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Assessing specificity and discordance between the tuberculin skin test and a whole-blood interferon-γ release assay for the detection of *Mycobacterium tuberculosis* infection among United States Navy recruits.

By

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Master of Public Health

Epidemiology

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Bachelor of Science in Biology

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University of Utah

2007

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An abstract of

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Abstract

Assessing specificity and discordance between the tuberculin skin test and a whole-blood interferon-γ release assay for the detection of *Mycobacterium tuberculosis* infection among United States Navy recruits.

By Jason Matthew Lempp

Purpose

Military application of tests for the detection of M*ycobacterium tuberculosis* infection requires accuracy to limit unnecessary treatment and prevent the development and spread of active tuberculosis. This study sought to estimate specificity of the tuberculin skin test (TST) and the whole-blood interferon-gamma release assay QuantiFERON®-TB Gold In-Tube (QFT-GIT) and identify factors associated with test discordance.

Methods

Cross-sectional data on US Navy recruits tested with TST and QFT-GIT is assessed through secondary analyses using univariate and multivariate methods.

Results

Among 787 recruits with determinate TST and QFT-GIT results, 5.3% of TST indurations were ≥ 10mm, 2.9% of TSTs were ≥ 15mm, and 1.7% of QFT-GITs were positive. Assuming recruits at low risk for tuberculosis exposure were not infected, estimates of TST specificity were 99.0% (95% confidence interval [CI]: 98.2–99.9%) using a 15mm cutoff, and 98.2% (95% CI: 97.1–99.4%) using a 10mm cutoff. Estimated QFT-GIT specificity was 98.8% (95% CI: 97.9–99.8%). Recruits born in countries with a high prevalence of tuberculosis were 18 to 34 times more likely to have TST-positive but QFT-GIT-negative discordance than recruits born in low-prevalence countries. Half (18/37) of the recruits with this discordance type had TST ≥ 15mm.

Conclusions

The specificity of QFT-GIT was high and similar to TST at either cutoff. Test discordance observed in recruits with increased risk may be due to lower TST specificity, lower QFT-GIT sensitivity, or both. Negative QFT-GIT results for recruits born in countries with high–TB prevalence and whose TST is ≥ 15mm suggest that QFT-GIT may be less sensitive than TST. Additional studies are needed to determine the risk of developing TB when TST and QFT-GIT results are discordant.

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Tables and Figures

Introduction

Tuberculosis (TB) is a leading cause of death and illness worldwide. The causative agent of TB is the *Mycobacterium tuberculosis* bacillus. *Mycobacterium tuberculosis* infection (MTBI) occurs in humans, who act as its primary reservoir. Approximately 2 billion people worldwide have quiescent or latent *M. tuberculosis* infection (LTBI) and are at risk of developing TB (1). Due to effective treatment and control measures, TB incidence and prevalence rates are declining in the United States (US) and most developed regions of the world (2, 3). However, elimination of TB remains elusive, even in high-resource countries because of continued transmission in groups at high risk of MTBI and progression to TB, difficulties in detecting MTBI, and programmatic complacency (4). Around 4% of the US population is thought to have LTBI, and is a constant source for future TB and MTBI transmission (5). There is continued pressure to improve diagnostic and screening methods for detecting MTBI, including LTBI and infection manifesting as TB (6-8). Identifying and treating LTBI among those at high risk of developing TB is an important component for TB control and elimination in low-TB incidence regions, such as the US and Canada (9, 10).

No method exists for accurately detecting LTBI. Historically, the only way to diagnose LTBI was the tuberculin skin test (TST), which involved measuring immunologic delayed hypersensitivity to an intradermal injection of purified protein derivatives (PPD) made from *M. tuberculosis* (tuberculin PPD) (11). The TST has limitations in detecting LTBI in some settings. For example, where the prevalence of LTBI is low, the positive predictive value (PPV) of TST is hindered by cross reactions induced by sensitization to other mycobacteria (11).

The development of interferon-gamma release assays (IGRA) offered an alternative to the TST and addressed some limitations in the TST (12, 13). MTBI typically induces an immune T-lymphocyte response which produces the cytokine interferon gamma (INF-γ) when the T-cells encounter mycobacterial antigens (13). Whole-blood IGRAs such as the 2nd generation, QuantiFERON®-TB Gold test (QFT-G) (Cellestis Limited, Carnegie, Victoria, Australia) measure the amount of INF-γ released when blood is stimulated with specific *Mycobacterium* antigens (14). In 2007, the US Food and Drug Administration (FDA) approved the 3rd generation of IGRA for the detection of MTBI, the QuantiFERON®-TB Gold In-Tube test (QFT-GIT) (Cellestis Limited, Carnegie, Victoria, Australia) (15). Like the TST, positive QFT-GIT results are highly associated with factors that historically contribute to MTBI (15, 16). However, QFT-GIT can exhibit discordance when compared to TST results and other IGRAs (15, 17-19).

Absence of a "gold standard" to confirm MTBI limits IGRA assessments of accuracy and allows only estimates of sensitivity and specificity (15, 16). Approximations of sensitivity have been achieved by comparing the results of diagnostic tests to culture-confirmed cases of TB (15). Previous studies have assessed IGRAs among "assumed negative" populations at low-risk of MTBI, to better approximate specificity, with some consideration of test discordance (20-22). Further research has been suggested to understand the factors associated with discordant test results (6, 15, 23).

In light of the need for more research to understand IGRA accuracy and discordance, this study on QFT-GIT is part of a series of IGRA studies (21, 24-26). This study was conducted to add to the current discourse on the interpretation of LTBI screening results among low-risk populations when considering multiple testing methods. The analysis of cross-sectional data obtained from US Navy recruits in 2004 will quantify QFT-GIT specificity for a population at low-risk for MTBI. Additionally, this study will identify recruit characteristics and estimate their associations to discordant results between the QFT-GIT and TST. Results for QFT-GIT will also be compared to other IGRA results, performed at the same time (21).

Chapter I

Background

Epidemiology of Tuberculosis

Tuberculosis is the 7th leading cause of global mortality and the 2nd most frequent cause of death from a single infectious disease agent, after the human immunodeficiency virus (HIV) causing acquired immune deficiency syndrome (AIDS) (27). TB causes an estimated 1.5 million deaths per year, with approximately 9.4 million new cases occurring annually, amounting to a global prevalence of 12 to 16 million people with active disease (1). One third of the world's population (more than two billion persons) may be infected with bacteria that cause TB (1). The US is a low-TB incidence country, with a decreasing TB rate of 3.8 cases per 100,000 population in 2008, with civilian LTBI prevalence estimated at 4.2% (over 11 million) in 2000 (2, 5). The US military has a lower rate of reported pulmonary TB than the US average (0.65 cases per 100,000 persons from 1998 to 2007) (28). Even when adjusting for underreporting, the military's low TB incidence (0.87/100,000 person-years) can be defined as a "low-incidence" population (29). Estimates of LTBI in the military, determined by TST reactivity, range from 1 to 5%, depending on the service branch (30). Despite the low incidence, military populations may still be at risk of Mtb

transmission due to exposure to populations at increased risk of MTBI and congregate settings (31-33).

Most TB is caused by *M. tuberculosis*, but other species of *Mycobacterium* may cause TB, including *M. bovis*, *M. africanum*, *M. canettii*, and *M. microti*. These genetically similar species of mycobacteria are collectively referred to as the *Mycobacterium tuberculosis* complex (Mtb) (34). Despite their similarities, the characteristics of specific Mtb species vary. *M. africanum* is primarily concentrated in western Africa, where it accounts for nearly half of TB cases, yet is absent throughout much of the world. This geographic isolation may be due to *M. africanum* being outcompeted in other environments, possibly due to slower progression to transmittable TB disease compared to *M. tuberculosis* (35). Infection by *M. bovis* can cause TB in humans, cattle and other mammals. It can be zoonotically transmitted via an aerosol or the ingestion of contaminated milk. The virulence of certain *M. bovis* strains may be greater than some *M. tuberculosis* strains (36). A mycobacterial vaccination was created by attenuating *M. bovis*, called the bacille Calmette-Guérin (BCG) vaccine, and is used as a protective measure against childhood MTBI (37). Non-tuberculous mycobacteria (NTM) can be pathogenic but their infections do not result in the development of TB. Examples include *M. leprae* that cause leprosy, and common environmental fauna, like *M. avium* causing opportunistic infection (34).

Mtb are aerobic bacilli, typically 1 – 5 μm in size, have a thick mycolic acid coating and a long replication time of 12 to 24 hours (9, 38). These features allow the bacteria to be transmitted through the air and manifests as a chronic infection (34, 38). TB is most commonly a respiratory disease contracted through the inhalation of aerosolized droplet nuclei containing the organism, by a susceptible host (34). Droplet nuclei are small particles of secretions which contain Mtb when expelled from an individual with active pulmonary or laryngeal TB, through coughing, sneezing, talking or singing (34, 39). MTBI may occur when these infectious floating nuclei are inhaled into the alveoli of the lungs (9). The factors which determine the likelihood of airborne transmission of Mtb are: the number of organisms expelled; the size of the droplet nuclei expelled, air space volume, ventilation, duration of exposure, the immune status of the exposed individual; and virulence of the Mtb strain (39, 40).

Pathogenesis of *Mycobacterium tuberculosis* Infection

Once the Mtb are in the alveoli, the host's alveolar macrophages attempt to consume the bacilli (39). The responding macrophages trigger an immunologic cascade that activates various lymphocytes and promotes secretion of cytokines, such as IFN-γ (41, 42). The release of IFN-γ is central to both innate and adaptive immune responses to certain infections, including Mtb (43). IFN-γ has the ability

to inhibit bacterial replication through activation of macrophages and production of reactive oxygen and antimicrobial intermediates; it also stimulates further immune response pathways (41, 42, 44). The localized response to Mtb involves innate or non-specific immune mechanisms that are not detectable by current TB diagnostic tools designed to detect responses to particular antigens (40, 41).

If the initial local immune defenses fail to clear the infection, the macrophages which phagocytized the bacilli may become the site of bacterial replication or dormancy (39-41, 45). Slow Mtb replication, as compared to other bacteria, may explain delays in the development of cellular immune response to Mtb (39, 46). During this phase, Mtb and infected macrophages can disseminate through the bloodstream to other extra-pulmonary sites. If specific immunity to Mtb is slow to develop, military TB may occur where multiple organs are diseased simultaneously (45, 47, 48). The adaptive immune response progresses when infected macrophages migrate to the lung's draining lymph nodes and present Mtb antigens (44). This activates naïve T-cells to differentiate into Mtb antigen specific T-cells, such as IFN-γ secreting CD4+ and CD8+ T-lymphocytes (40, 41, 46). These cells then enter blood circulation and are recruited to the infected tissue, where they continue the inflammatory cytokine response to control infection in the vast majority of the infected (40, 44).

CD4+ T-lymphocytes are effector cells and release more IFN-γ at primary infection, compared to CD8+ T-lymphocytes which are memory cells. CD8+ T-lymphocytes facilitate the development of a more rapid immune response with secondary infections (41, 49). The slow buildup of antigen specific cells and IFN-γ production in the lungs may take up to 20 days to disrupt log-phase bacterial growth (46). Detection of antigen specific immune responses may not be possible for 2 to 8 weeks while Mtb sensitization is developing (50, 51). Currently available indirect tests for MTBI that detect immune responses are frequently negative during this "window period" (52).

Within the alveolar macrophages, Mtb replication may slowly progress until macrophages burst and trigger further inflammatory immune response (47, 53). Additional immune defense cells (activated lymphocytes, phagocytes, neutrophils and fibroblasts) from the bloodstream then attempt to contain the MTBI and necrotic cells through the production of granulomas, or interlocking envelopes of fibrosis, inflammatory cells and calcification (45). These granulomas help to contain the bacteria by segregating the infection and concentrating the immune response; however, granulomas can also house live bacilli and maintain the MTBI for years as LTBI (39, 45, 54, 55). LTBI is a subclinical infection without symptoms, typically not apparent radiographically (39). Without treatment, the outcome of LTBI may be that Mtb become sterilized

by host defenses, remain viable but dormant in the host for their entire life, or reactivate to cause active TB disease (41, 45, 56).

The nature of LTBI is difficult to study due to its complex interaction with the human immune system and imperfect replication in animal models. An estimated 5% of people with untreated LTBI will develop TB within two years of being infected (39). An additional 5% of immunocompetent individuals with LTBI develop active TB in more than 2 years after being infected. The lifetime risk of TB increases to 30% among diabetics. The estimated annual risk for people with HIV infection is 10% per year (11, 57). The dormancy model of LTBI suggests that granuloma-encased Mtb enter a state of non-replicating persistence, with the potential for future activation. The Mtb may undergo a metabolic transformation due to decreased oxygen hypoxia to remain viable but in stasis (58). Additional studies indicate that prolonged Mtb growth may reach a state of non-cultureable viability in the absence of certain nutrients, where when exposed to exogenous resuscitation factors, the Mtb can activate (58).

Dynamic models of LTBI propose that LTBI involves cycles of Mtb growth and migration against the immune response control to maintain a subclinical bacterial load. Immunosuppressive events allow for an imbalance in the homeostasis in bacillary load, where growth continues to eventually manifest as TB (59). Mtb may still enter a decreased metabolic state or non-replicating

status within infected macrophages. However frequent "drainage" of Mtb into the interalveolar space and alveolar fluid aerosols, allow for reaerosolization within the alveoli. Mtb may be transported to a new location in the lung to again form a granuloma or proliferate to TB, enacting endogenous reinfection (56) Exogenous infection, or subsequent exposure to TB in the environment after initial MTBI, has been shown to lead to increased risk of TB when reinfected (60). In high-TB prevalence areas, exogenous reinfection may be more likely than previous estimates of LTBI activation (61). Reinfection, via endogenous reactivation or exogenous recent infection, may elicit a stronger inflammatory response, increasing tissue necrosis leading to activation of existing LTBI or providing more suitable conditions to rapid development of TB (56, 59-63). The occurrence of TB due to reinfection and activation contribute to estimates of lifetime risk (45, 59). However, the extent of "acute resolving infection" and subsequent exogenous reinfection versus a waned immune response from dormant LTBI and subsequent endogenous reactivation, remain unclear (41, 44). The natural history of LTBI, the activity of the Mtb during latency, Mtb interaction with the immune response and optimal LTBI treatment are all current areas of active research (64, 65). Indirect immunologic tests such as IGRAs can be used to add clarity to the discussion on LTBI natural history (41, 56).

Diagnostics for *Mycobacterium tuberculosis* Infection

TB often requires presumptive diagnosis to initiate therapy, due to the time required for confirmatory testing. Such diagnosis requires consideration of multiple clinical factors. Symptomatic individuals with TB usually present with a productive cough; other signs include fever, weight loss, or night sweats (39, 66). Indirect tests for MTBI such as the TST and IGRAs may provide support when TB is suspected, but do not differentiate LTBI from TB disease (15, 66). Individuals suspected of TB based on clinical findings, or MTBI based on immunodiagnostic testing, are also typically evaluated by chest radiography (CXR) for the presence of cavitations, infiltrates, scarring or other abnormalities (39, 67). The CXR is an important diagnostic tool for TB as nearly all pulmonary TB causes abnormalities on the chest film (39). A normal CXR with no TB symptoms has a high negative predictive value (NPV) to discount TB (67). Those suspected to have TB may have sputum examined for the presence of acid-fast bacilli (AFB) by microscopy (9, 39). Within weeks, growth of Mtb by culture of sputum or other sources typically confirms MTBI and is the "gold standard" for diagnosing clinically active TB (39, 68). Susceptibility testing of recovered Mtb can aid in selection of treatment regimens and is useful when considering antibiotic resistant strains of Mtb (9, 39).

Unfortunately, the methods used for diagnosis of TB are not effective for diagnosing LTBI. Individuals with LTBI have immune systems which are effectively containing the infection; therefore, they are symptom-free and non-infectious (39). These individuals do not expel Mtb and cannot be diagnosed by laboratory smear or culture (39). Typically results from CXR also appear normal due to the small bacterial load and limited tissue damage by the immune response (11, 69-71). LTBI diagnosis may require a normal CXR, a negative AFB smear and a negative culture to rule out TB, but these findings do not confirm a diagnosis of LTBI (57). The inability of the above methods to adequately diagnose or exclude LTBI, places a reliance on immunodiagnostic tests for MTBI. Both the TST and IGRAs indirectly test for infection by detecting an immune response, to mycobacterial antigens (44, 72). A positive response to either of these tests may indicate the presence of MTBI (either LTBI or active TB), resolved MTBI (due to an effective host immune system or treatment), or an erroneous false-positive (e.g. due to sensitization with BCG or cross reactivity to tuberculin PPD) (11). As these diagnostic tools are unable to reliably identify viable Mtb, the diagnosis of LTBI is left without a "gold standard" by which to measure diagnostic accuracy (22).

Various methods are applied to address the uncertainty inherent in MTBI testing and improve test accuracy. "Confirmatory" testing uses two tests with the same result, double-positive or double-negative, to support the presence or

absence of infection. Retesting people at low-risk of MTBI who initially test positive can decrease the number of people diagnosed with infection if only those with positive results on both tests are considered infected. This assumes people with discordant results and those initially negative are uninfected. This strategy has been used to develop testing algorithms for MTBI detection (73). Another method assesses response to PPD from different species of mycobacteria by skin testing. For example, selection of the "dominant" or larger reaction produced by either tuberculin PPD or Batty PPD (PPD from *M. intracellulare*) helps to differentiate reactivity due to MTBI versus tuberculin reactivity due to cross-reactive sensitization to NTM (74-76). A method of serial testing the same individual establishes a pretest likelihood of either a positive or negative result for subsequent tests. Assuming the accuracy of the baseline result, this can be used to monitor recent Mtb transmission. However, variations in the immune response, measured by subsequent tests, can lead to reversions from positive to negative or conversions from negative to positive, unrelated to MTBI (77-79). Yet another method to improve test accuracy is to adjust the threshold separating positive and negative results to match the risk and pretest probability of infection. This can be particularly effective if the likelihood of infection and pretest probability of a given population, or a segment of the population, is known. This logic applies to screening and targeted testing for only those at increased risk of MTBI. This

assumes the pretest probability for those without increase risk of infection, is so low that a positive result will most likely be false (11).

