 1955 and 1963, two tuberculin testing surveys have been carried out in Cyprus by World Health Organization (WHO) teams. The research done in 1955 made it possible to compare the transmission pattern of TB infection with the survey done in 1960, which was WHO/UNICEF (United Nations International Children's Emergency Fund)-assisted TB pilot project. Testing for tuberculin is

valuable for epidemiological purposes in Cyprus because of the tuberculin susceptibility was almost completely absent on the island. Also, since tuberculin allergy to the BCG vaccine is not common in the island, it has been easy to find population samples not affected by the BCG vaccine. The tuberculin test survey carried out in 1955 was conducted in primary schools in Nicosia, so the study carried out in 1960 has also done in primary schools. There was no "scar review" in the initial survey, as there was no BCG vaccination in Cyprus before 1955. After 1955, there was a decrease seen in the risk of TB infection in Cyprus. Therefore, the effect of the new anti-tuberculosis drugs did not appear to lead to an increase in infection transmission (Geser, 1964). From Romania, Bulgaria, Poland and Greece immigrants have increased in number which came to work at South side of Cyprus when joined to the European Union in 2004. This has also caused an influx of refugees from neighboring countries such as Syria and Libya. Although immigration had a positive effect on the economy, it caused a resurgence of TB in Cyprus. While most of the registered cases were Cypriot citizens in 1999, it was found that the cases registered were mostly patients from other countries, in 2012 (Petrou, 2017).

Table 1.

Positive TB cases reported from 1980 to 1990 in Southern Cyprus

Year	**1980**	**1981**	**1982**	**1983**	**1984**	**1985**	**1986**	**1987**	**1988**	**1989**	**1990**
Positive Cases	69	69	86	73	39	61	48	35	39	23	29

The number of positive TB cases in Southern Cyprus between 1980 and 2005 was reported in the WHO database. 86 patients were recorded with the higher positive cases seen in 1982. While an increase was seen from 1980 to 1982, a decrease was observed in the positive cases between 1983 and 1990. The number of cases, which an increase has detected again in 1991, decreased after 1992 till 1996, but then increased almost twice in 1997. Between 1998 and 2002, a decrease

has achieved and 20 positive patients were recorded as the lowest number of cases in 22 years after 1980. From 2003 to 2005, an increase was observed in the cases but it was considered stable on average (WHO, 2020).

According to the positive TB cases in Northern Cyprus mentioned in a study of Atasoy (2011), it is seen that more positive cases were recorded in Northern Cyprus (Table 3) between 1997 to 2001, then the number of cases in Southern Cyprus mentioned below (Table 2).

Table 2.

Positive TB cases reported from 1991 to 2005 in Southern Cyprus

Year	**1991**	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**
Positive Cases	43	39	37	37	36	24	47	45	39

Table 2. (Continued)

	Year	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**
	Positive Cases	33	40	20	35	30	34

Table 3.

Positive TB cases in Northern Cyprus between 1997 to 2001

Year	1997	1998	1999	2000	2001
Positive Cases	54	46	43	38	44

According to the data from the Turkish Republic of Northern Cyprus (TRNC) Notifiable Diseases statistics, positive cases from 2014 to 2020 are given in the Table 4. Although, TB cases seen in Turkish citizens has decreased, it can be seen that, foreign students and employees from abroad increased the number of positive cases on the island.

Table 4.

Positive TB cases reported between 2014-2020 in Northern Cyprus

Year	TRNC citizen	TR citizen	Other Nationalities	Total
2014	17	8	2	27
2015	21	2	11	35
2016	6	2	6	14
2017	3	2	5	10
2018	6	1	4	11
2019	11	5	7	23
2020	1	3	24	28

Foreigners, who have a work permit in the island, undergo a health check including a chest X-ray for TB and a blood test for HIV/AIDS (Acquired Immuno Deficiency Syndrome) in their first work permit application, and the positive results are deported (Güven-Lisaniler, & et al., 2005). The emergence of multi-drug resistant strains of *M. tuberculosis*, which has a higher prevalence, is among the most important challenges in TB control. WHO has published an updated global

TB strategy for the "post-2015 period" with the goal of "ending the global TB epidemic" by 2035 (Sulis, & et al., 2014).

WHO has defined three lists of high burden countries as TB, MDR (multi drug resistant)-TB and TB/HIV, each containing 30 countries for the 2016-2020 period. In 2016, 6,3 million TB cases were reported. 10 million new TB cases were reported in 2017. As in 2017, 10 million people were reported in 2018 and also in 2019, but deaths caused by TB, which was 1.6 million in 2017, decreased to 1.5 million in 2018 and then to 1,4 million in 2019. The most noticeable change is a significant reduction in the number of people were diagnosed with TB from 7,1 million in 2019 to 5,8 million in 2020 (WHO, 2020).