The Tuberculin Skin Test

The TST measures *in vivo*, delayed-type hypersensitivity to mycobacterial proteins in tuberculin PPD, injected intradermally. Antigen-specific lymphocytes and inflammatory cytokines accumulate at the site of injection, of individuals sensitized to *M. tuberculosis*, but not for those without Mtb infection or sensitization (72). Currently, the Mantoux method is preferred for administering tuberculin PPD (39). The test requires two patient visits. At the first visit, 0.1 ml, 5 tuberculin units, of PPD is injected intradermally into the forearm. The injection site is examined at the second visit, 48 – 72 hours later, for induration which is measured in millimeters (mm) (11).

Risk based interpretation of results can improve TST accuracy. Applying a higher threshold of TST induration to separate positive and negative results for those with less risk, decreases the likelihood that a low-risk person will have a positive result. The corollary is that use of a lower threshold of TST induration to separate positive and negative results for people who are at increased risk of infection are likely to have disease, increases the likelihood that these people will

test positive. This is helpful because there is considerable overlap in the induration seen in people who are infected and those who are uninfected. In addition to risk-based interpretations, another approach to improve TST accuracy is targeted testing of those determined to be at increased risk of MTBI. This increases the pretest probability that a positive result is a true positive and identifies someone actually infected with Mtb.

Guidelines by the US Center for Disease Control and Prevention (CDC) for induration interpretation are established by considering the prevalence of TB in different groups (11). Individuals at high risk of developing TB if infected (the immunocompromised and recent TB contacts) and those suspected of TB, should be considered positive at an induration $\geq$ 5 mm. Those not at high risk, but at increased risk of exposure or of developing TB are interpreted as positive at indurations $\geq$ 10 mm. Factors for increased-risk include: recent immigrants from high-TB prevalence countries; intravenous drug users (IDU); residents and employees of high-risk congregate settings such as institutional or health care facilities (prisons, hospitals, shelters, etc.); mycobacteriology laboratory personnel; persons with chronic, high-risk conditions; children younger $\leq$ 4 years old; and adolescents exposed to high-risk adults. Individuals with none of these risk factors for TB are considered TST positive at indurations $\geq$ 15 mm (11).

Current TST and tuberculin PPD were developed from methods for TB vaccination discovered in 1890 by Robert Koch, which involved a subcutaneous injection of filtrates from *M. tuberculosis* cultures (80). Koch's "Old Tuberculin" led to the development of tuberculin, a concentrated solution of heat-sterilized filtrate from cultured *M. tuberculosis* (80). Methods for precipitating proteins from Mtb culture filtrate were developed in 1939 to improve the reproducibility and purity of the TST antigens (81). Tuberculin PPD for commercial use is standardized through comparing master batches against a controlled international reference (82). The mixture of proteins present in the tuberculin PPD are not specific to *M. tuberculosis*, as many of the proteins are also present in *M. bovis* BCG and some NTM (83, 84).

Accuracy of the TST is limited by a number of factors. True test sensitivity and specificity cannot be determined because there is no way to confirm LTBI. TST PPV and NPV depend on sensitivity and specificity, positivity cutoff values and the prevalence of MTBI in the population (80, 85). Estimates of sensitivity and specificity are therefore required, but are subject to misclassification bias regarding who is and who is not infected. The specificity of a screening test is the probability that the test will be negative given the individual is free of disease, i.e. a specificity of 100% would indicate that all uninfected individuals had negative results. TST specificity is < 100%, allowing false-positive results (a positive result, but not Mtb infected) due to cross-reactions with

tuberculin PPD following sensitization to environmental NTM and with BCG vaccination. Proteins produced by these organisms are also in tuberculin PPD (11, 81, 86).

Serial testing is often necessary among TB contacts, health care workers (HCW), military personnel and others with ongoing risk of infection (9, 87). Injection of PPD can cause boosting of the immune response measured with subsequent tests for MTBI. A positive TST due to boosting may occur following a recent TST in persons where response to prior Mtb, NTM or BCG has waned, erroneously resembling recent transmission (9, 78). Country of birth and international residence can further complicate MTBI screening accuracy, as the prevalence of MTBI varies between countries, as does the prevalence of NTM sensitization (88, 89). Furthermore, many countries routinely apply infant BCG vaccination and may repeat BCG vaccinations into adolescence or later. The number of BCG vaccinations and their proximity to TST placement have been shown to impact TST results (86, 90-92). All of these factors can greatly reduce TST specificity and PPV, especially in populations at low-risk for TB (11, 78, 80, 86).

The sensitivity of a screening test is the probability that the test will be positive if the person is infected, i.e. a sensitivity of 100% would indicate that all infected individuals had positive results without any false-negative results (a

negative result in a person with MTBI). False-negative results reduce sensitivity and can be caused by immunosuppression, a waned or delayed immune response, and errors in TST techniques (9, 93). The ability to mount an immune response to MTBI and react to tuberculin PPD requires the mobilization of antigen-specific T-lymphocytes. Decreases in CD4+ T-lymphocytes among those infected with HIV/AIDS may cause selective cutaneous anergry and reduce or inhibit the expected immune response (39, 94). Impediments to immune function due to other coinfections (including severe TB), malnutrition, certain medications, and chronic illnesses may also interfere with responsiveness to tuberculin PPD (39). A waning immune response to remote or treated LTBI has been seen among older adults, resulting in TST reversion (78, 95-97). Individuals recently infected with TB may not become TST positive for 8 to 10 weeks while immune responsiveness is developing (50). Faulty techniques, measurement error, and within-subject variability can also lead to false-negative results (80, 98). The particular cutoffs used for TST interpreting will directly affect test sensitivity and specificity (11).

The TST also faces operational challenges. Administration of the TST requires a trained health care professional, practiced at placement and measuring for consistent results (39). Repeat visits 2 to 3 days after TST placement allows for high rates of loss to followup. Low adherence to TST schedules have been a considerable drawback among certain high-risk populations such as the homeless,

substance abusers, the HIV infected, recent immigrants, and among the poor in resource limited countries and settings. (7, 99-103). Screening programs for MTBI that rely on TST may have considerable costs associated with treatment of individuals with false-positive results and expense associated with resheduling and retesting for additional follow-up testing (76, 104, 105). Despite these limitations, the TST is still widely used to diagnose LTBI and intiate isoniazid (INH) prophylaxis. Treatment of MTBI diagnosed by TST can reduce the risk of developing TB by 60% to as much as 93% when adhering to prophylaxis for 12 months. (13, 39, 65).

Interferon-gamma Release Assays

The development of IGRAs in the 1990's, offered an alternative to TST for detecting MTBI, and addressed many TST limitations (13). IGRAs assess response to Mtb antigens secreted by circulating T-lymphocytes present in whole blood, sensitized to Mtb in response to ongoing or recent MTBI (13, 106). IGRA's do not boost IFN-γ responses measured in subsequent tests or serial testing because the antigen is not injected. IGRA testing requires a whole blood sample obtained from a blood draw during a single patient visit. Because antigen stimulation must occur while blood cells are viable, blood should be mixed with antigens within 12 hours of collection, and the mixture should be incubated for 16

to 24 hours (13, 107). For IGRAs, incubation time plays an important role in allowing the targeted lymphocytes (CD4+ efffector T-cells) time to recognize the mycobacterial antigens and produce measuarble IFN-γ (108). Incubation less than 24 hours allows for the detection of IFN-γ released by circulating effector CD4+ T-lymphocytes. Detection of IFN-γ released only after incubation of over 36 hours suggests activation of CD8+ central memory cells (109, 110). Once stimulated by Mtb antigens, the lymphocytes produce IFN-γ and can be detected through diagnostic laboratory techniques.

Since 2001, the Food and Drug Administration (FDA) has approved four IGRAs to assist in the diagnosis of MTBI (15). Two different techniques are used to detect IFN-γ release by sensitized T-lymphocytes. The first technique measures differences in IFN-γ concentration ([IFN-γ]) in plasma, from blood incubated with Mtb antigens vesus without. [IFN-γ-] is measured useing an enzyme-linked immunosorbent assay (ELISA). The QFT-G is one example of three tests which uses a "sandwhich" ELISA (14, 107, 111-113). For ELISA, plasma is added to different wellss of an ELISA plate along with conjugated antibody. The bottom of the wells are coated with murine anti-human IFN-γ monoclonal "capture" antibodies. The IFN-γ attaches to the antibodies and is bound to the bottom of the well. The conjugated antibody also bind to the IFN-γ and serves as the other "bookend" to the assay. The conjugate consists of a murine monoclonal anti-human IFN-γ antibody bound to horseradish peroxidase

(HRP). Mouse serum is included to supresses non-specific binding of the conjugate, or plasma heterophile antibodies, to the capture antibodies. Wells are washed in a buffer solution to remove unbound components. A chromogen substrate, *3,3'*,5,5' Tetramethylbenzidine (TMB), reacts with HRP in the presence of H_2O_2. The color change reaction that occurs is dependent on the amount of IFN-γ present. Optical densities (OD) or absorbance values for each sample are generated based on the amount of color that is present in the well. The OD of each plasma sample is compared to an IFN-γ standard curve, generated for each plate to determine the sample [IFN-γ] in international units (IU/ml) (14, 107, 112, 114).

The second IGRA technique measures the number of T-lymphocytes that release IFN-γ, using an enzyme-linked immunospot assay (ELISpot). For this approach, pripheral mononuclear cells (PMMC) are recovered from blood and incubed with and without Mtb antigens in an ELISpot plate. The bottom of the plate is coated with murine anti-human monoclonal antibodies. Lymphocytes from PMMC are stimulated with antigen to produce IFN-γ, which is bound to the base of the assay plate by the IFN-γ specific antibodies. Antigen and lymphocytes are then removed and the plate is washed with enzyme-labeled detection antibodies and chromogenic compounds which bind to the IFN-γ. Colored spots are produced on the plate where cytokine producing cells secreted IFN-γ, which can be counted and compared to positive and negative controls

(115, 116). The T-Spot®.TB test, (T-Spot) (Oxford Immunotec Limited, Abingdon, United Kingdom) uses ELISpot and is the most recent commercially available IGRA, FDA approved in 2008 (117). T-Spot incubates *M. tuberculosis* specific peptides simulating early secreated antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) with the extracted white blood cells to innumerate effector T-lymphocytes sensitized to Mtb (118).

Whole-Blood Interferon-gamma Release Assays

The QuantiFERON®-TB test (QFT) (Cellestis Limited, Carnegie, Victoria, Australia) was the first FDA approved IGRA, and uses ELISA to measure differences in [IFN-γ] in blood stimulated by Mtb antigen and controls (112, 119). QFT assesses the amount of IFN-γ released in response to tuberculin PPD, a mitogen positive control, an unstimulated "Nil" negative control, and an avian PPD (PPD produced from *M. avium*) as a control for NTM sensitization. The mitogen control contains phytohaemagglutinin (PHA), which non-specifically stimulates T- lymphocytes to release cytokines, therefore the concentration of IFN-γ found in the mitogen well ([Mit]) is a quality control for sample viability. Saline is added to the Nil control well to adjust for background, including heterophile antibody effects, and baseline IFN-γ in blood samples. IFN-γ concentrations from the nil ([Nil]) are subtracted from the other [IFN-γ]s. Ratios

of IFN-γ released in response to tuberculin PPD, avian PPD, and mitogen are used to interpret the QFT results (112). Studies investigating the QFT reported similar or inferior specificity to TST (13, 112, 120-122). Cross reactions still occurred from prior BCG vaccination (123). There also remained concerns about IGRA boosted reactions from recent applications of TST (124, 125). The test was discontinued after the release of a second generation of ELISA, targeted at improving specificity.

Through genetic sequence comparisons of *M. tuberculosis* and *M. bovis* BCG, chromosomal regions of difference (RD) were identified and used to search for specific antigens unique in Mtb (126). Low-molecular mass antigens, such as immunogenic protein-64 (MPT-64), ESAT-6 and CFP-10 were identified in the first assessed RDs. ESAT-6 and CFP-10 were most promising for sensitive and specific testing for MTBI, due to only being present in *M. tuberculosis*, and absent in most *M. bovis* BCG vaccine strains (72, 123, 127-131). However, these antigens do occur in some NTM species, such as *M. kansasii*, *M. marinum*, *M. szulgai*, and *M. riyadhense*, potentially causing NTM cross-reactions (72, 132-134). Synthetic overlapping peptide chains representing whole ESAT-6 and CFP-10 antigens were also found to be highly specific to identify Mtb sensitization (135, 136).

The QFT-G was FDA approved in 2004 for detection of MTBI and replaced the QFT (137). The antigens used for QFT-G include two cocktails of overlapping peptides simulating the antigens ESAT-6 and CFP-10 (14). QFT-G also includes a mitogen and Nil control. Interpretation of results requires comparisons among four [IFN-γ] components, the [Nil], [Mit] and the highest IFN-γ concentration from plasma stimulated with ESAT-6 or CFP-10 ([Tb]), independently. The TB Response is calculated as [Tb] – [Nil] and Mitogen Response as [Mit] – [Nil]. A positive interpretation requires the TB Response to be $\geq$ 0.35 IU/ml of and $\geq$ 50% of the [Nil]. Negative interpretations require a [Nil] $\leq$ 0.7 IU/ml and a Mitogen Response of $\geq$ 0.5 IU/ml. Indeterminate results can occur when the Mitogen Response is < 0.5 IU/ml in an otherwise negative result, or when the [Nil] is > 0.7 and the TB Response is < 50% of the [Nil] (14, 15).

QFT-G improved specificity in detecting MTBI, over tests using tuberculin PPD antigen (16, 21, 124, 131, 138). However, estimates of QFT-G accuracy vary (120, 138). Precise, overall measures of accuracy are difficult to determine due to heterogeneity in IGRA methodology and interpretations, populations studied, case definitions, (culture-confirmed versus clincal diagnoses), and misclassification bias (15, 139). For example, individuals at low-risk for MTBI may be truly infected due to an unknown exposure unrelated to common risk factors, but would still be considered negative in estimations of

specificity. Comparisons between studies are also challenging when testing is conducted in different populations or with heterogenous prevalence rates of TB, BCG vaccination and NTM exposure.

Some reviews estimate QFT-G sensitivity ranging from 70 to 89% among culture-confirmed cases, with a pooled sensitivity at 78% (95% CI: 73 – 82%) (16, 140-142). Other reviews find higher ranges of sensitivity (83 – 91%) in low-TB burden populations, and poorer sensitivity (69%) among high-TB burden populations (14, 143). This descrepancy may occur due to anergy from severe TB found in high-TB burden countries, as well as HIV/AIDS or malnutrition, which can cause false-negative IGRA results (16). Similar to high-TB prevalence countries, a high pre-test probability of infection (contact investigations) may reduce the NPV of the QFT-G (144). Overall, QFT-G sensitivity is considered at least as sensitive as the TST (138, 140, 145). Reported specificity of QFT-G is more consistent than estimates of sensitivity. Specificity is best assessed among populations with a low risk of having MTBI. Most reviews of QFT-G find specificity values around 97-99% (138, 146);Menzies, 2007 1903 /id;Lalvani, 2007 1859 /id}. QFT-G specificity is consistantly high in BCG vaccinated populations, where TST specificity can fall to below 50% (16, 147). The advent of the QFT-G showed the commercial utility of specific antigens in MTBI detection and the possibility for new advances in accuracy of LTBI testing (13, 124, 148).

In 2007, the QFT-GIT was approved by the FDA (149). The QFT-GIT included enhancements to the mix of simulated antigens to induce IFN-γ release, as well as a change in the method which exposes the antigens to the whole blood. Blood collected by other IGRAs require rapid transportation to laboratory settings for stimulation. Exposure of blood to the antigens must occur within hours of collection, to maintain the viability of the white blood cells and reduce the likelihood of indeterminate or false-negative results (14, 15, 141, 150, 151). QFT-GIT addresses these preanalytic limitations by including blood collection tubes internally coated with dried peptides representing select *M. tuberculosis* proteins, along with control tubes (107). The simplification of QFT-GIT to only one step requiring in-lab blood handling is a logistical advantage (145, 152). Per the manufacturer's specifications, QFT-G samples require stimulation with antigen within 12 hours of collection, whereas QFT-GIT samples are already exposed to antigen in the tube and for stimulation upon reaching incubation within 16 hours (107, 112). Immediate incubation of tubes after blood collection may further improve test accuracy by maintaining a higher number or greater activity of viable immune cells (153).

In addition to the antigens ESAT-6 and CFP-10, which were present in QFT-G, QFT-GIT includes part of the Mtb TB7.7 protein, as an antigen. The gene for TB7.7, Rv2654, was discovered in RD11 and is suspected to be a "phage insert" into the genome of a relatively recent ancestor of the Mtb; it is present in

some Mtb species (*M. tuberculosis*, *M. africanum*, *M. microti*, and several *M. bovis* isolates), but is not found in *M. canettii* (126). IFN-γ response to TB7.7 proteins were found to be highly specific for MTBI (97%) and specificity was not affected by BCG vaccination (154). Sensitivity of TB7.7 was similar to either ESAT-6 or CFP-10 alone. A portion of the protein (peptide 4) is unique to Mtb, but other portions are also present in some NTB (Gerald Mazurek, US CDC, personal communication, 2011). The inclusion of TB7.7 protein 4 in QFT-GIT was an attempt to improve sensitivity, synergistically with ESAT-G and CFP-10 (152, 154) (Gerald Mazurek, US CDC, personal communication, 2011).

Sample processing and interpretation for QFT-GIT were changed from the previous generation IGRA, QFT-G. The test requires three vacuum blood collection tubes: a tube containing heparin and a mixture of 14 peptides represting the amino acid sequence of ESAT-6, CFP-10 and part of TB7.7 (TB antigen tube); a tube containing heparin alone (Nil tube); a tube containing heparin and PHA (mitogen tube). Blood in these tubes are shaken to facilitate mixing of the tube contents with whole blood. The tubes with blood are incubated for 16 to 24 hours. The amount of IFN-γ in plasma for each tube is measured by ELISA. After ELISA, the TB Response and Mitogen Response are calculated by subtracting the [Nil] from the [IFN-γ] in the TB antigen tube or mitogen tube. Positive results require a [Nil] $\leq$ 8.0 IU/ml and a TB Response of $\geq$ 0.35 IU/ml and $\geq$ 25% of the [Nil]. Negative interpretations require a Mitogen Response of $\geq$

0.5 IU/ml, a [Nil] $\leq$ 8.0 IU/ml, and a TB Response < 0.35 IU/ml or < 25% of [Nil]. Indeterminate results occur when [Nil] is > 8.0 IU/ml; when a Mitogen Response is < 0.5 IU/ml, and the TB Response is < 0.35 IU/ml or < 25% of the [Nil] (15, 107).

Estimates of the accuracy of the QFT-GIT are variable. Sensitivity of the QFT-GIT in early reviews found a pooled range between 67 to 78%, which overall performed worse than the TST (70 to 85%) (16, 155, 156). More recent reviews of QFT-GIT sensitivity find closer agreement with a pooled range of 80 to 81%, with greater sensitivity when considering only culture confirmed TB cases (83%) and studies in developed countries (84%) (15, 157, 158). Lower sensitivity was seen in high-TB prevalence countries (74%), even when a lower threshold is used to define the TB Response (64-77%) (157, 159). QFT-GIT sensitivity was greater than pooled TST sensitiviy (65 to 70%) in some reviews (157, 158). While pooled TST sensitivity (89%) was significantly higher than QFT-GIT sensitivity in at least one review (15).

Specificity of QFT-GIT remains high across most reviews, with a range in pooled specificity of 98 to 100% (15, 16, 157, 160). However, some reviews have found lower specificity (64 to 79%) (104, 104, 158, 161). Regardless of the specificity found, QFT-GIT still has a considerably higher specificity than TST in most settings (55 to 97%) (15, 16, 155, 156, 158, 160-162). The differences in

sensitivity and specificty between the IGRAs and TST, and the variability in agreement among IGRAs, suggests a high level of uncertainty in these estimates. Assessing discordant results between these tests helps to explain some uncertainty in test accuracy (15, 163).

Discordance in Results for the detection of *M. tuberculosis* Infection

An understanding of discordance between tests for MTBI is clinically relevant. LTBI cannot be confermed by any test, therefore uncertainty due to discordant test results may result in misdiagnoses. Misdiagnosis may lead to unneccessary prophylaxis, a missed opportunity to prevent TB activation, and allow transmission of MTBI. Discordant results are not infrequent, with pooled frequencies seen in 10 to 40% of results (145, 156, 164, 165, 165).

The most frequent form of discordance seen is positive TST but negative IGRA results (TST+/IGRA-) (21, 25, 120, 145, 156, 164, 166). This is not surprising because IGRAs that use select Mtb antigens are more specific than TST, and have less false-positive results due to BCG vaccination and NTM sensitization (13). Many studies have found BCG to be associated with TST results and not IGRA results in direct comparisons (6, 120, 167, 168). Populations with high BCG vaccination coverage have higher rates of

TST+/IGRA- discordance, espeically where BCG vaccination occurs at older ages and multiple times (16, 18, 156). In a small review of QFT-GIT, BCG vaccination or foreign birth accounted for 96% of TST+/QFT-GIT- discordance. Among those BCG vaccinated, 85% with discordant results had received BCG vaccination $\leq$ 10 years prior to screening (169).

Agreement between TST and IGRAs is higher in non-BCG vaccinated populations, but discordance still occurs (130, 156). Sensitization to NTM can cause false-positive TST results and discordance with IGRAs (47, 86, 170, 171). NTM sensitization is expected to cause greater false-positive TST results when a 10mm cutoff is used (75, 172). The species and prevalence of NTM sensitization in foreign countries or various populations is difficult to quantify (89, 173). In addition to high-TB prevalence, the prevalence of NTM sensitization in Asia may support the higher reates of discordance seen among those of Asian or Pacific Island ethnicity (21, 174-176). However, certain Asian subpopulations have been shown to have reduced IGRA sensitivity in culture-confirmed cases of TB (177).

In addition to the factors known to cause false-positive TST results, various other factors have been associated with TST+/IGRA- discordance. Increased age, healthcare position (such as nursing, laboratory staff, or attending physician) and years in the healthcare profession have been found associated with TST+/IGRA- (166, 178). A study of IGRA use among prisoners, a population at

increased risk of MTBI due, in part, to the congregate setting, found significant discordance. African American race, foreign birth and history of incarceration were significant to TST+/QFT-G- discordance. Here TST positivity may point to remote infection or sensitization due to BCG vaccinaton. Among the US born population, only African Amerian race was significantly associated to discordance (179). Anergy, advanced age, severe disease, HIV/AIDs profile, and immunosuppressive therapy have been shown to cause false-negative IGRA results, and influence TST+/IGRA- discordance (174, 180).

The accuracy and ability of IGRAs to detect a different spectrum of the immune response to MTBI may also affect discordance. One hypothesis for this discordance is that TST is positive for old or resolved TB infections but QFT-GIT is negative (164). IGRAs may be more sensitive at detecting rececent infection, with a reduced sensitivity for detecting remote LTBI . IGRAs may become negative during and after effective MTBI treatment, while TST results remain positive. With treatment, Mtb sensitized CD4+ T-lymphocytes may disappear from circulating blood due to the reduction in Mtb (181, 182). TST reactivity may persist after treatment causing discordance (156, 174). A CXR indicative of prior TB infection has been associated with TST+/QFT-GIT- in household contact investigations in a high-TB incidence country (183).

Discordance where TST induration is $\geq$ 15 mm but IGRA results are negative, may suggest lower sensitivity of the IGRA (144, 163, 184-186). Remote, resolved or treated MTBI may produce large indurations that are not detected by IGRAs (165, 183, 187, 188). Age and foreign birth can be indicators of remote infection and have been associated with this TST+/IGRA- discordance (79). Induration sizes of $\geq$ 15 mm are more likely to be the result of MTBI than prior BCG vaccination, especially if the subject was immunized in infancy and the vaccination occurred more than 10 prior to IGRA testing (86, 189). Large induration from NTM sensitization can occur (170, 190, 191). However, most large TST reactions are assumed to be due to MTBI (172).

Theres is less supportive evidence to explain negative TST but positive IGRA discordance (TST-/IGRA+), possibly due to its less frequent occurrence (100). This discordance may be attributed to a higher sensitivity of IGRAs over TST, depending on the population (145, 174, 174, 185). Using T-Spot and risk-based TST in a prison population, age and IDU was associated with TST-/T-Spot+ discordance (192). MTBI tests on elderly populations also yield discordant results. Response to tuberculin PPD can wane with age (39, 96). In another IGRA study, the size of the TST reaction decreased with advanced age and TST-/QFT-GIT+ discordance was more frequent (164). Discordant participants were older and more similar in age to concordant positive participants. In similar

situations, MTBI prevalence may be highest among the oldest age groups due to the cohort effect seen in countries with decreasing TB prevalence (193).

Factors that inhibit the immune response, such as older age, viral coinfection, alcoholism, liver and renal failure, steroid therapy, or cancer, may cause false-negative TST results. These factors may also impact TST-/IGRA+ rates (194). Immunosuppression and the use of tumor necrosis factor-alpha inhibitors have been associated with this form of discordance (167, 174). Severe cases of TB have also been associated with TST+/GIT- results (195). HIV/AIDS is established as a serious limitation to TST use in the immunocompromised (196). Circulating CD4+ T cell counts are decreased to a varrying degree in patients with HIV/AIDS. The [IFN-γ] of QFT-GIT has been shown to be negatively correlated to the number of cicrulating CD4+ T cells in HIV infected patients (197). TST-/T-Spot+ may occur among high HIV prevalent populations due to T-Spot ennumerating lymphocytes prior to performing the ELISpot, ensuring enough cells are present to allow IFN-γ release (197).

Additional evidence suggesting that TST-/IGRA+ discordance is due to false-negative TSTs comes from studies among contacts of people with TB. Exposure to TB was more closely correlated with IGRA results than TST results (145, 166, 183, 187, 198, 199, 199). Intesity of TB exposure (e.g., household contacts, prolonged contact, and close contact) is associated with higher rates of

TST-/IGRA+ discordance (52, 166, 183, 187, 200, 201). However, any increase in risk factors for MTBI has been shown to uniformly increase the odds ratio for either TST or QFT-GIT positivity (167).

Treatment of TB or LTBI may lead to reversion of either TST or IGRA results (202). Since TST has may be more associated to remote infection than IGRAs and IGRAs more sensitive to recent infection, TST+/IGRA- discordance may be more likely (203). Recent or repeated TST testing may contribute to increases in T-cell circulation, IFN-γ production, and the rate of TST and IGRA positivity. Some authors suggest that this effect may be greater for IGRA's and cause TST-/IGRA+ discordance (204, 205). Tests conducted during the "window period" may have greater discordance due to the kinetics of developing an immune response to MTBI and tests detecting different a different immune response (51).

Weakly positive IGRA results, where the [IFN-γ] is just above the 0.35 IU/ml cutoff may contribute to discordant results. Reversions, conversions and non-specific subject variabiltiy (seen in serial testing) may occur more frequently about a range of uncertanty surrounding the positive threshold (206-209). Variation in the cutoffs used for either TST or IGRA can also result in increases or decreases in discordance by altering test sensitivity and specificity (152, 165, 187). Additional untested factors may contribute to false-positive IGRA results,

as head to head comparisons with IGRAs in low-risk populations identify different individuals as positive (156, 190, 210).

Few studies directly address discordance between IGRAs. Some studies find QFT-GIT to have higher sensitivity (93%) compared to QFT-G (81%), which accounts for some positve QFT-GIT but negative QFT-G discordance (152). However, the opposite finding is also seen; two reviews found a pooled QFT-G sensitivity of 80%, greater than that for QFT-GIT (pooled 67% and 74%) (142, 156). This difference could explain some negative QFT-GIT but positive QFT-G discordance (156). QFT-G agreement with QFT-GIT was also low (0.5), which may imply poor reproducability of these tests in field conditions, higher sensitivity in QFT-GIT due to the addition of TB7.7, or QFT-GIT's technical simplicitiy over QFT-G (156). The proportion of discordant results from QFT-G or T-Spot to TST results (23% versus 26%) are similar (156). When tested together both IGRAs have moderate agreement (kappa = 0.57 – 0.70). Other studies find higher agreement with QFT-GIT and T-Spot (71%, 90%) with no significant discordance between the tests (178, 211). Variation between the second and third generation of whole-blood IGRAs and T-Spot may be due to higher T-Spot sensitivity or lower T-Spot specificity (157, 187, 212).

Interpreting discordance which includes unequivocal or indeterminate results between IGRAs is also a challenge. QFT-GIT has been shown to have a

pooled indeterminate rate of 2.1% (95% CI: 2.0 – 2.3%), which is significantly less than T-Spot (3.8%), and both have improved indeterminate rates compared to the QFT-G (11%) (157, 212) Young age, immunosuppression and variation in testing procedures may be associated with indeterminate results (145, 153, 157, 213).

Diagnosing MTBI when test results disagree requires an assessment of additional individal clinical factors. The CDC's 2010 guidelines for using IGRAs to detect MTBI, suggest considerations when interpreting discordant results. Healthy persons at low-risk of MTBI or progression to active disease may more likely be negative, regardless of the type of discordance found. TST induration size, MTBI treatment history, and BCG vaccination history can be a guide. Individuals at increased risk of MTBI, progression to active disease, or severe outcome should be considered positive from either type of discordance, especially in the presence of other clinical signs of infection or compromized immune function (15).

Limitations to Interferon-gamma Release Assays

Logistical issues exist for the use of IGRAs in the place of TST. A major limitation of IGRAs in low-resource settings, is the need for laboratory

infrastructure (148). Rapid delivery of collected blood requires safe handeling and transportation from remote locations. The acquisition and processing of blood samples increases HCW's risks of occupational blood-borne pathogen exposure. In some patients, especially children or the very ill, collection of sufficeint quantities of blood for IGRA may be difficult, or blood testing may be refused (188, 214). However, IGRAs do not induce adverse reactions, as can occur in instances of allergic or extreme reactions to the TST (215).

IGRAs require a shifting of costs from the clinic to the laboratory. The cost of establishing a laboratory is high, and the individual cost of each IGRA is more than a TST (214). Although expensive as a single test, comparative cost-benefit analyses seem to indicate overall cost-savings through IGRA use, where laboratory infrastructure already exists (216). QFT-G screening prior to CXR was more cost effective and identified more MTBI than TST prior to CXR among immigrants to the United Kingdom from high-TB prevalence countries (217). Dual screening with IGRAs and TST may result in lower costs, when using TST first and confirming with IGRAs, but may vary in the number of MTBI cases detected (104, 218). Screening for LTBI among low-risk HCW found QFT-G and QFT-GIT to be more effective at identifying MTBI and less expensive than TST screening, regardless of the HCW's BCG status (219).

Unfortunately, IGRAs can not differentiate between active and latent infection (44, 220, 221). IFN-γ may be present at all stages of the immune response. This may be what impedes the ability of IFN-γ based testing to consistantly differentiate different forms of MTBI or severity of TB (220). There are uncertainties with the predictive ability of IGRAs to determine who may be at the highest risk to progress to active TB from LTBI. Increasing evidence shows that individuals who are IGRA positive, with a large TB Response, may be at increased risk of progressing to culture-confirmed TB, as indicated in various contact investigation and follow-up studies (162, 221). There is further evidence to suggest that discordant TST-/IGRA+ subjects are at lower risk to develop TB (162, 222). However, there is contention on the issue of the efficacy of IGRAs in various low- and middle-income settings with high-TB or HIV burden (223, 224). The appropriate TB Response thresholds for seperating positive and negative IGRA results for various settings need additional prospective, longitudinal studies.

Despite the drawbacks to IGRA use in some settings, their differences from TST regarding logistics, accuracy and option for controls, represents welcome progress for MTBI diagnostics in many populations (6, 124, 221, 222). Healthcare workers are at increased risk of MTBI through exposure to TB patients, TB suspects or Mtb samples and cultures. Serial screening among HCWs in low-TB incidence countries has shown associations between positivie

IGRAs and MTBI risk (223). However, high rates of reversion and conversion with serial testing may limit the utility of these assays (225).

Similar to HCW's, military personnel are at increased risk for MTBI transmission. Military personnel are at increase risk due to congregate settings, travel to TB endemic regions and activities with heterogeneous populations of variable MTBI prevalence (31) Many militarys are comprised of a high proportion of BCG vaccinated members. TST's ability to accurately reflect risk of LTBI may be reduced in those who receive BCG vaccination after 2 years of age and receive multiple vaccinations (226). This presents an opportunity for tests with increased specificity to prevent unneccessary INH prophylaxis (226, 227). Military personnel are frequently screened for MTBI as part of routine health evaluations. Positive TST results are frequently encountered among low-risk military recruits, resulting in considerable costs for treatment INH treatment (228). Katsenos et al. conducted a comparison between QFT-GIT and TST among BCG vaccinated recruits into the Greek army. They found significant TST+/QFT-GIT- results, likely attributable to the high proportion of post-infancy BCG vaccination. The use of dual screening with this population decreased potentially unnecessary treatment with INH (227). IGRA's targeted use in the US military, with improved specificity over TST, may decrease unnecessay and potentially dangerous chemoprophylaxis, while improving logistical health efforts (33).

Tuberculosis and the United States Navy

Control and prevention of TB has long been a chief health concern for the US military, especially for the US Navy (229, 230). The incidence of hospitalization among Navy personnel for pulmonary TB at the turn of the 20th century exceeded 600 cases per 100,000 persons (230). This burden of disease prompted the Navy's development of tuberculosis control measures, including exclusionary recruitment screening of tuberculous individuals and those with chest-cavity abnormalities (230). Continuing improvements to Navy TB control programs included the extensive use of CXR beginning in the 1940's; universal and routine Mantoux tuberculin PPD testing initiated in 1948; and TB contact investigations with LTBI INH chemoprophylaxis adopted in 1960's (230). Since the late 1900's, the Navy and US military's incidence of TB has been steadily declining, as has the rate of TB in the general US population. Irregularities in the decline of TB incidence occurred surrounding World Wars I and II, the Korean and Vietnam Conflicts and the combined effects of the HIV epidemic and decreased programmatic TB-control in the late 1980's through early 90's (33, 229-233). From January 2005 to October 2010, 13 cases of active TB were reported among US Navy personnel in the Defense Medical Surveillance System by the Armed Forces Health Surveillance Center (MAJ Cecili K. Sessions, US Air Force, personal communication, 2011).

Navy service can expose individuals to elements associated with risk of MTBI transmission. Prolonged exposure to the shipboard environment of close working conditions, confined berthing quarters, poor air volume circulation and closed system ventilation have all been associated with past MTBI outbreaks (234-236). Congregate occupational risk exists throughout military life, such as in recruit barracks, mess-halls and marching formations (33). Exposure to populations with a higher prevalence of TB than the US average is also a concern for increased transmission, for example interactions with foreign nationals at stations abroad or humanitarian aid and peacekeeping work with high risk groups (31, 237-239). However, recent studies on foreign deployment alone have not shown a significant association to increased risk of MTBI, among military populations (32, 240). A US Army study has found that risk of developing TB while on active military duty was most related to factors existent prior to accession into the military and unrelated to military service, specifically foreign birth and Asian ethnicity (32).

Beginning in the late 1980's, the rate of identified TB among the Navy and US Marine Corps (USMC) stabilized at below 2 cases per 100,000 population, while TB-related hospitalization steadily declined (231, 241, 242). The improvement in detection of TB prior to requiring hospitalization has limited the usefulness of military TB-hospitalization data to reflect TB incidence. Despite this decrease in TB cases, transmission remains a concern, as a single TB

event can have severe effects on military health. A 1998 outbreak investigation aboard the USS Wasp documented 712 new TST reactors (LTBI cases), 21 new TB cases, and 4 subsequent progressions to TB from treated LTBI cases, stemming from a single source patient with TB (241). 1998 was the only year the Navy-USMC TB incidence rate surpassed that of the general US population, between 1988 and 2001 (241). In addition to the closed shipboard environment, this outbreak was attributed to incomplete adherence to PPD screening, initial misdiagnosis the index case's respiratory illness, delays in the contact investigation, and poor compliance to INH LTBI treatment (237, 241). Future recommendations to limit shipboard transmission include engineering control measures of UV irradiation or HEPA filtration within ship ventilation systems, and the construction of less crowded berthing areas (237).

Owing to its rigorous TB control program, recent Navy shipboard outbreaks have been few (242). A 2003 outbreak of new TST conversions aboard the USS Iwo Jima found new LTBIs among 48 troops (1.8%), without identifying an active case of TB (237). A thorough investigation for the index case included further testing of TB suspects with respiratory symptoms, those with prior TB treatment, prior TST conversion, those infrequently exposed to local populations en route, PPD lot inspections and training adequacy of TST issuing personnel. Despite these standard surveillance measures, the index case remained undetected.