2.3. *Mycobacterium* species

*Mycobacteriacea*e is one of the family members of the order *Actinomycetales* and only comprises the genus *Mycobacterium*. Acid-fastness and the presence of mycolic acids are two distinctive traits of the genus *Mycobacterium*. The cell wall of *Mycobacterium* species is thicker than many other bacteria cell wallsand is waxy, hydrophobic, rich in mycolic acids/mycolates and is made up of two layers of hydrophobic mycolate layer and a peptidoglycan layer bound together by a polysaccharide, arabinogalactan.They are free-living aerobic microorganisms found in soil and water, which are appear as a thin, rod-shaped and non-spore forming gram-positive bacilli under the microscope. Another characteristic of *Mycobacteria* is their slow growth rate which may take around 20 hours, and therefore it is possible for isolation and identification to take up to 6 weeks (Percival & Williams, 2014).

2.3.1. *Mycobacterium tuberculosis complex*

Mycobacterium tuberculosis complex (MTC) is the infectious agent of TB disease and each member of the MTC is pathogenic (Alli, & et al., 2011). The MTC includes eight subgroups known as; *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium caprae*, *Mycobacterium canettii*,

Mycobacterium mungi, *Mycobacterium pinnipedii* and *Mycobacterium microti*. The two more separate branches of the MTC phylogenetic tree are named as *Mycobacterium dassie* and *Mycobacterium oryx* which cause TB in the animal species. However, both are not yet officially classified as a separate taxon and have not been linked as a human disease (van Ingen, & et al., 2012).

2.3.2. *Mycobacterium tuberculosis*

M. tuberculosis is a member of the MTC, which specifically causes human TB, and the first cause of death worldwide due to a single pathogen. When a *M. tuberculosis* infected person sneezes, coughs, spits up, or speaks, droplets of saliva including tubercle bacilli are spread into the air and enters the body when inhaled by a nearby person (Bañuls, & et al., 2015). In addition, people living with HIV has a greater chance of developing latent TB, and it is the top cause of mortality among HIV-positive patients worldwide (Cudahy & Shenoi, 2016).

2.3.3. *Mycobacterium bovis*

Mycobacterium bovis causes TB infection especially in cattle and it supports zoonotic transmission. Human TB of *Mycobacterium bovis* can be seen in countries where bovine TB infection is not under control. Farm workers can become infected through direct contact with infected cattle and infection can be transmitted to their families. There is also a possibility of transmission of *Mycobacterium bovis* infection from infected food products like drinking a contaminated milk. Additionally, the epidemic of HIV infection could make it a serious public health threat for those at risk for zoonotic TB (Cosivi, & et al., 1998).

2.3.4. *Mycobacterium caprae*

Mycobacterium caprae is mainly recognized in Central Europe (Rodríguez, & et al., 2011). In most countries, the major reservoir of *Mycobacterium caprae* infection are cattle and goats, as a specie *Mycobacterium bovis*. While both infections cause TB in a wide range of wild and domestic animals, they can also be seen as zoonotic TB in humans. (Gormley, & et al., 2014).

2.3.5. *Mycobacterium africanum*

Mycobacterium africanum West African 1 and *Mycobacterium africanum* West African 2 are two phylogenetically separate lineages of *Mycobacterium africanum*. In West Africa, these *Mycobacterium africanum* strains are responsible for half of all human pulmonary TB cases (Jong, & et al., 2010). The clinical presentation and disease course of *Mycobacterium africanum* is same as that of *M. tuberculosis*. *Mycobacterium africanum* is known to be less lethal in animal models of TB, whereas it has been reported that individuals infected with *Mycobacterium africanum* are more likely to be HIV positive than patients infected with *M. tuberculosis* (Wood, 2014).

2.3.6. *Mycobacterium canettii*

TB caused by both *Mycobacterium canettii* and *Mycobacterium africanum*, is known as a disease that usually occurs in African patients or patients of African descent (Velayati & Farnia, 2017). The geographic distribution of *Mycobacterium canettii* is restricted to the Horn of Africa and especially to Djibouti which causes lymph nodes and pulmonary TB in patients (Conan, & et al., 2018).

2.3.7. *Mycobacterium pinnipedii*

Mycobacterium pinnipedii is known as the causative agent of TB in marine mammals. It is thought to cause TB in a variety of species, including non-marine mammals and even humans (Macedo, & et al., 2020). Cases of cattle may occur through direct or indirect contact with seals, grazing on the beach, or while accessing waterways that connect to the ocean. Also, it can be spread through contact with humans and domesticated species (Roe, & et al., 2019).

2.3.8. *Mycobacterium mungi*

Northern Botswana and Northwestern Zimbabwe are home to the only known populations of *Mycobacterium mungi*-infected banded mongooses. *Mycobacterium mungi* did not appear to be spread by primary aerosol or oral routes.

In the study conducted on the Chobe and Zambezi Rivers in Botswana, environmental transmission routes have been described, including the emergence of a new *Mycobacterium mungi* pathogen transmission mechanism (Alexander, & et al., 2016).