Identification of the index case may be difficult. A false-negative result by TST may avert suspicion of TB, as seen in the 2006 MTBI outbreak aboard the USS Ronald Regan (242). When the patient's symptoms first presented, a false-negative TST result was obtained, and over 4-months lapsed before the patient was diagnosed and treated for TB. During this deployment, nearly 5,000 sailors and over 1,000 civilians had been aboard the USS Reagan. The contact investigation used risk-based screening of nearly 5,000 individuals to identify limited Mtb transmission to 13% of the patients close contacts who became TST positive.

TB control measures currently employed by US military services include multiple screenings throughout the duration of service, using either the TST or FDA approved IGRAs (243). In 2009 the Navy began to change its approach to MTBI screening. Annual universal screening for all active personnel was replaced with a testing approach based on risk of infection and disease, similar to the approach recommended by CDC guidelines (230, 244, 245). Routine testing occurs: at accession into Navy service for a baseline screening for MTBI of new recruits; within 6 months of receipt of orders to serve on a commissioned vessel; prior to separation from Navy service; and as determined appropriate by TB exposure risk questionnaires given at regular health assessments. For each TST induration $\geq$ 5 mm, Navy risk criteria are applied to determine test interpretations. High risk criteria considered positive at reactions $\geq$ 5 mm are close contact to a

known active TB case, fibrotic or other changes to CXR consistent with prior TB, or patients suspected of active TB. Medium risk criteria considered positive at reactions ≥ 10 mm include immigration from a high TB prevalence country in the past 5 years, mycobacteriology laboratory personnel and those with clinical conditions that increase the risk of developing MTBI. Other individuals with no risk factors for TB are considered positive at reactions ≥ 15 mm. Increase in induration of ≥ 10 mm, over 2 years indicates a TST conversion (243). Navy protocol prior to 2008 considered all TST reactions ≥ 10 mm as positive, regardless of risk (245). Those with TST reactions ≥ 5 mm, a positive IGRA, or prior TB history have CXR performed. Those with symptoms of TB or CXR's suggesting TB have additional examinations that typically include evaluation of sputum for presence of Mtb (33). Those with positive TST or IGRA results and no other evidence of active TB are typically diagnosed with LTBI. Troops diagnosed with LTBI are recommended to receive chemoprophylaxis of 9 months of INH 5mg/kg daily or 15 mg/kg twice weekly. Active TB cases are treated based on the individual susceptibility and severity of the infection (243).

Studies of TST screening among operational Navy personnel show that conversion rates have increased in recent years, with statistical significance. New TST positive result rates have risen from 1.35% in 1999 to 1.61% in 2003, with smaller amphibious ship's crews having a 1.76% increased risk of conversion over those aboard aircraft carriers (246). Despite these shipboard rates being

below the conversion rate for other Navy and Marine Corp personnel, any increase in TST conversion is a concern, considering the risk of Mtb transmission by a single TB case in congregate settings (235, 241, 246).

Latent Tuberculosis Infection in Military Recruits

Identifying LTBI for treatment and establishing baseline MTBI status among recruits is a key component to military TB-control programs. One factor that impacts the overall TST conversion rate for the military is the amount of positive TSTs identified upon recruit screening during accession into the armed services. The US Navy has seen recent increases in the proportion of recruits with LTBI. The average rate of recruit LTBI determined by TST between 1980 and 1986 was 1.6% (33, 232). Estimates of LTBI prevalence increased to 2.5% in 1990, 3.5% in 1998, 6.4% in 2003 and 5.1% in 2004 (21, 33, 247, 248). Navy recruits are screened prior to enlistment for chronic health conditions, HIV infection, illicit drug use and criminal records (249). These exclusionary conditions reduce the proportion of Navy personnel who are at risk of LTBI and TB (247, 249, 250). Military recruits also tend to be healthier than the general population, which is referred to as the "healthy-soldier" and "healthy-warrior" effects. Military enlistment selects for healthy individuals, and active duty status with frequent deployment mandates that troops maintain a strong physical

condition with frequent medical examinations, providing health benefits to the overall military population (32, 251).

The increasing rate of Navy recruits with positive TST results may be attributed to an increasing proportion of recruits born outside the US. The proportion of foreign-born recruits has increased from 5.0% in 1990 to 9.4% in 1998 (247, 248). In 1998, foreign-born recruits had an 8 fold increase in the odds of having MTBI, over US-born recruits (248). Between 1990 and 1998, the prevalence of infection within either group did not change significantly; indicating that frequency of foreign-born recruits was influencing the odds of MTBI. Of the foreign-born with positive TST results, 70% had indurations $\geq$ 15 mm, implying that many may have MTBI, rather than cross-reaction to BCG vaccination. Asian or Pacific Island ethnicity and birthplace in regions of high-TB prevalence, such as Africa or the Philippines, were also highly associated with positive TST results $\geq$ 15 mm. Similarly, studies in other military settings find that TST positivity among recruits and other personnel are highly associated to foreign birth and emigration from endemic regions (21, 105, 252, 253).

Increasing LTBI among recruits is likely to continue, as national and military demographic trends indicate rises in high risk foreign-born populations. The US has experienced increased immigration over the last four decades, with current estimates of the foreign-born population accounting for 12.5% (38.5

million) of the 2009 population (254). The most common country of origin among foreign-born immigrants into the US is Mexico, accounting for 30%, while China, India, and the Philippines, are the next most frequent, each accounting for 5%. WHO estimates of TB prevalence rates in 2009 for China, India the Philippines, and Mexico are 138, 249, 520, 19 cases per 100,000 population, respectively, compared to the US TB prevalence of 4.5 cases per 100,000 population (1). The 2009 US rate of TB cases among foreign-born individuals remains well above that of US-born persons (18.6 versus 1.7 cases per 100,000 persons) (2). Of the 6,806 reported cases of TB among the US foreign-born in 2009, Mexico, India, the Philippines and Vietnam accounted for over 50% of the patient origins, with 1,574, 523, 799 and 514 cases, respectively (2). Emigration within two years from a foreign country is also associated with an increased risk for developing TB, especially from sub-Saharan African and Southeast Asian countries (88, 255). Paralleling the rise in immigration in the general population, recent years have seen steady increases of the enlisting of noncitizens into the US military (256). Mexico is the birthplace of the most non-citizens joining the US military, with the Philippines being the second most common source, and highest among Navy enlisted non-citizens (257). These demographic changes within the military are likely to continue to increase estimates of LTBI among recruits and may lead to increased Mtb transmission among the enlisted.

The rise in TST positive results among Navy recruits, may not be entirely indicative of rising LTBI levels, but also may reflect an increase in NTM sensitization and false-positive results. Recent US Army studies have seen that universal testing of low-risk personnel result in false positive conversions and resulting pseudoepidemics due to TST product-related quality, variability and errors in test administration, host variability (such as boosting) and cross-reactivity due to NTM and BCG vaccination history (105). Studies among BCG-naïve, Dutch Armed Forces personnel found increased exposure to NTM during deployments to TB endemic regions, which considerably increased post-mission TST conversions, but was not associated to QFT-GIT positivity (240, 258). An increase of *M. scrofulaceum* sensitization in among Royal Netherlands Army recruits was used to suggest withholding INH treatment for LTBI until multiple positive TST reactions are demonstrated (259). A recent study of MTBI screening among military recruits found dominant skin test induration to Battey PPD was associated to TST indurations $\geq$ 10 mm, but not to QFT-GIT or T-Spot positivity (210). In a post-deployment study of previously TST negative military personnel, the authors proposed that TST conversion and TST+/IGRA- discordance at $\geq$ 15 mm were likely due to NTM sensitization during deployment and not recent Mtb transmission (258).

However, there is still debate as to the effects of NTM sensitization upon tests for MTBI. A review by Farhat et al. of studies where simultaneous testing

with NTM and tuberculin PPD antigens were conducted, concluded that false-positive TST results from NTM cross-reaction are infrequent (86). The range of positive TST indurations, between 10 to 14 mm, due to NTM cross-reaction is 0.1 to 2.3%, seen in highly sensitized NTM populations. IGRAs are expected to respond differentially to NTM sensitization, depending on the species (15, 173). Both the prevalence of MTBI and NTM sensitization affects the rate of false-positive results encountered (250). Selection of a higher TST cutoff (e.g. $\geq$ 15 mm) decreases the effect of NTM sensitization on discordance (75). Where the expected prevalence of true MTBI is below 1%, (e.g. among US-born military recruits) and TST specificity is < 99% (e.g. when TST induration is 10 to 14 mm), over 50% of positive results will be false-positive (86).

TST screening among the military recruits will continue to be impacted by NTM epidemiology. NTM exposure and sensitization is not rare and is anticipated to rise in the US (260). Sensitization to *M. intracellulare* has increased, both among US-born and foreign-born individuals, from an estimated 10.9% and 11.7% in 1971-2, to 16.2% and 26.7% in 1999-2000, respectively (89). The southern and southeastern regions of the US have permissive environments for NTM, with higher rates of infection and sensitization than northern states (261, 262). US-born Naval recruits from southern, central and eastern seaboard states have historically had higher rates of tuberculin reactions and NTM sensitization (172). Current estimates of the odds of TST positive results among

Navy recruits are significantly increased among those born in the southwest compared to the northwest (248). Florida has had rising levels of non-AIDS, NTM associated hospitalizations, with a prevalence much higher than compared to New York or California (263). Florida has been one of the states with the highest Navy recruitment (33).

The rate of presumed false-positive TST results among US Navy recruits overall, highlights the need for a specific diagnostic test to detect MTBI. IGRAs offer the possibility of reducing the number of false-positive results and limiting the number of people needing unnecessary INH preventive therapy. However, increasing numbers of foreign-born recruits at high-risk of MTBI are enlisting in the military, highlighting the need for accurate testing. With sensitivity similar to the TST, IGRAs are a realistic alternative to TST for detecting MTBI among Navy recruits. Screening, followed by targeted IGRA testing of those at increased risk of MTBI also is expected to improve test accuracy. IGRAs have been found to be costly and effective when targeted to increased risk populations.

Despite an increasing body of literature regarding the use of IGRAs, QFT-GIT assessments of specificity and discordance are still few. Results of QFT-GIT require assessment from various populations to address the complexities inherent in testing for MTBI. Discordant results occur often and may lead to different interpretations of results, depending on the population. This need for further

research is evident when considering the application of QFT-GIT in special populations. Military testing for MTIB has been demonstrated to require certain considerations regarding the likelihood of infection among recruits and the consequences of a misdiagnosis.

The objective of this study is to assess the use of QFT-GIT in a military recruit population to profile the test's accuracy. The prevalence of LTBI will be estimated based on QFT-GIT, TST and previously assessed IGRAs. QFT-GIT specificity will be determined among low-risk recruits. Discordance between TST and QFT-GIT results will be assessed to identify factors associated with discordance and improve the future diagnostic utility of QFT-GIT among military recruits.

Chapter II

Manuscript

Title Page

Specificity and Discordance between the Tuberculin Skin Test and an Interferon-γ Release Assay for the Detection of M*ycobacterium tuberculosis* Infection in US Navy Recruits

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Introduction

Tuberculosis (TB) is a leading cause of death worldwide, and is transmitted by the spread of *Mycobacterium tuberculosis* (Mtb) bacilli. The number of new TB cases has increased to 9.4 million in 2009, according to World Health Organization (WHO) estimates [1]. Approximately 2 billion people worldwide have latent *M. tuberculosis* infection (LTBI) and are at risk of progressing to active TB and spreading further infection [1].

M. tuberculosis infection (MTBI), both LTBI and TB, are threats to the health of military personnel. Mtb transmission may increase during periods of conflict or crisis due to: reactivation of LTBI from stress, malnutrition and other co-morbidities; disruption of TB treatment and prevention efforts; migration of infected individuals; and over-crowding [2–6]. Military personnel in settings of conflict and indigent support are at increased risk of MTBI from exposure to groups with a high prevalence of infection [7]. For militaries in TB-endemic regions, vigilant control programs to identify TB can limit disruption from illness and contact investigations [8–11]. Military personnel in countries with a low prevalence of TB may still be at risk Mtb transmission, and close screening is required to detect MTBI [12,13].

The United States (US) military, particularly the US Navy, has historically had high levels of MTBI. Hospitalization of Navy personnel for pulmonary TB at

the turn of the 20th century exceeded 600 cases per 100,000 persons [14]. Navy estimates of LTBI were over 400 infected per 100,000 persons into the 1960's [15]. However, the prevalence of MTBI in the military has been dropping, and the rate of decline has been faster than the decline seen in the general US population [16–19]. The rate of pulmonary TB in the US military from 1998 to 2007 was 0.65 cases per 100,000 person-years, well below the age adjusted US rate of 4.3 cases per 100,000 person-years [20,21]. Estimates of the rate of LTBI in US military personnel are also low, where 2 to 5% of members test positive [22]. However, since the 1980's, the rate of LTBI appears to be increasing among US Navy recruits [17,23–26].

The US military's low incidence of TB may be due to a lower baseline risk of MTBI among recruits. This reduced risk may be linked to the military's bias of selecting young, athletic recruits without chronic-illness, and emphasizing good health during military service [20,27,28]. Additionally, the US military may be comprised of less foreign-born individuals, than the general US population [20,24,29]. Both of these elements select against groups that are at increased risk of having MTBI or developing TB [30,31]. However, as Mtb transmission may still occur due to risk from congregate settings, such as aboard Navy ships, travel to TB-endemic regions, and activities with people with high TB prevalence [7,32–36].

Another practice which may account for the low prevalence of MTBI among US military personnel is their ongoing TB control programs. These programs actively identify individuals with LTBI for the initiation of treatment to prevent progression to TB [37]. Updates to the US Navy practices for MTBI screening are working to incorporate Centers for Disease Control and Prevention (CDC) guidelines for LTBI detection and control [31,38,39]. As the prevalence of TB in the US has decreased, these revisions encourage targeted testing in place of universal serial testing, such as annual screening for most Navy personnel [40]. Current Navy TB protocols require MTBI screening upon entry into service, within 6 months prior to serving aboard a commissioned vessel, upon exiting Navy service and as warranted by interim risk assessments [41]. Despite the use of targeted testing, complete and accurate detection of MTBI remains an elusive goal.

The presence of LTBI cannot be confirmed by any diagnostic tool. Therefore, tests for MTBI do not have well-defined accuracies, as test sensitivity and specificity can only be estimated in the absence of a "gold standard" to confirm MTBI. For example, approximations of specificity have been achieved by assessing test results among persons at low-risk of MTBI and assumed to free of infection [23,42,43]. Historically, the tuberculin skin test (TST) has been the preferred test for detecting MTBI. TST requires an intradermal injection of purified protein derivative (PPD) produced from *M. tuberculosis* (tuberculin PPD)

and subsequent measurement of induration [31]. TST specificity is reduced by false-positive results due to cross-reactions with the tuberculin PPD following sensitization to environmental nontuberculous mycobacteria (NTM), and with bacille Calmette-Guérin (BCG) vaccination, both of which can contain the same antigens present in tuberculin PPD [31,44,45]. Tests measuring the release of interferon-gamma (IFN-γ), a cytokine critical for the control of MTBI, can be used to quantify cell mediated immune responsiveness [46–48]. IFN-γ release assays (IGRA) offer the potential to detect MBTI with greater specificity than the TST by assessing sensitization to specific Mtb antigens as compared to the assessing of sensitization to the complex mixture of proteins included in tuberculin PPD [49,50].

In 2001, the QuantiFERON®-TB test (QFT) (Cellestis Limited, Carnegie, Victoria, Australia) became the first IGRA approved by the Food and Drug Administration (FDA) for the detection of MTBI [51]. QFT uses an enzyme-linked immunosorbent assays (ELISA) to measure the amount of IFN-γ released in response to tuberculin PPD, compared to controls [52]. One QFT control includes PPD produced by *M. avium* (avian PPD) to aid in discriminating MTBI from NTM sensitization [53]. QFT specificity was found to be less than TST [49,50,52].

In an attempt to improve specificity, subsequent generations of IGRAs use manufactured peptides that represent specific *M. tuberculosis* antigens such as early secreted antigenic target–6 (ESAT-6) and culture filtrate protein–10 (CFP-10) [50]. ESAT-6 and CFP-10 are released by pathogenic Mtb and are highly antigenic [54,55]. These antigens are present in Mtb, deleted from BCG, and absent from most NTM [56–58]. Such antigens allow the specific detection of MTBI, without being affected by BCG vaccination [54,59–61].

The QuantiFERON®-TB Gold test (QFT-G) (Cellestis Limited, Carnegie, Victoria, Australia), was FDA approved in 2004 and contains peptide cocktails simulating ESAT-6 and CFP-10 [62,63]. Several studies demonstrated that the specificity of this commercial assay was higher than the specificity of QFT and at least as specific as TST using tuberculin PPD antigen [23,64–67].

The QuantiFERON®-TB Gold In-Tube test (QFT-GIT) (Cellestis Limited, Carnegie, Victoria, Australia) was FDA approved in 2007 and is performed using a method different from the previous IGRAs [68]. Blood collected for other IGRAs require transportation to a laboratory for stimulation with antigens within hours of collection, while blood cells are viable [50,63,69–71]. QFT-GIT addresses this preanalytic limitation by including blood collection tubes internally coated with peptides representing ESAT-6, CFP-10 and a third Mtb protein, TB7.7, or control reagents [72–75]. QFT-GIT has been used to detect MTBI in

various populations, including those at high- and low-risk of MTBI, those who are immunocompromised, and children [42,50,76,77]. Reviews of QFT-GIT have found the assay to be highly specific with comparable sensitivity to TST [50,67,78–81]. Comparisons between QFT-GIT to TST and other IGRAs have found variable degrees of discordance [79,82–85]. Understanding disagreement between QFT-GIT and TST is clinically important to prevent misdiagnosis based on erroneous test results.

This study assesses the prevalence of MTBI in US Navy recruits based on TST and QFT-GIT results and compares these estimates to a previous assessment based on QFT-G. Recruits categorized as having low-risk of *M. tuberculosis* infection are used to estimate test specificity. Historical factors related to *M. tuberculosis* exposure and positive TST results are assessed for their association to test positivity and discordance between QFT-GIT and TST.