2.3.9. *Mycobacterium microti*

It is known that *Mycobacterium microti* has relatively low pathogenicity to humans and commonly seen in wild boar, domestic pig, cattle, goat, wild and pet ferrets, South American camelids, dogs, cats, badger, wood mice and meerkat (Pigoli, & et al., 2021).

2.3.10. *Mycobacterium dassie and Mycobacterium orygis*

Mycobacterium dassie is a pathogenic bacteria found in free-living dassies that can cause pulmonary pathogen disease (Parsons, & et al., 2008). Another new member of the MTC is *Mycobacterium orygis*, previously known as oryx bacillus. Human TB cases have been generally detected in patients of South Asian descent and also been proven to infect animals (Dawson, & et al., 2012). Moreover, it has been detected mostly from hoof stock in eastern Africa and the Arabian Peninsula (Love, & et al., 2020).

2.4. Laboratory diagnosis of *Mycobacterium tuberculosis*

People with symptoms that may be associated with pulmonary TB are often suffer from cough, pain in the chest, fever, weakness, breathlessness, blood in sputum, loss of appetite, weight loss and night sweat. In general, further bacteriological examinations are performed after the tuberculin testing and X-ray examination (Banerji and Andersen 1963). *Mycobacterium* species can be diagnosed with methods including conventional mycobacterial dignosictic techniques, immunological techniques and molecular techniques to develop effective control and preventive solutions for patients with TB (Tassew, 2018).

2.4.1. *Microscopic examination*

Microscopy-based diagnosis is fast, simple, cheap, and highly specific in TB -endemic locations to identify the most infectious patients (Steingart, & et al., 2006). Two techniques used to diagnose TB are known as conventional and fluorescence microscopy. In fluorescent microscopy, the acid-fast fluorochrome dyes which are known as auramine O or auramine-rhodamine are applied and a halogen or high-pressure mercury-vapor lamp is used as an intense light source. The samples to be examined by conventional microscopy are stained with acid-fast stains, Ziehl-Neelsen or kinyoun, and with a use of conventional artificial light source. One of the differences between Ziehl-Neelsen and fluorescent microscopy is that fluorescent microscopy uses a lower power objective lens (25x), while conventional microscopy uses a higher power objective lens (100x). Also, it is known that fluorescent microscopy is about 10% more sensitive than a conventional microscope (Costa Filho, & et al., 2015). The auramine-rhodamine staining for fluorescent microscopy analysis, firstly the auramine-rhodamine dye is applied to the slide and then rinsed. Secondly, acid-alcohol added on the slide as a decolorizing agent and then rinsed again. Lastly, potassium permanganate applied, rinsed and slide is allowed to dry (Wanger, & et al., 2017).

In the Ziehl-Neelsen staining technique, the smear is first coated with carbol-fuchsin dye and heated at a constant rate. Afterwards, it is rinsed and the colour is removed by applying acid-alcohol. Finally, methylene blue is added to the slide and then rinsed to allow dry. In the Kinyoun dyeing technique, only the heating process is excluded and the same steps are applied as in Ziehl-Neelsen staining technique. Organisms appear as rods, with a length of 2 to 4 µm and 0,2 to 5 µm wide when the stained smear is analysed with the conventional microscopy. Any biological fluid or material, such as gastric lavage fluid, pleural fluid, cerebrospinal fluid and urine can be directly prepared and stained to examine. However, thin liquids are prepared after sedimentation centrifugation to be examined (Fitzgerald, & et al., n.d.).

The healthcare workers should be correctly trained and the patients should also be provided with fully understood instructions by the nurses, to ensure that the best and most accurate specimens taken to be examined for the diagnosis of TB. In addition, after making certain that healthcare workers and others are safe, aerosol-generating processes such as sputum collectioncan be done. The sputum that accumulates slowly in the patient's respiratory tract during the night improves the quality and yield of the morning sample. Therefore, WHO recommends to test both morning sample and a spot sputum sample for microscopic laboratory diagnosis of TB (Parsons, & et al., 2011).

2.4.2 *Culture*

The culture method is regarded as the gold standard for TB infection laboratory diagnosis (Asmar, & et al., 2015). The Löwenstein Jensen (LJ), buffered egg-potato medium is the most commonly used egg-based media. The advantages of egg-based media have included a low cost of preparation and it has a long-dated shelf life up to one year when refrigerated. For agar-based culture methods, Middle brook 7H10, Middle brook 7H11 and Dubose oleic-albumin agars are suggested (Kedir, & et al., 2018).

LJ tubes should be incubated at 37°C after the sample cultured and reported as negative if the cultures showing no growth after 8 weeks of incubation (Kassaza, & et al., 2014). Colony morphology is evaluated by analyzing when growth occurs on LJ medium. Cultures showing rough with a cream-colored colonies are generally take into account as *M. tuberculosis* positive (Monteiro, & et al., 2003). Furthermore, detecting *Mycobacteria* using the radiometric Becton Dickinson (BD) BACTEC 460 TB system increases isolation and reduces time (Rodrigues, & et al., 2009).

The improved BACTEC TB system is a fast, sensitive and eficient method used in the clinical laboratories (Anargyros, & et al., 1990). Samples taken for TB screening are prepared for culturing under the appropriate conditions. Firstly, sodium hydroxide and N-acetyl-L-cysteine (NaOH/NALC) were used to purify the

specimens and then concentrated by centrifugation for 15 min at 3,000 RPM (revolutions per minute). After discarding the supernatant, 2 ml of sterile phosphate buffer is added on the sediment obtained to resuspended. 0,5 ml of prepared suspension then added into the BACTEC MGIT (Mycobacterium Growth Indicator Tube) 960 culture tube. The BACTEC MGIT 960 culture tube includes an antibiotic mixture of amphotericin B, polymyxin B, trimethoprim, azlocillin and nalidixic acid to suppress the development of other microorganisms. Additionally, 7 ml of the culture tube is the Middle brook 7H10 broth base which is an enrichment supplement containing dextrose, catalase, albumin, and oleic acid. The tubes were then inserted to the BD BACTEC MGIT 960 system. The culture tube also contains a fluorescent sensor which detects the oxygen concentration in the culture medium. The purpose of including the fluorescent sensor is to identify the positive tubes when a specified amount of fluorescence is attained by automatically monitoring at every 60 minutes. Negative tubes are retained for a maximum of 42 days (Diriba, & et al., 2017).

2.4.3. *Differentiation of Mycobacterial Species*

PPD, also known as the Mantoux test, is the "gold standard" immunological test, performed as a diagnostic screening for newly formed or asymptomatic MTC infections. PPD is the most utilized test to detect infection due to *M. tuberculosis*, although there is a possibility of false positive and false negative reactions based on tuberculin reading and cross-reactivity among mycobacterium species such as *Mycobacterium bovis* BCG and *Mycobacterium avium*. The patient should return for a control reading of the test site within 48 to 72 hours and there is also the possibility of a modulation of skin response due to underlying disease or immunosuppression (Katial, & et al., 2001). The Interferon gamma release assays (IGRA), QuantiFERON-TB Gold In-Tube test and the T-SPOT.TB test are especially performed to detect occult TB infection in children, immunocompromised children and adults, as well as people recently came from the countries with a high TB burden (Auguste, & et al., 2017).

If a patient has *M. tuberculosis* antigens, the secretion of Interferon gamma IFN-γ is detected while antigen-specific T cells ($CD4^+$ and $CD8^+$) are activated in patients' blood with the IGRA tests. With only T cells responding during the night, and IFN-γ releasing, assays show positivity (Whitworth, & et al., 2013).

2.4.4. *Detection of Drug Resistant Mycobacterium tuberculosis*

After the introduction of the BCG vaccine in 1921 and the use of antimicrobial drugs such as streptomycin, isoniazid and rifampicin, the incidence of TB decreased. However, due to the HIV pandemic and the appearance of antibiotic resistance, the prevalence of TB increased once again in the 1980s. The first antibiotics discovered in the 1950s and 1960s are rifampicin and isoniazid which are still used for first-line drugs for TB medication. The emergence of completely drug-resistant and MDR TB strains other than rifampicin and isoniazid resistance make the management of TB very difficult. Several stages with different antibiotic combinations have been recommended by the WHO to control the problem of drug resistance (Bañuls, & et al., 2015). However, two new drugs called bedaquiline and delamanid are widely used for drug-resistant TB, and added to the first-line drugs, rifampicin and pyrazinamide, to shorten the treatment time (Murray, & et al., 2015).

There are two different methods that are helpful in determining drug susceptibility for *M. tuberculosis*. One of these methods is the phenotypic method, which assesses the inhibition of growth of *M. tuberculosis* despite the use of antibiotics, while also determining resistance based on the organism's response to drug exposure. The other method is known as the genotypic method. With this method, resistant genes and mutations are detected with Xpert MTB/RIF, which is the most widely used and easiest test. With this test, rifampicin resistance detection results can be obtained within short hours. Rifampicin resistance is another reliable way of detecting MDR TB as it is associated with isoniazid resistance (Dunn, Starke, and Revell 2016).

Generally, the detection of drug resistance in *M. tuberculosis* has been performed by culture-based methods that assess the growth of mycobacteria in the presence of the drug. GenoType MTBDR Plus (Hain) Lifesciences and the Line Probe Assay (INNO-LiPA Rif TB Assay, Innogenetics) are also used for simultaneous detection of rifampicin and isoniazid resistance (Palomino, 2009).