Materials and Methods

This study is a continuation of a larger study of IGRAs [23,86–88]. The study was conducted at the Recruit Training Command, Great Lakes, Illinois (RTC) after Institutional Review Board approval. All US Navy recruits enter boot camp at RTC and have a comprehensive medical assessment. All recruits

receive a baseline TST, excluding those with documented prior positive TST result or a history of MTBI treatment [41]. Recruits with TST indurations $\geq$ 5 mm and those excluded from TST testing received further evaluation and a chest radiograph. At the time of data collection, Navy recruits with TST induration $\geq$ 10 mm were considered to have MTBI regardless of risk [40,89]. Current Navy Tuberculosis Control Program practices use risk-based criteria for interpreting TST reactions [39,41].

Recruits scheduled for TST between January 31 and February 12, 2004, were asked to participate in the parent study and when possible provide additional blood for QFT-GIT. Written informed consent was obtained and subjects completed a questionnaire about risk of MTBI. Information was collected on prior TST, BCG vaccination, and symptoms compatible with tuberculosis. Information about chest radiograph, mycobacterial cultures, and tuberculosis related treatment were abstracted from medical records. Recruits were categorized as: 1) "tuberculosis suspects" if they reported a cough, fever, or unintentional weight loss of more than 2 weeks duration, and had a chest radiograph suggestive of TB; 2) "increased risk for *M. tuberculosis* infection" if they did not meet the "tuberculosis-suspect" criteria, but reported contact with someone with tuberculosis, birth (or residence > 1 month) in a country where estimated tuberculosis prevalence exceeded 20 cases per 100,000 population [90], or having resided, worked, or volunteered > 1 month in a homeless shelter, prison,

drug rehabilitation unit, hospital, or nursing home; or 3) "low-risk for *M. tuberculosis* infection" if they were neither suspects nor at increased risk of MTBI.

Blood for QFT-GIT was collected when possible for this study, after blood was collected for other routine and investigational tests, including QFT and QFT-G [87]. Blood was collected prior to applying TST. The QFT and QFT-G were performed and interpreted as described previously [87]. For QFT-GIT, approximately 1 ml of blood was collected into tubes containing heparin alone (Nil control tube); heparin, dextrose, and phytohaemagglutinin (PHA) (positive mitogen control tube); and heparin, dextrose, and a single mixture of peptides representing ESAT-6, CFP-10, and part of TB7.7 (TB antigen tube) [72]. Blood was mixed with other tube contents within 12 hours of collection and incubated for 16 to 24 hours at 37˚C prior to centrifuging and harvesting plasma. The concentration of IFN-γ in 50 µl of each plasma sample was determined by ELISA as previously described for the QuantiFERON®-TB Gold test [87]. The Mitogen Response was calculated by subtracting the IFN-γ concentration in plasma from unstimulated blood ([Nil]) from the IFN-γ concentration in plasma from mitogen stimulated blood. The TB Response was calculated by subtracting [Nil] from the IFN-γ concentration in plasma from blood stimulated with the cocktail of peptides

representing ESAT-6, CFP10, and TB7.7. QFT-GIT was interpreted as described in published guidelines [50].

TST was administered by the Mantoux method using 0.1 ml (5 TU) of Tubersol tuberculin PPD (Connaught Laboratories, Inc., Toronto, Canada) according to published guidelines [31]. TST was interpreted stratified by risk where indurations $\geq$ 5 mm among "tuberculosis-suspects", $\geq$ 10 mm among those at increased risk for *M. tuberculosis* infection, and $\geq$ 15 among those at low risk for *M. tuberculosis* infection were considered positive [31]. TST induration was interpreted by risk stratification unless otherwise stated that the cutoff for a positive reaction was 15 mm or 10 mm.

Statistical analyses were conducted using SAS (Ver. 9.2, SAS Institute, Cary, NC, USA). Differences between groups of recruits were compared by the Wilcoxon Rank Sums Exact Test, for continuous variables and Fisher's Exact Chi-square test for categorical variables. P-values $\leq$ 0.05 were considered significant. Subjects categorized as "low-risk for *M. tuberculosis* infection" were assumed to be uninfected, and specificity was estimated among low-risk recruits with determinate test results by dividing the number with negative results by the number tested. Prevalence estimates were based on the number of positive results out of all tested recruits. Estimates of specificity and prevalence were compared using McNemar's Exact Test for correlated proportions [91,92]. Excluding

samples with incomplete or indeterminate results, overall test agreement, was calculated as the number of samples with concordant results divided by the number of samples tested. Positive agreement was calculated as the number of samples with concordant positive results divided by the number of samples with concordant positive or discordant results. Negative agreement was calculated as the number of samples with concordant negative results divided by the number of samples with concordant negative or discordant results [93,94]. Kappa statistics (*k*) were also calculated [95].

Discordance was categorized as "TST positive but QFT-GIT negative" or "TST negative but QFT-GIT positive" using a 10 or 15 mm cutoff. Subjects in each category of discordance were compared to the remaining subjects, consisting of those with concordant results and those with the opposing category of discordance. Univariate analyses were used to identify factors associated with TST results, QFT-GIT results, and with each type of discordance. Risk factors for test positivity and discordance were evaluated by exact maximum likelihood estimation (MLE) using stratified logistic regression. Odds ratios with confidence intervals not containing 1.0 and p values < 0.05 were considered significant. Factors evaluated included the covariates age, sex, and race/ethnicity, with variables for the estimated tuberculosis prevalence in country of birth, greatest tuberculosis prevalence in countries of residence exceeding 1 month other than place of birth, history of exposure to someone with tuberculosis, BCG status, TST

in the prior year, and reactivity to *M. avium* PPD by QFT. US-born recruits without foreign residence were classified as "not having received BCG." Estimated tuberculosis prevalence for countries of birth and residence were based on World Health Organization estimates for 1990 [90]. Prevalence by country of birth and residence were categorized as low "< 20 cases per 100,000 population", medium "20 through 100 cases per 100,000 population", or high "> 100 cases per 100,000 population" levels of prevalence [23].

Multivariate models used MLE logistic regression analysis to identify factors associated with test discordance. Model refinement included assessments for dependent variable collinearity, interaction by likelihood ratio chi squared tests, and comparative tests for confounding.[96,97] Dependent variable collinearity was assessed using regression diagnostic variance inflation factors, condition indices and variance decomposition proportions (VDP) [96]. Variables and interactions expressing collinearity were removed hierarchically when condition indices and VDPs were high (> 20 and > 0.5, respectively). Covariate-exposure and select exposure-exposure interactions were considered in model specification, when appropriate. Variable interactions were assessed through manual backwards elimination using the likelihood ratio chi squared test [97]. Confounding was assessed through comparisons of regression coefficients and effect measures.

Results

Of 866 recruits who consented, 10 were excluded from all analysis (Figure 1). Of the 856 who had TST placed and blood collected, 838 (97.9%) had TST completed; 805 (805/856%) had QFT-GIT completed with determinate results; 787 (91.9%) had TST completed and determinate QFT-GIT results. Of these 787 recruits, 510 (64.8%) were categorized as being at low-risk for MTBI and 277 (35.2%) were categorized at increased risk for MTBI. Subject characteristics are presented in Table 1. Collectively, the 69 recruits with incomplete or indeterminate QFT-GIT or TST results were younger than those with determinate results (mean age 19.8 versus 20.5 years, $p = 0.027$) and were less likely to have risk for tuberculosis infection (23.2% versus 35.2%, $p = 0.047$), but otherwise did not differ.

The outcome of TST and QFT-GIT are presented in Table 2. Among the 787 with TST and determinate QFT-GIT results, detectable TST induration was observed (i.e. > 0 mm) in 52 recruits and ranged from 6 to 50 mm. Detectable induration was observed more frequently in recruits at increased risk for *M. tuberculosis* infection than in recruits at low risk for infection (13.0% versus 3.1%; $p < 0.001$), and was larger (median = 14.5 mm versus 10.0 mm, $p = 0.005$). While 42 (5.3%) of the 787 recruits had TST induration $\geq$ 10 mm, 38 (4.8%) were interpreted as positive based on risk and TST size (the method recommended by

CDC for the general US population). TST induration was ≥ 15 mm for 23 (2.9%) of these recruits and QFT-GIT results were positive for 14 (1.8%). TB Response ranged from -1.51 to 12.29 IU/ml. Positive QFT-GIT results were no more frequent among recruits at increased risk of prior *M. tuberculosis* infection than among recruits at low risk for infection (2.9% versus 1.2%; p = 0.095) and the median TB Response by QFT-GIT was not significantly larger (1.68 IU versus 0.68 IU; p = 0.491). QFT demonstrated *M. avium* reactivity for 62 (9.5%) of the 654 recruits with negative QFT results for MTBI. Of the recruits who reacted to avian PPD, 10 had a TST induration > 10 mm, 4 of which had TST ≥ 15, and one was positive by QFT-GIT. Among all recruits tested with TST, 53 had induration ≥ 5 mm, all received a chest radiograph, and 1 was interpreted as "abnormal but not typical of tuberculosis". TST induration for this recruit was ≥ 15 mm and positive for QFT-GIT.

Agreement between TST based on risk-stratified interpretation and QFT-GIT was 94.7% (k = 0.17), positive agreement was 19.2%, and negative agreement was 97.2%. Based on a 10 mm TST cutoff, agreement was 94.2% (k = 0.16), positive agreement was 17.9%, and negative agreement was 97.0%. Based on a 15 mm cutoff, agreement was 96.6% (k = 0.25), positive agreement was 27.0%, and negative agreement was 98.2%.

TST specificity was estimated among low risk recruits to be 98.2% (95% CI = 97.1 – 99.4%) using a 10 mm cutoff, and 99.0% (95% CI = 98.2 – 99.9%) using a 15 mm cutoff. QFT-GIT specificity was estimated to be 98.8% (95% CI = 97.9 – 99.8%). The differences between QFT-GIT specificity and TST specificity (using either a 10 or 15 mm cutoff) were not significant ($p = 0.581$ and > 0.999, respectively). If limited to the 500 low-risk recruits with determinate QFT-G, QFT-GIT, and TST results, estimated QFT-G specificity was 99.8% (95% CI = 99.4 – 99.9%) and QFT-GIT specificity was 99.4% (95% CI = 98.7 – 99.9%). The difference between the QFT-G and QFT-GIT specificity was not significant ($p = 0.500$). TST specificity at either a 10 or 15 mm cutoff was unchanged by the exclusion of the 10 indeterminate or incomplete QFT-G results within the low risk population. Prevalence estimates by QFT-GIT were lower than TST using a risk-based criteria and at 10 mm cutoff ($p < 0.001$) but no different than TST at 15 mm ($p = 0.087$).

The outcomes of QFT-GIT were compared to QFT-G (Table 3). Among those who had QFT-GIT and QFT-G tests completed (807), QFT-GIT gave less frequent indeterminate results (0.6% versus 2.0%; $p = 0.019$). Agreement between determinate QFT-GIT and QFT-G was 99.2% ($k = 0.62$), positive agreement was 62.5%, and negative agreement was 99.6%. Among the 769 recruits with TST results and determinate QFT-GIT and QFT-G results, 5 were positive by all three tests, with TST indurations ranging from 15 to 27 mm

(Figure 2). Of those 5 recruits, 4 were at increased risk of MTBI. Of the 42 recruits with a TST induration $\geq$ 10 mm, 37 (88.1%) were negative by both IGRAs, and 29 (69.0%) of these were at increased risk. If limited to the 500 low-risk recruits, 1 was positive by all three tests. Of the 9 low-risk recruits, with a TST induration $\geq$ 10 mm, 8 were negative by both IGRAs. Of the 489 low-risk recruits with no detectible TST induration, 2 were positive by QFT-GIT and 0 were positive by QFT-G. The prevalence estimate by QFT-G was lower than that by QFT-GIT ($p = 0.004$).

Characteristics associated in univariate analysis with TST induration $\geq$ 15 mm, $\geq$ 10 mm, and positive QFT-GIT results, are shown in Table 4. For example, TB prevalence in the country of birth was associated with TST positivity using either a 15 mm or 10 mm cutoff, and QFT-GIT positivity. The odds of having TST induration $\geq$ 10 mm were 30.1 times greater for recruits born in high-TB prevalence countries and 12.0 times greater for recruits born in medium-TB prevalence countries, than for those born in low-TB prevalence countries. The odds of QFT-GIT positivity was 6.6 times greater for recruits born in high-TB prevalence countries compared to those born in low-TB prevalence countries. TST induration $\geq$ 15 mm and $\geq$ 10 mm were associated with BCG vaccination (both p values < 0.001), but QFT-GIT results were not ($p = 0.252$). Of the 18 recruits who reported being vaccinated with BCG, 7 (38.9%) had TST induration $>$ 0 mm; all were $\geq$ 10 mm, 5 of the 7 were $\geq$ 15 mm and 1 was positive by QFT-

GIT. Of the 61 recruits with unknown BCG status, 9 (14.8%) had detectable TST indurations; all were $\geq$ 10 mm, 7 of the 9 were $\geq$ 15 mm and 2 were positive by QFT-GIT. The proportion of recruits with detectable TST reactions was less among recruits with unknown BCG vaccination than among those known to be BCG vaccinated ($p = 0.0421$).

Characteristics associated in univariate analysis with discordance between TST interpretations using a 10 mm cutoff and QFT-GIT results are shown in Table 5. While 37 recruits had TST induration $\geq$ 10 mm but a negative QFT-GIT result, 9 recruits had TST induration < 10 but positive QFT-GIT results. TST $\geq$ 10 mm but QFT-GIT negative discordance was associated with age, race/ethnicity, TB prevalence in country of birth, BCG vaccination status, and *M. avium* reactivity by QFT. Characteristics associated in univariate analysis with discordance between TST interpretations using a 15 mm cutoff and QFT-GIT results are shown in Table 6. While 18 recruits had TST induration $\geq$ 15 mm but a negative QFT-GIT result, 9 recruits had TST induration < 15 but positive QFT-GIT results. *M. avium* reactivity was no longer associated with positive TST but negative QFT-GIT discordance using the 15 mm cutoff. Other characteristics associated with discordance using a 10 mm TST cutoff, remained significant using a 15 mm cutoff. No characteristics were significantly associated with negative TST but positive QFT-GIT discordance, regardless of TST cutoff used.

Characteristics associated in multivariate analysis with positive TST results, using either a 10 or 15 mm cutoff, but negative QFT-GIT results are shown in Table 7. For modeling involving the 10 mm cutoff, all considered interactions were removed through collinearity and significance testing. Hospital exposure is retained due to the log-likelihood significance ($p = 0.048$) during backwards model specification. The multivariate model examining TST $\geq$ 10 mm but negative QFT-GIT discordance retained TB prevalence in country of birth, *M. avium* reactivity by QFT, and hospital stay or employment exceeding 1 month. Recruits born in high- and medium-TB prevalence countries had adjusted odds of discordance 26.6 times and 14.6 times greater, than those from low-TB prevalence countries, respectively. The adjusted odds of discordant results for those with *M. avium* reactivity by QFT were 6.1 times greater than for those who were not reactive. For the multivariate model involving the 15 mm cutoff, all exposure variables and covariates were initially considered. Interaction terms could not be reliably assessed due to the low frequency of discordance using the 15 mm cutoff. Age was removed prior to testing for confounding and association due to collinearity to the intercept (condition index of 24.6; VDP = 0.5795). The multivariate model examining TST $\geq$ 15 mm but negative QFT-GIT discordance retained TB prevalence in country of birth and race/ethnicity. Recruits born in high- and medium-TB prevalence countries had adjusted odds of discordance 18.1 and 11.7 times greater than those from low-TB prevalence countries, respectively. Race and ethnicity categories

were not significantly associated with discordance in this model (all p values $\geq$ 0.2), but were retained due to confounding. Discordance with negative TST results, using either the 10 or 15 mm cutoff, but positive QFT-GIT results, was assessed and no associations were found to contribute significantly in these models.

Discussion

This study of US Navy recruits compares the outcome of QFT-GIT to other tests for MTBI. It supplements the comparison of QFT and QFT-G with TST in essentially the same population [23]. Absence of a "gold diagnostic standard" to confirm the most common forms of MTBI (LTBI and culture negative TB) limits assessments of accuracy of tests for MTBI. This limitation can be addressed by estimating specificity in persons presumed uninfected by Mtb [23,42]; by estimating sensitivity in persons with culture-confirmed TB [98–100]; by examining factors associated with discordant test results [23,101]; and through long term follow-up studies to determine the negative and positive predictive value of tests for MTBI [76]. This study estimated specificity among low-risk US Navy recruits. The specificity of QFT-GIT was high, approaching 99%, with no significant difference from TST specificity. Discordance between tests was also assessed. QFT-GIT and QFT-G results are compared to TST indurations, stratified by risk of MTBI. Factors historically associated with an increased risk of infection were found to be associated with positive TST results and, to a lesser

extent, positive QFT-GIT results. Discordant results were few but not uncommon. TST positive but QFT-GIT negative discordance was most highly associated with an increasing prevalence of TB in the country of birth, possibly indicating a lower sensitivity or higher specificity of the QFT-GIT, compared to TST, or both. Recruits at increased risk of MTBI with a TST > 15 mm but a negative QFT-GIT may be more likely to have remote MTBI, as QFT-GIT may be more sensitive to recent infection [101,102]. Results from both tests highlight the need for screening of US Navy recruits from areas where TB is highly prevalent.

This study demonstrates that the prevalence of MTBI among Navy recruits is low, regardless of the test used. However, significant differences were observed in the proportion of recruits positive by QFT-GIT compared to other tests. Results were positive for 1.6% of recruits by QFT-GIT, 0.6% by QFT-G, and 2.8% and 5.0% by TST at 15 mm and 10 mm cutoffs, respectively. The estimates of LTBI prevalence based on TST alone are comparable to the increase seen in LTBI among Navy recruits since the mid 1980's, from 1.2% in 1986 to as high as 6.5% by the mid 2000's, attributable by some to an increase in foreign-born persons [17,23–26]. However, estimates based on QFT-GIT indicate a significantly lower prevalence of LTBI than TST by using a risk based interpretation or 10 mm cutoff, (1,635 versus 4,439 and 5,023 per 100,000 persons, respectively). QFT-GIT estimates for the prevalence of LTBI among

Navy recruits is similar to the US national estimate of LTBI for 15 to 24 year olds (2,393.8 per 100,000) [103].