2.4.5. *Molecular Diagnosis and Drug Resistance of Mycobacterium tuberculosis*

For infection prevention and antimicrobial treatment selection, differentiating *M. tuberculosis* from non-tuberculous mycobacteria is important. Various rapid diagnostic methods fot detection and identification of *Mycobacteria* have been developed. Some of these methods are DNA (deoxyribonucleic acid) probes, nucleic acid amplification tests, and PCR based tests which have ability to rapid diagnosis (Shrestha, & et al., 2003). In PCR amplification assays of various clinical specimens, it has been reported that 16S rRNA is the target for detection of *Mycobacterium* spp. and are frequently used to identify various specific microorganisms (Fukushima, & et al., 2003). IS6110 is a insertion element present only in MTC members, and as a result of this uniqueness, it has become a significant diagnostic tool for MTC species identification. Moreover, the element's existence in several copies and at various locations across the genome has offered an ideal way for genotyping strains (Coros, & et al., 2008).

CHAPTER III

Materials and Methods

3.1. Sample and Patient Information

The samples used in the study were examined in a Mycobacteriology Laboratory at the Near East University (NEU) Hospital in Northern Cyprus between 2016 and 2020. After informing all suspected *M. tuberculosis* infection case patients who came to the hospital, their consent was obtained and recorded. All samples were sent to the laboratory from different departments of hospital, and samples which were not suitable for transfer or were not approved by the center expert were excluded from the study.

3.2. Microscopic examination

3.2.1. *Preparation of Smears*

After wearing a protective mask and gloves, the sample was opened in a biological safety cabinet. A small amount of the sample was spread directly on a sterile microscopic slide as a thin layer with a use of sterile swab and then fixed with fire or kept in dry air.

3.2.2. *Ziehl-Neelsen Staining*

The smear preparations were stained with the Ziehl-Neelsen staining method (Figure 2). First, carbol fuschin dye was applied to the slides and slightly heated until steam rose for about 5 minutes. Slides were then rinsed thoroughly until no color was visible in the water and 3% v/v acid alcohol was added for 2 minutes. Slides were washed again with clean water and the smears were covered with methylene blue dye for about 3 minutes. Finally, slides were rinsed with water and left to air dry.

Figure 2.

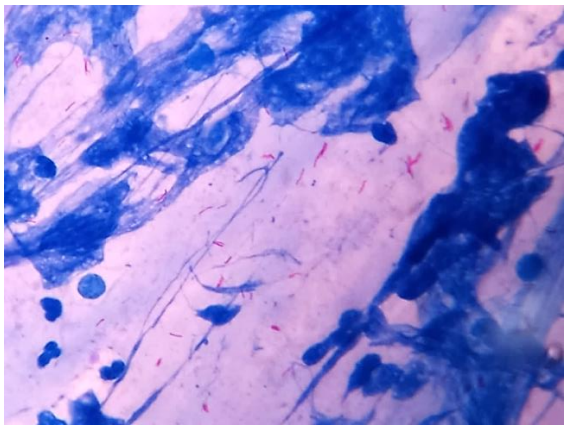
Ziehl-Neelsen Stains

3.2.3 *Evaluation of Smear Samples*

Immersion oil was dropped on the air-dried smear slides with using a pasteur pipette and examined with 100x objective light microscope. Positive patient samples were detected as red rod-shaped bacilli on the blue colored surface under the microscope as seen in Figure 3.

Figure 3.

Acid-fast bacilli under the microscope (100x objective)



3.3. Tuberculosis Culture

3.3.1. *NALC/NaOH Solution*

BD BBL MycoPrep reagent was used for the homogenization and decontamination of TB culture samples. This solution, known as N-acetyl-cysteine - 4% NaOH – 2,9% sodium citrate (NALC-NaOH), contains 20 gr of NaOH, 14,5 gr trisodium citrate and 0,375 gr NALC in the ampule found in the reagent bottle (Figure 4). Before using the solution, the ampule inside is broken with a hand and shaken well. After that, in a biological safety cabinet, 5-10 ml of culture samples were mixed with an equal amount of NALC-NaOH solution put in the sterile, 50 ml centrifuge falcon tubes and mixed well up to 30 seconds with a vortex mixer. For 15 mins, the falcon tubes were kept at room temperature.

Figure 4.

NALC-NaOH solution

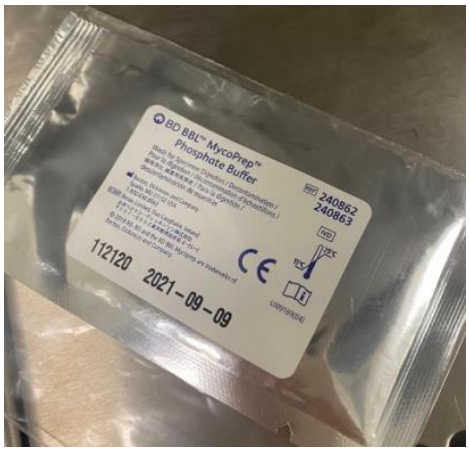
3.3.2. *Phosphate Buffer*

BBL MycoPrep Phosphate Buffer containing 2,37 gr of Disodium Phosphate and 2,27 gr Monopotassium Phosphate was used to achieve a constant pH level (0,067 M, pH=6,8), (Figure 5). For its preparation, phosphate buffer package was emptied into a 1000 ml glass laboratory bottle and distilled water was

added to it. After autoclaving at 121°C for 15 minutes, it was used when it came to room temperature. Prepared phosphate buffer added up to 50 ml centrifuge falcon tubes in a biological safety cabinet, which have a mixture of the patient sample and NALC-NaOH solution. The mixture then centrifugated at 3,000 RPM for 15 mins and the obtained sediment was diluted again with 1-2 ml of phosphate buffer (pH=6,8) after the centrifugation.