Agreement between QFT-GIT and TST was slight (*k* ranged from 0.16 to 0.25), and percent positive agreement was low (17.9% to 27%). QFT-GIT and QFT-G had higher agreement (*k* = 0.62) than either had compared to TST. However, of the 19 recruits positive by either IGRA, only 5 (26%) were positive by both tests. Of the 14 low-risk recruits positive by any test (QFT-GIT, QFT-G, or TST at either 15 or 10 mm induration), only 1 recruit was positive by 2 or more tests (IGRAs or TST). Comparable results among US Army recruits using head-to-head comparisons of IGRAs find similar proportions of positive results, and the majority of individuals with positive results were also different between tests [104]. These findings indicate that the majority of IGRA positive results among recruits at low- and increased risk of MTBI may be false-positive results.

QFT-GIT had less indeterminate results than QFT-G (0.6% versus 2.0%). The proportion of QFT-GIT indeterminate results was less than expected, based on the results of some studies, but similar to the manufacturer's findings [72,79]. The criteria for an indeterminate result are not the same for QFT-GIT as QFT-G. A QFT-GIT [Nil] > 0.7 IU/ml and $\leq$ 8.0 IU/ml can produce a negative result but the same value for QFT-G would be interpreted as indeterminate. In addition, people with a TB Response $\geq$ 0.35 IU/ml and $\geq$ 25% of the [Nil] but < 50% of the

[Nil], with a [Nil] of > 0.7 IU/ml are interpreted as positive by QFT-GIT but indeterminate by QFT-G [50]. Allowance of higher baseline levels of IFN-γ may have improved the ability of QFT-GIT to produce determinate results.

Indeterminate results may be helpful when considering clinical interpretations and IGRA efficacy. Studies have found indeterminate results for QFT-GIT and QFT-G associated with subject characteristics, such as advanced age, underlying disease or depressed immune status [42,77,105–107]. Such conditions are unlikely causes of indeterminate results in the present study because subjects were selected from among military recruits who are young and healthy.

Preanalytic factors, such as blood sample processing, may cause variation in indeterminate rates. Delaying blood incubation, even within the limits established by the manufacturer, may affect the number of indeterminate results obtained. Herrara et al. noted that immediate incubation of QFT-GIT blood tubes produces fewer indeterminate results than those incubated within 6 to 12 hours, for samples collected from the same subjects [71]. Interestingly, the manufacturer recommends incubating blood for QFT-G within 12 hours of collection, but allows up to 16 hours for QFT-GIT [63,72]. For this study, blood for QFT-GIT and QFT-G were collected at the same time, and plasma from the same person was analyzed on the same ELISA plate for both QFT-GIT and QFT-G.

Therefore, the only difference in sample handling was how the blood was stimulated for QFT-GIT and QFT-G.

Logistical features of QFT-GIT may reduce the likelihood of encountering indeterminate results, as compared to QFT-G. For example, QFT-GIT exposes blood with antigen "in-tube" immediately upon collection, as opposed to the delay in blood exposure to antigen by QFT-G. QFT-GIT includes a peptide representing the antigen TB7.7, not included in QFT-G. Although TB7.7 was included in an attempt to improve test sensitivity, no concrete evidence has been published to support this claim. However the inclusion of TB7.7 may induce greater release of IFN-γ and may account for samples which were indeterminate by QFT-G to be classified as positive by QFT-GIT. Additional field variations in sample processing methods for QFT-GIT, from the volume of blood collected to tube shaking, may further impact indeterminate rates [108,109].

Among low-risk recruits, there were no significant differences in estimates of specificity for QFT-GIT (98.8%) or TST (99.0%) using the 15 mm cutoff. Further limiting the assessment of specificity to low-risk recruits with determinate QFT-G results, 9 indeterminate results by QFT-G were excluded, 3 of which were positive by QFT-GIT. Specificity of QFT-GIT (99.4%) increased in this population, but was not significantly different from QFT-G (99.8%). QFT-GIT specificity is similar to that found by other studies conducted in low-risk

populations. One review of such studies found pooled QFT-GIT specificity (99.4%; 95% CI: 97.8 – 99.9%) to be higher than pooled TST specificity (88.7%; 95% CI: 84.6 – 92.0%) [80]. In the review, TST specificity was reduced by prior BCG vaccination or confirmed NTM infection. Lower specificity of IGRAs are found in studies involving low-risk subjects in countries with higher background prevalence of MTBI [100,110]. Studies assessing the specificity of tests for MTBI among controls other than those at low-risk of MTBI, for example patients who are TB negative by culture, are estimating the specificity to detect TB and may be estimating the background prevalence of LTBI in the population [78,81]. Such reported estimates of specificity are not comparable to low-risk specificity estimates for MTBI detection, as they are measuring different degrees of infection.

Recruits at increased risk of MTBI had positive TST results more frequently than recruits at low-risk, and the average widths of induration, when present, were larger. TST positivity was associated with the following subject characteristics: age, Asian or Pacific Island ethnicity, Hispanic ethnicity, high- and medium-TB prevalence in the recruits' country of birth, hospital stay or employment, BCG vaccination and reactivity to *M. avium* by QFT. Asian or Pacific Islander ethnicity and birth in a high-TB prevalence country were the variables most strongly associated to positive TST results. These factors have

been previously seen in military personnel to be associated with positive TST results [20,24,111–114].

While risk classification was associated with TST results, it was not associated with QFT-GIT results. Despite the lack of association between positive QFT-GIT results and an overall increased risk of MTBI, individual characteristics, i.e. birth in a high-TB prevalence country and Asian or Pacific Islander ethnicity, were associated with positive QFT-GIT results. While BCG and reactivity to avian PPD by QFT were associated with positive TST results, these factors were not associated with positive QFT-GIT results. Similar findings in other military populations have been reported [13,104,111].

TB prevalence in the country of birth was the strongest predictor of TST results, QFT-GIT results, and discordant TST positive but QFT-GIT negative results. Recruits born in high-TB prevalence countries were 7 times more likely to have a positive QFT-GIT result, 44 times more likely to have a TST induration $\geq$ 15 mm and at least 18 times more likely to have TST positive but QFT-GIT negative discordant results. Birth in a country with high-TB prevalence is most strongly associated with having LTBI and development of TB. Therefore, QFT-GIT may not be as sensitive for LTBI as TST.

As a representation of the Navy recruit population, 10.3% of study recruits were foreign-born, which represent a considerable population of at risk recruits. In

this population, recruits born in high-TB prevalence countries are at the greatest risk of having LTBI, and may be at increased risk of developing TB. The length of residence in the US for these recruits was not determined. Individuals arriving into the US within 2 years from a highly TB endemic region, such as sub-Saharan Africa and Southeast Asia, are at high risk of developing TB, with estimates over 250 cases per 100,000 population [29]. Cumulatively, 17.8% of recruits were either born in or resided in a country of medium- or high-TB prevalence, although living outside the US for more than 1 month was not associated with positive TST or QFT-GIT results. However, living with someone born outside the US has been associated with increased evidence of LTBI [21]. Determining an approximate date of entry into the US and asking about co-habitation with foreign-born individuals in future studies may further characterize the relationships between MTBI test positivity, discordance and risk of exposure to MTBI.

A substantial number of Asian recruits were foreign-born. Of these 35 recruits, 34 were born in high-TB prevalence countries and comprise at least 64% of that high risk category. Asian recruits in the US Navy have historically had high rates of MTBI and high rates of TB [16,24]. Among US military personnel, Asian ethnicity was among the strongest predictors for development of active TB between 1990 and 2006 [20]. Among the WHO's 22 high-burden countries of TB, accounting for 80% of global TB, half of the countries have populations that are predominantly Asian or Pacific Islander ethnicity [1]. Asia accounts for over

50% of the world's reported TB cases in 2009, where China and India account for 35%. The risk of MTBI and progression to TB for individuals originating from Asia may differ by country. For example, individuals from the Philippines who immigrate to the US are at increased risk of developing TB, as compared to immigrants from other Asian or Pacific Island countries [115]. This is despite higher estimated TB prevalence rates in other Asian countries compared to the Philippines. The prevalence of NTM sensitization in Asia may further confound the relationship between test positivity, discordance and risk of infection [116]. Identifying recruits by individual countries of origin may be necessary to help target LTBI screening.

Concordant negative results were the most common finding. Recruits with discordant results accounted for 46 (5.8%) of those obtained from TST and determinate QFT-GIT. TST positive but QFT-GIT negative discordance and TST negative but QFT-GIT positive discordance appears to occur by different mechanisms. TST positive but QFT-GIT negative discordant results occurred more frequently than TST negative but QFT-GIT positive discordance (37 versus 9). Multivariate analysis of discordant results where TST $\geq$ 15 mm but QFT-GIT was negative found increasing prevalence of TB in recruit country of birth as the only significant factor, when accounting for race and ethnicity. Race and ethnicity may confound the relationship between test discordance and TB prevalence in the country of birth as evidenced by its strong correlation between

foreign-birth and non-white ethnicities. Discordance where TST $\geq$ 10 but QFT-GIT was negative in multivariate analysis found associations with TB prevalence in the country of birth, avian PPD sensitization and residing or working in a hospital. Discordant results may be due to false-positive results, false-negative results, or differences in sensitivity or specificity in either test [50,113].

Sensitization to avian PPD was associated with TST positive but QFT-GIT negative discordance in both univariate and multivariate analyses using a 10 mm cutoff, but not using a 15 mm cutoff. Reactivity by avian PPD as measured by QFT has been used to demonstrate sensitization to NTM [23,117]. NTM sensitization has been shown to cause false-positive TST results, especially at indurations < 15 mm [14,118]. The prevalence of NTM sensitization is difficult to establish, but within the US it is not rare and may be increasing [21,119,120]. Studies with Battey PPD (PPD made from *M. intracellulare*) suggest that NTM sensitization is common among US-born Navy recruits, especially those from the southern and central US [121]. The antigens ESAT-6 and CFP-10 are not present in *M. avium*, *M. intracellulare*, and most other NTM, with the exceptions of *M. kansasii*, *M. marinum*, and *M. szulgai* [57,58]. IGRAs using ESAT-6 and CFP-10 have remained negative despite infections with NTM [122,123]. Thus, this study's evidence of NTM sensitization with discordant results between QFT-GIT and TST at 10 mm but not 15 mm is not surprising. High rates of discordant TST positive but QFT-GIT negative results, using a 10 mm cutoff, among US Army recruits was

associated with Battey PPD reactivity by skin test [104]. Additional military studies suggest that some TST reactions ≥ 15 mm may occur due to NTM exposure [111,112]. However, it is likely that most recruits with TST induration ≥ 15 mm are due to MTBI and not NTM sensitization [124]. In this cohort of recruits, most TST reactions ≥ 15 mm were among recruits at increased risk of MTBI infection. However, most had negative QFT-GIT results.

In univariate analyses, BCG vaccination was not associated with positive QFT-GIT results. It was associated with positive TST results and TST positive but QFT-GIT negative discordance using either 10 or 15 mms cutoffs. BCG vaccination is known to cause false-positive TST results, especially < 15 mm [45]. Some studies have found that BCG vaccination after infancy or within 10 years of MTBI testing may cause TST indurations ≥ 15, normally indicative of MTBI [110,125–127]. However, use of antigens ESAT-6 and CFP-10, not present in any BCG vaccination strain, eliminate BCG as a cause of false-positive results for QFT-GIT [57,67,74]. BCG vaccination was not present in the multivariate models of discordant TST positive but QFT-GIT negative results. BCG vaccination may have been overshadowed by the TB prevalence in the country of birth due to the high coverage of BCG vaccination in countries with high TB endemnicity. BCG status did not have a considerable confounding effect on the association between discordance and other exposures; however a low number of recruits reported having been BCG vaccinated. We were unable to accurately assess BCG vaccination, due

to recall bias by the recruits, lack of vaccination documentation and variability in scarring. Additional information regarding age at BCG vaccination could be helpful in examining discordant test results.

No factors were found associated to TST negative but QFT-GIT positive discordance in univariate or multivariate analyses. Studies in similar populations also did not identify any factors associated with negative TST but positive IGRA results [23,104,112]. Other studies including subjects with immunosuppression have encountered false-negative TST results and discordant TST negative but IGRA positive results [50,77,128–130]. In these studies, conditions such as HIV/AIDS, advanced age, severe or chronic illness, and certain medications can be associated with this type of discordance. However, the military's exclusionary criteria reduce the prevalence of such immunosuppressive factors among recruits to limit conditions which may be infectious or prove as limitation to military assignments [28].

Using risk-based interpretation of TST, 38 recruits would have been diagnosed with LTBI and likely be prescribed treatment with isoniazid (INH). Using a 10 mm induration cutoff, 43 recruits would been treated with INH. QFT-GIT identified 14 recruits for INH treatment, a reduction of 63% and 67% from TST interpreations by risk or 10 mm cutoff, respectively. Use of QFT-GIT alone would have identified 9 recruits for INH treatment who had TST induration of 0

mm, 5 of whom were at low risk for prior MTBI. If QFT-GIT results are falsely positive, INH prophylaxis would be unneccessary. QFT-GIT would not have detected 10 of 15 recruits at greatest risk of MTBI, those who had a TST induration $\geq$ 15 mm and were born in high-TB prevalence countries. Of those recruits, 4 denied BCG vaccination and were nonreactive to avian PPD negative or QFT was not performed. Their lack of detection by QFT-GIT may indicate a lower sensitivity than TST.

As long as TB remains a problem globally, military personnel will continue to be at risk of Mtb transmission due to activities in congregate settings and interactions with people at increased risk of TB. QFT-GIT remains a viable alternative to TST for military screening and targeted testing for MTBI and offers potential logistical advantages. Cost effectiveness studies suggest that although IGRAs are more expensive than TST, cost saving may occur where laboratory infrastructure exists and unneccessary INH preventative treatment is reduced [77,131,132]. Without additional studies on the positive predictive value of QFT-GIT using various military screening and testing algorithms and tracking INH adherence, accurate cost effectiveness of QFT-GIT would be difficult to establish [20,133].

Limitations and Strengths

This study was limited due to the inability to confirm MTBI and by factors related to the study design. The lack of a confirmatory test for MTBI presents a challenge when comparing test results because there is no standard to verify accuracy. However, young, US-born military recruits reporting no history of increased risk for MTBI, are among those with the lowest prevalence of MTBI in the US, and may be presumed almost entirely free of infection [103]. The analysis was limited in power due to the small number of positive test results and discordant results. Larger studies may be able to detect smaller associations and additional interactions to TST and QFT-GIT discordant results. Differences in interpretation criteria between QFT-G and QFT-GIT may allow the same blood sample to be interpreted differently. Similarly, the single cut-point used by QFT-GIT to interpret positive results has been described with some uncertainty, especially regarding subjects with IFN-γ concentrations near the 0.35 IU/ml threshold [134,135]. Adjusting the positivity threshold for QFT-GIT, i.e. using a TB Response of less than 0.35 IU/ml to be interpreted as positive may be more appropriate to identify MTBI in low-TB prevalence populations [136]. However in this study, among recruits with a TB Responses < 0.35 IU, the 99th percentile for TB Response was 0.18 IU/ml, signifying most recruits had a TB Response far from the threshold of positivity.

This study used cross-sectional data on subjects to investigate tests for LTBI. Without performing a longitudinal study or reanalysis of positive results, the cross-sectional design may underestimate the agreement between TST and QFT-GIT due to false-positive results or reversions in QFT-GIT [42,111,137]. Variability within subjects may change result interpretations by subsequent tests, and is unrelated to infection. Similarly, false-positive TST results due to BCG vaccination and NTM sensitization cannot be corrected for in cross-sectional analysis. However, the increased adjusted odds of discordant TST positive but QFT-GIT negative, at a 10 mm cutoff, among recruits reactive to avian PPD by QFT compared to those who are not, supports that cross-reaction of tuberculin PPD by NTM sensitization reduces agreement between QFT-GIT and TST. Additional follow-up after 1 year to assess the development of TB may have been helpful, but such results may be biased due to recent transmission and progression to disease occurring after participation in the study. Furthermore, the small number of recruits identified as positive by any test, the low prevalence of TB in the military, and the reduced likelihood of progression to TB after INH treatment, makes it unlikely that any cases of TB would be identified among this study sample, during a reasonable follow-up.

In making head-to-head comparisons between TST, QFT-GIT and QFT-G, we excluded up to 10% of recruits, depending on the comparison. Subjects excluded from the primary analysis between TST and QFT-GIT were slightly

younger and at lower risk to have MTBI. It is not likely that their exclusion would have made significant changes in the study findings. Such subjects account for the vast majority of the study population, and being at lower risk and younger implies these recruits would more have been negative by QFT-GIT and TST.

Age was associated with TST positive results and TST positive but QFT-GIT negative discordance. Despite this finding, age was removed from multivariate analyses due to collinearity association to a constant which introduced instability into logistic significance testing. This complication is likely due to the narrow age range of the subjects (75% were > 17 and ≤ 21 years old). This limited range notwithstanding, age has been significantly associated with TST positivity in previous Navy recruit studies [23–25]. Ignoring the effects of collinearity, and proceeding with the same aforementioned multivariate modeling techniques, age is retained in both models, but does not considerably alter the findings. The logistic model for discordance at TST $\geq$ 10 mm retained age as a confounder (OR: 1.1; p = 0.069) and allowed the removal of hospital exposure (p = 0.076). Countries of birth at medium- (OR: 12.6; 95% CI = 3.1 – 44.6) and high-TB prevalence (OR: 24.5; 95% CI = 9.9 – 62.3) and *M. avium* response by QFT (OR: 5.9; 95% CI = 2.0 – 16.9) remained significant. The logistic model for discordance at TST $\geq$ 15 mm finds a significant odds of discordance with increasing age (OR: 1.2; 95% CI = 1.0 – 1.3) in birth countries at medium- (OR:

9.0; 95% CI = 1.1 – 61.9) and high-TB prevalence (OR: 14.9; 95% CI = 2.7 – 89.0), when controlling for the non-significant confounding effects of race and ethnicity. Although the age range of recruits in this population is narrow, increasing age may still indicate an increased risk of MTBI or sensitization to NTM, by having cumulatively more time to accrue exposures to the bacilli.

Recruits involved in this study originated from across the US and some from other countries. The findings based on these recruits may be generalizable to other US populations of young adults at low-risk of MTBI. This study had a large number of participants with most having multiple IGRAs performed on blood and completed TST. This allows QFT-GIT results to be compared to results for the same subject by TST, QFT-G and avian QFT, which is no longer commercially available. By assessing US-born military recruits, this study includes a substantial number subjects at very low-risk of MTBI, compared to other specificity studies [80]. While other studies estimating the specificity of tests for MTBI may limit their recruiting to only low-risk subjects, this study included recruits at increased risk of MTBI [138]. This allowed for estimations of MTBI prevalence and to identify factors associated with test positivity and discordance.

Conclusion

Overall, US Navy recruits have a low prevalence of LTBI regardless of the assay used to detect infection. Specificity for QFT-GIT was high and no different than TST specificity using a 15 or 10 mm cutoff. The factors most strongly associated with infection, detected by either QFT-GIT or TST, was prevalence of TB in the country of birth. This also was associated with TST positive but QFT-GIT negative result discordance. Test discordance observed in recruits at increased risk of MTBI may be due to lower TST specificity, lower QFT-GIT sensitivity, or both. Recruits born in high TB-prevalence countries with TST indurations $\geq$ 15 mm but negative QFT-GIT results suggest that QFT-GIT may be less sensitive than TST. Additional studies on tests for MTBI are needed to determine the risk of developing active TB when results are discordant.

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Tables

Table 1. Characteristics of US Navy recruits eligible to be tested with the tuberculin skin test (TST) and the QuantiFERON®-TB Gold In-Tube assay (QFT-GIT).

Characteristic	Recruits with determinate QFT-GIT and TST results (n = 787)	All tested recruits (n= 856)
Age, years		
Mean	20.5	20.4
Median (range)	20.0 (17-38)	19.5 (17-38)
Male Sex	94.5	94.3
Race / Ethnicity		
White (non-Hispanic)	59.7	59.7
Black (non-Hispanic)	16.3	16.5
Asian or Pacific Islander	6.6	6.2
Hispanic	14.5	14.8
American Indian / Alaskan Native, or other	2.9	2.8
TB prevalence in country of birth		
< 20 cases per 100,000 population	90.6	91.0
20 - 100 cases per 100,000 population	2.9	2.8
> 100 cases per 100,000 population	6.5	6.2
TB prevalence in country of residence[a]		
Not out of US or < 20 cases per 100,000 population	91.0	91.1
20 - 100 cases per 100,000 population	6.1	6.0
> 100 cases per 100,000 population	2.9	2.9
Foreign-Birth	10.3	9.9
Hospital stay or employment for > 1 month[a]	10.5	10.8
Correctional facility stay or employment > 1 month[b]	10.7	10.1
Reported TB exposure	2.9	2.8
Any symptom suggestive of TB	4.3	4.1
History of BCG vaccination		
No	90.0	90.3
Unknown	2.3	2.2
Yes	7.7	7.5

Note. Data are percentage of recruits, unless otherwise indicated. TB, tuberculosis; BCG, bacille Calmette-Guérin; [a] Unknown for 2 recruits; [b] Unknown for 1 recruit

Table 2. Outcomes of the tuberculin skin test (TST) versus the QuantiFERON®-TB Gold In-Tube assay (QFT-GIT) for all recruits and recruits stratified by risk for *Mycobacterium tuberculosis* infection.

TST induration,	QFT-GIT results				
recruit group	Negative	Positive	Indeterminate	Incomplete	All
< 5 mm					
All	726	9	5	45	785
Low-Risk	489	5	3	38	535
Increased Risk	237	4	2	7	250
5 - 9 mm					
All	10	0	0	0	10
Low-Risk	7	0	0	0	7
Increased Risk	3	0	0	0	3
10 - 14 mm					
All	19	0	0	0	19
Low-Risk	4	0	0	0	4
Increased Risk	15	0	0	0	15
> 15 mm					
All	18	5	0	1	24
Low-Risk	4	1	0	0	5
Increased Risk	14	4	0	1	19
Not Completed					
All	18	0	0	0	18
Low-Risk	12	0	0	0	12
Increased Risk	6	0	0	0	6
All					
All	791	14	5	46	856
Low-Risk	516	6	3	38	563
Increased Risk	275	8	2	8	293

Note. Data are number of recruits.

Cells represent the set of recruits with positive and negative, determinate QFT-GIT results and valid TST responses (n = 787).

Table 3. Outcomes of the QuantiFERON®-TB Gold assay (QFT-G) versus the QuantiFERON®-TB Gold In-Tube assay (QFT-GIT).

	QFT-GIT results				
QFT-G results	Negative	Positive	Indeterminate	Incomplete	Total
Negative	776	6	4	37	823
Positive	0	5	0	0	5
Indeterminate	12	3	1	1	17
Incomplete	3	0	0	8	11
Total	791	14	5	46	856

Note. Data are number of recruits.

Table 4. Univariate association between selected subject characteristics and tuberculin skin test (TST) results or valid QuantiFERON®-TB Gold In-Tube assay (QFT-GIT) results.

Characteristic	Total no. of recruits (n= 787)	TST Induration ≥ 15 mm		Positive QFT-GIT		TST Induration ≥ 10 mm[a]	
		No. of recruits (n = 23)	Odds Ratio (95% CI)	No. of recruits (n = 14)	Odds Ratio (95% CI)	No. of recruits (n = 42)	Odds Ratio (95% CI)
Age, years	…	…	**1.2 (1.1 - 1.3)**	…	1.1 (0.9 - 1.3)	…	**1.2 (1.1 - 1.3)**
Sex							
Male	744	22	1.0	13	1.0	38	1.0
Female	43	1	0.8 (0.0 - 5.1)	1	1.3 (0.0 - 9.3)	4	1.9 (0.5 - 5.7)
Race / Ethnicity							
White (non-Hispanic)	470	3	1.0	6	1.0	10	1.0
Black (non-Hispanic)	128	4	5.0 (0.8 - 34.6)	2	1.2 (0.1 - 7.0)	7	2.7 (0.8 - 7.9)
Asian or Pacific Islander	52	11	**41.0 (10.3 - 238.1)**	4	**6.4 (1.3 - 28.1)**	18	**24.0 (9.6 - 63.1)**
Hispanic	114	5	**7.1 (1.4 - 46.4)**	2	1.4 (0.1 - 7.8)	7	3.0 (0.9 - 9.0)
American Indian / Alaskan Native, or other	23	0	…	0	…	0	…
TB prevalence in country of birth							
< 20 cases per 100,000 population	713	6	1.0	9	1.0	16	1.0
20 - 100 cases per 100,000 population	23	3	**17.4 (2.6 - 88.9)**	1	3.5 (0.1 - 27.6)	5	**12.0 (3.1 - 39.5)**
> 100 cases per 100,000 population	51	14	**43.9 (14.8 - 147.6)**	4	**6.6 (1.4 - 24.8)**	21	**30.1 (13.5- 68.7)**
TB prevalence in country of residence[b]							
Not out of US or < 20 cases per 100,000 population	714	21	1.0	13	1.0	38	1.0
20 - 100 cases per 100,000 population	48	0	…	0	…	1	0.4 (0.0 - 2.4)
> 100 cases per 100,000 population	23	1	1.5 (0.0 - 10.2)	1	2.4 (0.1 - 17.7)	2	1.7 (0.2 - 7.4)
Hospital stay or employment for > 1 month[b]							
No	703	19	1.0	12	1.0	33	1.0
Yes	82	4	1.8 (0.4 - 5.7)	2	1.4 (0.2 - 6.6)	9	**2.5 (1.0 - 5.6)**
Correctional facility stay or employment > 1 month[c]							
No	702	23	1.0	13	1.0	40	1.0
Yes	84	0	…	1	0.6 (0.1 - 4.4)	2	0.4 (0.0 - 1.6)
Reported TB exposure							
No	764	22	1.0	13	1.0	40	1.0
Yes	23	1	1.5 (0.0 - 10.4)	1	2.6 (0.1 - 19.0)	2	1.7 (0.2 - 7.5)
History of BCG vaccination							
None	708	11	1.0	11	1.0	26	1.0
Unknown	61	7	**8.2 (2.6 - 24.2)**	2	2.1 (0.2 - 10.2)	9	**4.5 (1.8 - 10.6)**
Vaccinated	18	5	**23.9 (5.7 - 89.3)**	1	3.7 (0.1 - 28.4)	7	**16.5 (5.0 - 51.2)**
Mycobacterium avium PPD reactivity by QFT							
No	725	19	1.0	12	1.0	32	1.0
Yes	62	4	2.6 (0.6 - 8.1)	2	2.0 (0.2 - 9.2)	10	**4.2 (1.7 - 9.3)**

Note. Boldface font indicates statistically different odds ratios, in which the 95% CIs do not include 1.0. BCG, bacille Calmette-Guérin; TB, tuberculosis; PPD, purified protein derivative; QFT, QuantiFERON-TB.; [a] Induration ≥ 10 mm includes reactions ≥ 15 mm; [b] Unknown for 2 recruits; [c] Unknown for 1 recruit

Table 5. Univariate association between selected subject characteristics and discordance between QuantiFERON®-TB Gold In-Tube assay (QFT-GIT) results and tuberculin skin test (TST) results using a 10 mm cutoff.[a]

		TST ≥ 10mm but negative QFT-GIT		TST < 10mm but positive QFT-GIT		Total no. of recruits with discordant results (%) (n = 46)
Characteristic	Total no. of recruits (n = 787)	No. of recruits (n = 37)	OR (95% CI)	No. of recruits (n = 9)	OR (95% CI)	
Age, years	787	37	**1.1 (1.1 - 1.3)**	9	1.1 (0.8 - 1.3)	46 (5.8%)
Sex						
Male	744	33	1.0	8	1.0	41 (5.5%)
Female	43	4	2.2 (0.5 - 6.7)	1	2.2 (0.0 - 17.0)	5 (11.6%)
Race / Ethnicity						
White (non-Hispanic)	470	9	1.0	5	1.0	14 (3.0%)
Black (non-Hispanic)	128	7	3.0 (0.9 - 9.1)	2	1.5 (0.1 - 9.1)	9 (7.0%)
Asian or Pacific Islander	52	14	**18.6 (7.0 - 52.3)**	0	…	14 (26.9%)
Hispanic	114	7	**3.3 (1.0 - 10.3)**	2	1.7 (0.2 - 10.3)	9 (7.9%)
American Indian / Alaskan Native, or other	23	0	…	0	…	0
TB prevalence in country of birth						
< 20 cases per 100,000 population	713	15	1.0	8	1.0	23 (3.2%)
20 - 100 cases per 100,000 population	23	5	**12.8 (3.3 - 42.6)**	1	4.0 (0.1 - 32.1)	6 (26.1%)
> 100 cases per 100,000 population	51	17	**23.0 (9.9 - 54.2)**	0	…	17 (33.3%)
TB prevalence in country of residence[b]						
Not out of US or < 20 cases per 100,000 population	714	33	1.0	8	1.0	41 (5.7%)
20 - 100 cases per 100,000 population	48	1	0.4 (0.0 - 2.8)	0	…	1 (2.1%)
> 100 cases per 100,000 population	23	2	2.0 (0.2 - 8.6)	1	4.0 (0.1 - 32.1)	3 (13.0%)
Hospital stay or employment for > 1 month[b]						
No	703	29	1.0	8	1.0	37 (5.3%)
Yes	82	8	2.5 (1.0 - 5.9)	1	1.1 (0.0 - 8.2)	9 (11.0%)
Correctional facility stay or employment > 1 month[c]						
No	702	35	1.0	8	1.0	43 (6.1%)
Yes	84	2	0.5 (0.1 - 1.9)	1	1.0 (0.0 - 8.0)	3 (3.6%)
Reported TB exposure						
No	764	35	1.0	8	1.0	43 (5.6%)
Yes	23	2	2.0 (0.2 - 8.7)	1	4.3 (0.1 - 34.4)	3 (13.0%)
History of BCG vaccination						
None	708	24	1.0	9	1.0	33 (4.7%)
Unknown	61	7	**3.7 (1.3 - 9.3)**	0	…	7 (11.5%)
Vaccinated	18	6	**14.1 (4.0 - 45.0)**	0	…	6 (33.3%)
Mycobacterium avium PPD reactivity by QFT						
No	725	28	1.0	8	1.0	36 (5.0%)
Yes	62	9	**4.2 (1.7 - 9.8)**	1	1.5 (0.0 - 11.3)	10 (16.1%)

Note. Boldface font indicates statistically different odds ratios, in which the 95% CIs do not include 1.0. BCG, bacille Calmette-Guérin; TB, tuberculosis; PPD, purified protein derivative; QFT, QuantiFERON-TB.; [a] Induration ≥ 10 mm includes reactions ≥ 15 mm; [b] Unknown for 2 recruits; [c] Unknown for 1 recruit

Table 6. Univariate association between selected subject characteristics and discordance between QuantiFERON®-TB Gold In-Tube assay (QFT-GIT) results and tuberculin skin test (TST) results using a 15 mm cutoff.

		TST ≥ 15mm but negative QFT-GIT		TST < 15 but positive QFT-GIT		Total no. of recruits with discordant results (%) (n = 27)
Characteristic	Total no. of recruits (n = 787)	No. of recruits (n = 18)	OR (95% CI)	No. of recruits (n = 9)	OR (95% CI)	
Age, years	787	18	**1.2 (1.1 – 1.3)**	9	1.1 (0.8 – 1.3)	27 (3.4%)
Sex						
Male	744	17	1.0	8	1.0	25 (3.4%)
Female	43	1	1.0 (0.0 – 6.8)	1	2.2 (0.0 – 17.0)	2 (4.7%)
Race / Ethnicity						
White (non-Hispanic)	470	2	1.0	5	1.0	7 (1.5%)
Black (non-Hispanic)	128	4	**7.5 (1.1 – 84.0)**	2	1.5 (0.1 – 9.1)	6 (4.7%)
Asian or Pacific Islander	52	7	**35.8 (6.6 – 363.3)**	0	…	7 (13.5%)
Hispanic	114	5	**10.7 (1.7 – 113.5)**	2	1.7 (0.2 – 10.3)	7 (6.1%)
American Indian / Alaskan Native, or other	23	0	…	0	…	0
TB Prevalence in Country of Birth						
<20 cases per 100 k	713	5	1.0	8	1.0	13 (1.8%)
20-100 per 100 k	23	3	**20.9 (3.0 – 116.3)**	1	4.0 (0.1 – 32.1)	4 (17.4%)
>100 case per 100 k	51	10	**34.0 (10.1 – 133.0)**	0	…	10 (19.6%)
TB prevalence in country of residence[a]						
Not out of US or <20 cases per 100 k	714	16	1.0	8	1.0	24 (3.4%)
20-100 per 100 k	48	0	…	0	…	0
>100 case per 100 k	23	1	2.0 (0.0 – 14.0)	1	4.0 (0.1 – 32.1)	2 (8.7%)
Hospital stay or employment > 1 month[a]						
No	703	15	1.0	8	1.0	23 (3.3%)
Yes	82	3	1.7 (0.3 – 6.3)	1	1.1 (0.0 – 8.2)	4 (4.9%)
Correctional facility stay or employment > 1 month[b]						
No	702	18	1.0	8	1.0	26 (3.7%)
Yes	84	0	…	1	1.0 (0.0 – 8.0)	1 (1.2%)
Reported TB exposure						
No	764	17	1.0	8	1.0	25 (3.3%)
Yes	23	1	2.0 (0.0 – 13.9)	1	4.3 (0.1 – 34.4)	2 (8.7%)
History of BCG vaccination						
None	708	9	1.0	9	1.0	18 (2.5%)
Unknown	61	5	**6.9 (1.8 – 23.9)**	0	…	5 (8.2%)
Vaccinated	18	4	**21.8 (4.4 – 90.8)**	0	…	4 (22.2%)
Mycobacterium avium PPD reactivity by QFT						
No	725	15	1.0	8	1.0	23 (3.2%)
Yes	62	3	2.4 (0.4 – 8.8)	1	1.5 (0.0 – 11.3)	4 (6.5%)

Note. Boldface font indicates statistically different odds ratios, in which the 95% CIs do not include 1.0. BCG, bacille Calmette-Guérin; TB, tuberculosis
[a] Unknown for 2 recruits; [b] Unknown for 1 recruit

Table 7. Results of multivariate analysis examining discordance between negative QuantiFERON®-TB Gold in-Tube assay (QFT-GIT) results and tuberculin skin test (TST) results using a 15 mm or a 10 mm cutoff.[a]

		TST ≥ 15mm but negative QFT-GIT[b]			TST ≥ 10mm but negative QFT-GIT[c]		
Characteristic	Total no. of recruits (n = 785)[d]	No. of recruits (n = 18)	OR (95% CI)	p-value	No. of recruits (n = 37)	OR (95% CI)	p-value
TB Prevalence in Country of Birth							
<20 cases per 100 k	712	5	1.0		15	1.0	
20-100 per 100 k	23	3	**11.7 (1.5 - 74.9)**	0.018[e]	5	**14.6 (3.6 - 52.3)**	<0.001[e]
>100 case per 100 k	50	10	**18.1 (3.4 - 102.8)**	<0.001[e]	17	**26.6 (10.1 - 67.4)**	<0.001[e]
Mycobacterium avium PPD reactivity by QFT							
No	723	NR[f]	NR[f]	-	28	1.0	
Yes	62	NR[f]	NR[f]	-	9	**6.1 (2.1 - 17.1)**	<0.001[e]
Hospital stay or employment > 1 month							
No	703	NR[f]	NR[f]	-	29	1.0	
Yes	82	NR[f]	NR[f]	-	8	2.5 (0.8 - 6.6)	0.101

Note. Boldface font indicates statistically different odds ratios, in which the 95% CIs do not include 1.0. TB, tuberculosis; NR, not retained.

[a] No significant associations were found in multivariate analysis to predict the discordance between a positive QFT-GIT result and TST induration < 15 or < 10 mm.

[b] The discordance model at induration 15 mm includes the Race/Ethnicity variable as a confounder. All categories were non-significant ($p \geq 0.2$)

[c] Induration ≥ 10 mm includes reactions ≥ 15 mm

[d] Results for 2 recruits with unknown hospital exposure were excluded from both 15 mm and 10 mm multivariate analyses

[e] Data are statistically significant at a significance level of 0.05

[f] Data related to *M. avium* PPD reactivity by the QuantiFERON®-TB assay and hospital exposure > 1 month, were not retained in the final model examining discordance between QFT-GIT results and TST results using a 15 mm cutoff.