Figure 5.

Phosphate Buffer



3.3.3. *BD BACTEC MGIT 960 System*

0,8 mL of MGIT Growth Supplement/MGIT PANTA antibiotic mixture is added into the BACTEC *Mycobacterium* Growth Indicator Tube (MGIT-960, BD, Biosciences, Sparks, MD), (Figure 6). Afterwards, 0,5 mL of the decontaminated and concentrated with using a sterile pipette tip. Tubes then placed in the BACTEC MGIT-960 sample cultivated on into the MGIT device at 37°C (Figure 7) and incubated for an average of 6-8 weeks until the device gave a positive or negative result.

Figure 6.

MGIT

Figure 7.

BACTEC MGIT-960

3.3.4. *LJ Media Culture*

The negative button of the BACTEC MGIT-960 device gives a green alarm, if the result is negative and the tube was removed from the device and patient reported as negative whereas, positive samples were removed from the device and carefully cultured in LJ medium when the button of the device gave a red alarm.

Samples cultured on LJ media were kept in a laboratory incubator at 35-37°C up to 6-8 weeks (Figure 8).

Figure 8.

LJ media

3.4. Identification of *M. tuberculosis* from Clinical Specimen by Molecular Method

DNA was extracted from clinical samples by the nucleic acid extraction method using the RTP Mycobacteria Kit (STRATEC Molecular GmbH, Berlin Buch, Germany) according to the manufacturer's instructions. Primarily, 200 µl of the clinical sample and 200 µl of NAC buffer were mixed, kept at room temperature for 20 minutes and then centrifuged at 11,000 x g. The resulting supernatant was then mixed with 400 µl buffer R and transferred to an extraction tube. The next steps were performed according to the standard protocol. The purity and concentration of DNA extracted on the Nanodrop 1000 (Thermo Scientific) were determined. Samples were stored at -20°C until further analysis. Initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, amplification at 63°C for 30 seconds and extension at 72°C for 45 seconds, followed by 5 minutes at 72°C with a final extension, and at last the PCR protocol was completed. The PCR products were then electrophoresed on 1,5% agarose gels.

3.5. The QuantiFERON-TB Gold Plus

For each patient, 1 ml of whole blood is collected into four QuantiFERON-TB-Plus Blood Collection Tubes which are known as Nil tube, TB1 tube, TB2 tube and a Mitogen tube. Patients' blood taken into the tubes were incubated for 16 to 24 hours in the 37°C incubator. Plasma was collected after the tubes were centrifuged and tested for the production and release of IFN-γ by ELISA (enzyme linked immunosorbent assay) test. If one or both of the TB antigen tubes detected in the results were positive (TB1 and/or TB2), the patient was defined as positive. (The ll ES, 2018).

3.6. Statistical Analysis

SPSS (Statistical Package of the Social Sciences) Demo Ver 22 (SPSS Inc., Chicago, IL, USA) program was used for all statistical analysis of the data obtained in the study. Pearson Chi-square and Fisher's Exact tests were used to determine statistical significance and $p<0.05$ values were considered significant.

CHAPTER IV

Results

A total of 288 patients were examined in the study, of which 162 (56,3%) were male and 126 (43,8%) were female, with a mean age of 47.17±22.98 (0-95 age). Of the examined patient samples, 207 (71,9%) were sputum, 16 (5,6%) aspiration fluid, 27 (9,4%) urine, 33 (11,5%) blood and 5 (1,7%) were cerebrospinal fluid (Table 5). Since the patients applied from different departments of the hospital, the departments from which the samples came were examined in this way; 124 (43,1%) patients from the chest and allergy diseases department, 60 (20,8%) patients from the infectious diseases and clinical microbiology department, 35 (12,2%) patients from the internal medicine department, 16 (5,6%) from the pediatrics, 12 (4,2%) patients from the cardiology department, 8 (2,8%) patients from the emergency department, 7 (2,4%) patients from the neurology service, 6 (2,1%) patients from the urology service, also 6 (2,1%) from the intensive care unit, 3 (1,0%) from the otolaryngology department, 2 (0,7%) from general surgery and the last 2 (0,7%) patient came from the oncology department.

Table 5.

Distribution of the Samples

Samples	**Number**	**Percentage**
Sputum	207	71,9
Aspiration	16	5,6
Urine	27	9,4
Blood	33	11,5
Cerebrospinal fluid	5	1,7
Total	**288**	**100,0**

142 of the total patient samples were analyzed only with the Ziehl-Neelsen Staining method; 10 samples (3,5%) were found to be positive and 132 (45,8%) samples were found to be negative. The other 103 samples were only cultured and 7 (2,4%) samples were found positive whereas 96 (33,3%) samples were found negative. Only the QuatiFERON-TB GOLD PLUS test was run on 33 samples; that 8 (2,8%) of them were found positive and 25 (8,7%) were found negative. The last 20 samples were analyzed only by PCR; 2 (0,7%) samples were found positive and 18 (6,3%) samples were negative. Among the patient samples studied with different methods, 27 (9,4%) patients were found to be positive (Table 6). The mean age was 44.85±20.75 (10-82 ages) and at the gender analysis; 18 (66,7%) of the TB positive patients were male and 9 (33,3%) were female. There is no statistically significant difference between TB positivity and gender (p=0,252).

Table 6.

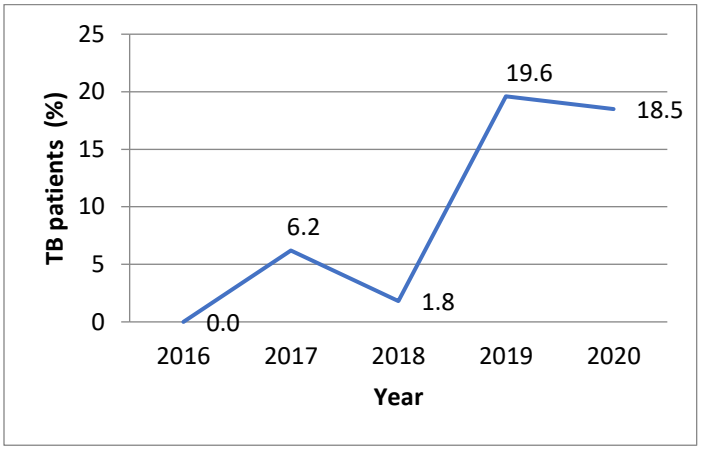
The Number of Patients Who Found as Positive for Any of The Samples

Results	Number	Percentage
Positive	27	9,4
Negative	261	90,6
Total	288	100,0

The distribution of years, total positive results obtained with different methods, it was seen that TB positive patients increased significantly between 2016 and 2020 (p=0,000). There was no any positive patients found in 2016. In 2017, 4 patients were positive then the number decreased to 1 in 2018. However the positive number of patients increased up to 10 patients in 2019 and to 12 patients in 2020 (Figure 9).

Figure 9.

Distribution over the years

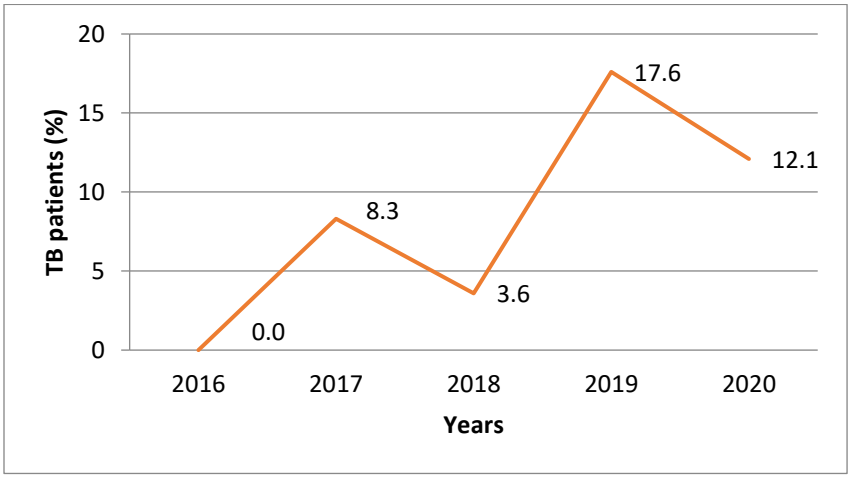


The patients who were positive for TB were also analyzed by nationality as; 14 (8,2%) TB positive patients were from Northern Cyprus, 4 (5,7%) TB positive patients were from Turkey, and the last 9 (18,8%) TB positive patients were from other nations. A statistically significant difference was found between TB positivity and nationalities. The prevalence of TB among foreign nationals appears to be higher than among other nationals (p=0,042).

There is a certain increase seen in the prevalence of TB positivity among Northern Cyprus citizens. The distribution of positivity over the years is also increased from 2016 to 2020 considerably (p=0,000). Nevertheless, this increase was not statistically significant (p=0,062) (Figure 10).

Figure 10.

Distribution of TB positivity in TRNC citizens by years



CHAPTER V

Discussion

Our study aimed to discuss the addition of BCG vaccine to the Northern Cyprus vaccination calendar by examining the prevalence of TB in the TRNC population. Total 288 patient results were examined as both retrospectively and prospectively. The majority of the samples belonged to male patients (56,3%). In this study, similar to other studies conducted in Turkey and in the world, it was found that the rate of men with TB is higher than women (Öztop, & et al., n.d.). TB is most common among the elderly and is mostly due to reactivation of a previous primary infection in developed countries, because of that, another factor in the TB epidemiology is the age distribution of TB patients. On the other hand, TB appears to affect all age groups especially teenagers and young adults in developing countries, so as the patients analyzed in our study were between 10 and 82 years old (Kılıçaslan, 2010). In addition to our results, there is no statistically significant difference between TB positivity and gender.

Mycobacteria can be isolated from different samples as; sputum, gastric aspirate, tissue biopsy specimens, cerebrospinal fluid, blood, bone marrow and urine, but sputum is the most commonly referred sample in suspected cases of TB disease (Shinnick & Good, 1995). In research of Kox et al. (1994), 218 different clinical samples tested for detection of MTC and the most the samples tested were sputum samples (n=145), and the rest were tissue cerebrospinal fluid, pleural fluid, biopsy samples, fluid from fistulae, blood, feces, and pus from a wound. In another research of MTC detection done by Clarridge et al. (1993), the study was also carried out on the most sputum samples. In comparison with other studies conducted in other countries I mentioned, most of the 288 samples tested in our study were also sputum samples (n=207).

According to the Global Tuberculosis Report 2020, the prevalence of the South-East Asia was 44%, Africa was 25%, Western Pacific was 18%, Eastern Mediterranean was 8,2%, the Americas was 2,9% and Europe was 2,5%. Also,

Voniatis et al. (2014) reported that South part of the Cyprus has virtually reached the elimination phase in its native population.

Compared to the prevalance result of Southern part of Cyprus, in 2019 it was reported as 5,3%, while the prevalence of Northern Cyprus was found as 9,4% in our study (WHO, 2020). The positive number of TB in TRNC citizens by years; none in 2016, 4 in 2017, 1 in 2018, 10 in 2019 and 12 patients reported in 2020.

Among the reasons for living of many other nationalities in Northern Cyprus that it has sunny beaches, being one of the frequent destinations for entertainment, as well as the casino tourism and also the island of the university campuses. According to our study results, TB positive patients from Northern Cyprus was 8,2%, 5,7% from Turkey and from the other nations TB positivity was 18,8%. Lillebaek et al. (2002), also stated that, people migrating from areas of high incidence to areas of low incidence are thought to have caused a resurgence of TB. The statistically significant difference was found between TB positivity and nationalities in this study (p=0,042). Besides, TB positive patients increased significantly between 2016-2020 (p=0,000) which shown at the distribution over the years in Northern Cyprus.

Since 1980, the whole country has been screened by the Ministry of Health of Northern Cyprus with the PPD test. Therefore, TB has not been declared a public health problem. However, population in Northern Cyprus is changing with globalization, tourists, immigrants, and the number of university students which are rising year by year. In addition, diversity started to increase by including people from different countries to the population of Northern Cyprus.

In the study data of Arbeláez, et al., (2000), it was stated that BCG vaccine is important for public health, especially in individuals with sufficient immunity for the prevention of extrapulmonary TB. BCG vaccine is effective against severe types of TB, but also prevents TB-related death every year (Luca & Mihaescu, 2013). The findings of Soysal et al. (2005), provided evidence that the BCG vaccine is an important factor in reducing the risk of TB infection, as well as protecting

against TB. As a result of their study, they showed that successful BCG vaccination programs reduce the risk of latent TB infection in children and the incidence of active TB in their country. Surveys of the study conducted in England and Wales in 1983 determined that the BCG vaccine given to individuals aged 15-24 years prevents against TB (Sutherland & Springett, 1987). Besides these, BCG vaccine is also known as its protective effiacy on other mycobacterial infections, such as leprosy and Buruli ulcer. It has also been shown for immunotherapy of certain types of cancer, particularly bladder cancer. In addition, health care workers' risk of getting TB infection should be also considered (Barreto, & et al., 2006).

Our results show that TB positivity is higher (18,8%) in other foreign nationals, while TB positivity among TRNC citizens has also increased over the years. Therefore, we suggested the question of 'Whether the BCG vaccine is necessary or not for North Cyprus' according to our results which should be reconsidered by the Ministry of Health.

CHAPTER VI

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Appendices

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 26.11.2020

Toplantı No **:** 2020/85

Proje No **:**1185

Yakın Doğu Üniversitesi SHYMO öğretim üyelerinden Doç. Dr. Meryem Güvenir'in sorumlu araştırmacısı olduğu, YDU/2020/85-1185 proje numaralı ve **"Bir Üniversite Hastanesi'ndeki Klinik Örneklerden *Mycobacterium tuberculosis* Saptanmasında Dört Farklı Testin Değerlendirilmesi"** başlıklı proje önerisi kurulumuzca online toplantıda değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Prof. Dr. Rüştü Onur

Yakın Doğu Üniversitesi

Bilimsel Araştırmalar Etik Kurulu Başkanı

(Ethical approval: The study was approved by Near East University Institutional Review Board (Project no: NEU/2020/85-1197)).

Curriculum Vitae

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