Figures

Figure 1. Diagram of study participants and testing for the detection of *Mycobacterium tuberculosis* infection. QFT, QuantiFERON®-TB assay; QFT-G, QuantiFERON®-TB Gold assay; QFT-GIT, QuantiFERON®-TB Gold In-Tube assay; TST, tuberculin skin test.

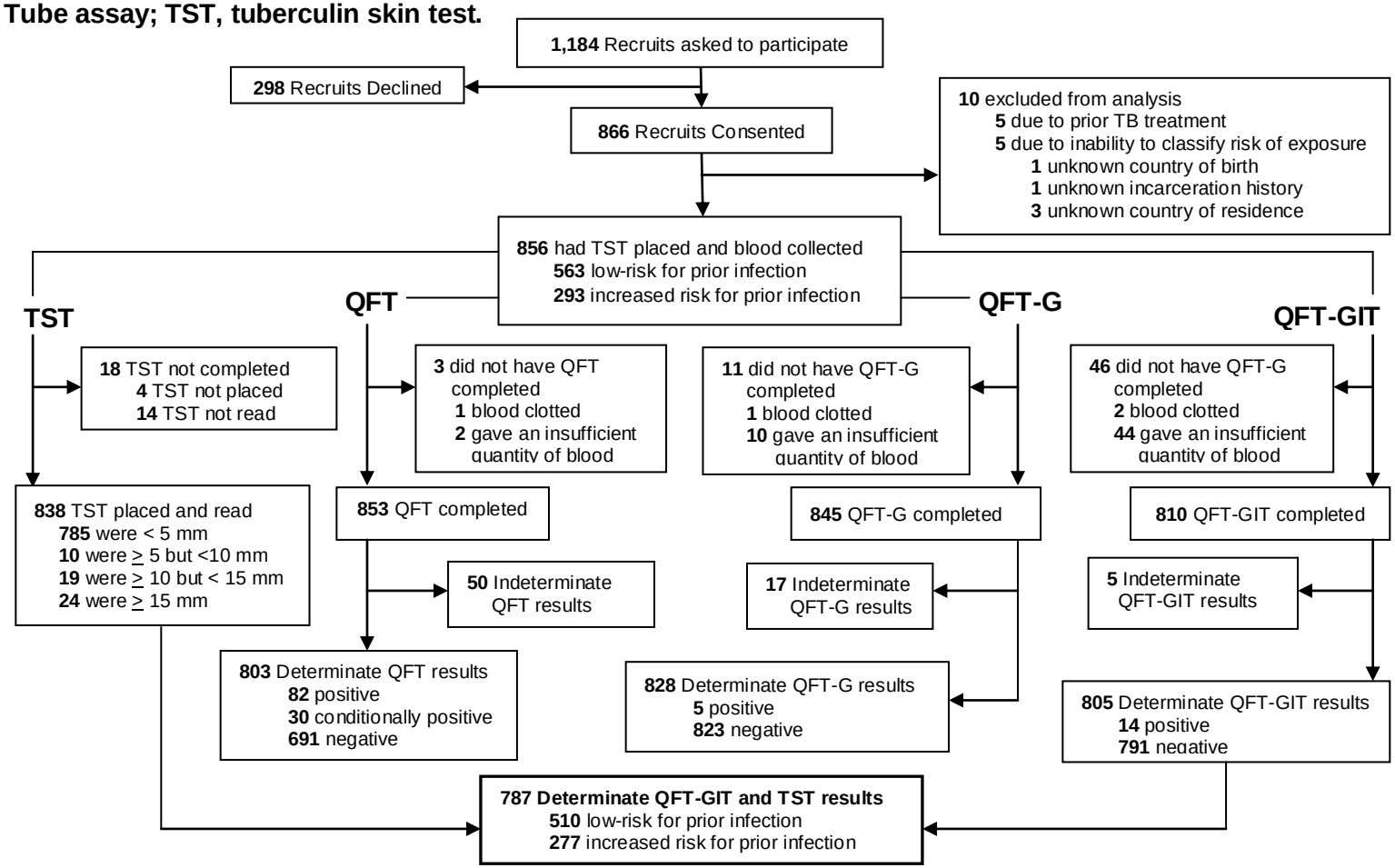
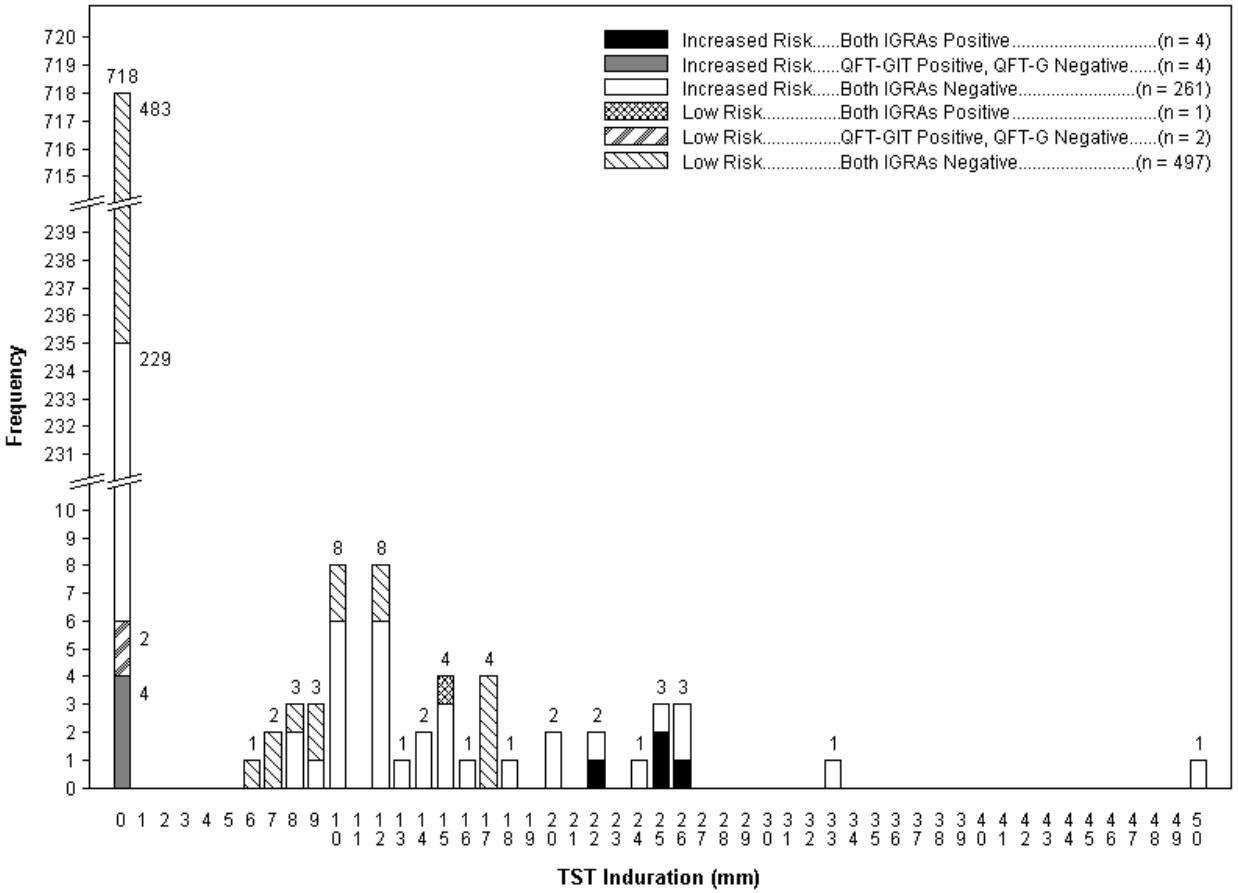


Figure 2. Comparison of the tuberculin skin test (TST) to Interferon-γ release assay (IGRA) interpretations[a] for QuantiFERON®-TB Gold (QFT-G) and QuantiFERON®-TB Gold In-Tube (QFT-GIT), by risk category[b] for infection by *Mycobacterium tuberculosis*.



a Total number of participants (N = 769) who had interpretable, positive or negative, results for both IGRAs and a valid TST result.

b *M. tuberculosis* risk of infection categories determined by exposure to someone diagnosed with tuberculosis (TB), having been born or lived for more than one month in a country with a TB prevalence of ≥ 20 cases per 100,000 population, or spending over 1 month in a homeless shelter, prison, jail, drug rehabilitation unit, hospital or nursing home.

Chapter III

Public Health Implications

Summary

This study assessed the use of tests for MTBI detection among US Navy recruits. This population is generally at low-risk of infection, but may undergo frequent MTBI testing in the future because of an increased risk due to military activities. The demographics of the participants in this study are similar to other Navy recruit studies and are likely representative of the general US Navy recruit population, enlisting around 2004. Recruit blood samples were tested by QFT-GIT and compared to QFT-G and TST, performed at the same time. The outcomes of QFT-GIT, QFT-G and TST were compared for test performance. TST results, determinate QFT-GIT results, and discordance between the two tests, were analyzed for associations with characteristics related to MTBI and test positivity. Risk factors historically associated with MTBI were used to approximate recruit likelihood of prior infection by Mtb. Results from recruits at low-risk of MTBI were assessed to estimate test specificity.

The estimated prevalence of MTBI in the study population was 5% or less and varied significantly depending on the test performed. TST results indicated the most cases of MTBI, followed by QFT-GIT then QFT-G. QFT-GIT had

fewer indeterminate results than QFT-G. The specificity of QFT-GIT was found to be high, approaching 99%, but no different than the specificity of TST or QFT-G. Factors known to cause false-positive TST results, such as BCG vaccination and sensitization to NTM were associated with positive TST results and discordant TST positive but QFT-GIT negative results, but not with either QFT-GIT positive results or discordant TST negative but QFT-GIT positive results.

TB prevalence in the country of birth was most highly associated with results by TST or QFT-GIT and discordant results where TST was positive but QFT-GIT was negative. The odds of having this type of discordance was at least 10 times higher for recruits born in countries where the TB prevalence is between 20 to 100 cases per 100,000 population, and almost 20 times higher for recruits born in countries with over 100 cases per 100,000, compared to recruits from countries with less than 20 cases per 100,000. Recruits from countries with high-TB prevalence were the most risk of having MTBI and may be at the greatest risk of developing TB. Discordant TST positive but QFT-GIT negative results at a 15 mm cutoff, among recruits born in high-TB prevalence countries, may indicate a lower sensitivity of QFT-GIT to detect remote Mtb infection.

Applications of the QuantiFERON-TB Gold In-Tube Assay

QFT-GIT performed well in this study of Navy recruits, especially among low-risk individuals. Military personnel and HCWs at increased risk of MTBI can use QFT-GIT for MTBI screening and serial testing, as the test appears to be approximately as effective as TST and does not cause boosting. The US CDC considers QFT-GIT as an acceptable alternative for diagnosing MTBI, in all situations where TST use is recommended. In situations where individuals have BCG vaccination, QFT-GIT may be preferred to TST.

QFT-GIT agreement to TST is particularly high where the majority of results are expected to be negative. However, individuals identified as positive by QFT-GIT may not be the same as those who are positive by other tests for MTBI, and vice-versa. Determinant factors for risk of MTBI, such as country of birth, may be useful considerations when interpreting QFT-GIT results and may guide efforts to target testing toward individuals at increased risk of TB.

The utility of QFT-GIT to serve as a prognostic tool among those suspected of MTBI to identify those at highest risk of progression to TB require further studies. In this study, QFT-GIT may have been less effective than TST at identifying remote cases of LTBI among foreign-born, US Navy recruits. This is evident in the number of recruits with TST induration $\geq$ 15 mm but negative QFT-GIT results. Traditionally, using TST, these recruits would be diagnosed

with MTBI and, without proper treatment, 10% would be expected to develop TB over their lifetime. However, as QFT-GIT results indicate a different immunologic response to different mycobacterial proteins than TST, the spectrum of infection identified by QFT-GIT may also differ. Quantitative estimates of exposure intensity (exposure duration and proximity) of contacts to TB cases have been shown to be more strongly associated with QFT-GIT results than TST results, and associated with TST negative but QFT-GIT positive discordance (183, 187). Identifying a different spectrum of MTBI may account for some evidence that suggests QFT-GIT may have a higher positive predictive value than TST for identifying those most likely to develop active TB in the future (162).

In situations where individuals at increased risk of MTBI are identified, and their pre-test probability of testing positive is high, a confirmatory or follow-up test may be useful for QFT-GIT results. Such repeat testing may help explain changes in IFN-γ response, where some individuals remain persistently positive, versus those who revert from positive to negative after recent exposure. Such reversions in IFN-γ levels for contacts may identify those who cleared infection without treatment and may be at less risk of developing TB than those who are persistently positive (220).

The logistical advantages of QFT-GIT allow for versatility in testing for MTBI. The “in-tube” component allows for remote blood collection, followed by

controlled temperature transportation to an incubator within 16 hours. However, some QFT-GIT logistical features require further investigation. The exact volume of blood collected for QFT-GIT may affect the amount of IFN-γ produced, due to the surface area of blood exposed to antigens on the inside of the tube or the concentration of IFN-γ released being diluted at higher blood volumes. These logistical advantages in MTBI testing represent the potential for novel advances in military field applications of QFT-GIT and may develop into simpler methods for remote testing and interpretation of QFT-GIT in non-laboratory settings.

Future Directions

IGRAs offer a fresh approach to diagnostic testing for MTBI. The current tests available are being studied to determine their most appropriate uses and to identify additional features of clinical value. However, new directions are also available using the components and format of IGRAs. Based on the improvement in specificity found in IGRAs using antigens ESAT-6 and CFP-10, new skin testing reagents are under investigation. A phase I clinical trial using ESAT-6 and CFP-10 in place of tuberculin PPD for skin testing has found no serious adverse events, some mild adverse events (264). Among 42 healthy volunteers who had negative QFT-GIT at baseline and received the novel skin test, 2 converted to positive QFT-GIT, at various follow-up intervals after placement of the skin test,

1 of which reacted positively to the skin test. The sensitization seen in these individuals may have been due to a boosted reaction to prior MTBI, prior sensitization to certain NTM cross-reactive with ESAT-6 or CFP-10, or may indicate recent transmission of Mtb. This new methodology still has the inherent limitation of requiring in-vivo injection of antigens which may confound future testing.

The discovery of additional antigens with unique properties may introduce more specific identification capabilities to future IGRAs. Genetic comparisons of species within the *M. tuberculosis* complex may find chromosomal regions of difference containing genes for additional antigens of clinical use. For example, antigens present in *M. africanum* but absent other *M. tuberculosis* complex bacilli. Being able to distinguish between species of Mtb causing sensitization or infection may lead to advances in understanding the natural history of LTBI for different species and provide new opportunities for different treatment strategies.

Finally, additional biomarkers, other than IFN-γ, may also have the potential to be developed as diagnostic tools for detection of MTBI. Interferon induced protein 10 (IP-10) and monocyte chemoattractant protein 2 (MCP-2) are two cytokines produced by monocytes and T-lymphocytes, in the immune response to Mtb infection. In a manner similar to QFT-GIT, stimulation of whole-blood to produce IFN-γ, IP-10 and MCP-2 has been performed in TB

patients and healthy unexposed controls (265). Both novel biomarkers expressed higher concentrations than IFN-γ, the antigen-stimulated levels were high in patients and the levels were low in un-stimulated blood and among controls. Larger studies on different biomarkers may provide evidence to create new diagnostic tools using these cytokines in conjunction or to replace IFN-γ. Such tests may improve current IFN-γ based tests due to the wider response seen to differentiate a positive TB case from a negative uninfected control. Tests using these biomarkers may be capable of identifying variations in the immune response to diagnose different phenotypes or points of MTBI, possibly distinguishing between LTBI and TB, or identifying those with MTBI most likely to progress to TB.

A diagnostic tool which can accurately identify cases of MTBI which will progress to TB before the development of disease is the "Holy Grail" of TB diagnostic research and continues to be an elusive challenge. In the interim, diagnosis of MTBI in the US can use QFT-GIT as a highly specific assay which performs at least as well as available alternative tests. QFT-GIT is an attractive programmatic tool with logistical advantages where laboratory infrastructure exists. Interpreting QFT-GIT results may still require additional subject information on individual risk of MTBI. Additional studies are needed to maximize the accuracy of IGRAs and understand the limits of their prognostic capabilities.

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Appendices

Military History Form from Study Protocol

Evaluation of New Blood Tests for TB: CDC IRB Protocol # 3390
Rev. Jan. 12, 2004
Military History Form (Form 15)

1. Date of birth: ☐☐-☐☐-☐☐☐☐
m m - d d - y y y y (at least y y y y)

2. Gender: ☐1 Male ☐2 Female

3a. Race: ☐1 White ☐2 Black
☐3 Asian/Pacific Islander ☐4 American Indian/Alaskan Native
☐5 Other _____

b. Ethnic Origin: ☐1 Hispanic ☐2 Non-Hispanic

4a. Have you ever had a TB skin test in the past?

☐1 Yes ☐2 No ☐9 Unknown

b. If tested, when was most recent? ☐☐-☐☐-☐☐☐☐ ☐9 Unknown
m m - d d - y y y y (at least y y y y)

c. If tested, what was the result of the most recent test?

☐1 Positive ☐2 Negative ☐9 Unknown

5. Have you been vaccinated against TB (e.g. received BCG vaccine)?
☐1 Yes ☐2 No ☐9 Unknown

6. Have you ever been around anyone who was sick with TB?
☐1 Yes ☐2 No ☐9 Unknown

7. Have you ever been told by a nurse or a physician that you have TB?
☐1 Yes ☐2 No ☐9 Unknown

8. Have you ever been treated for TB?
☐1 Yes ☐2 No ☐9 Unknown

9. Have you coughed up blood at any time during the past 2 **months**?
☐1 Yes ☐2 No ☐9 Unknown

10. Have you had a cough that has been present for more than the past 2 **weeks**?
☐1 Yes ☐2 No ☐9 Unknown

11. Have you had fevers and chills for more than the past 2 **weeks**?
☐1 Yes ☐2 No ☐9 Unknown

12 Have you had unexplained weight loss of more than 10 pounds in the past 2 **months**
☐1 Yes ☐2 No ☐9 Unknown

13. Have you ever lived, worked, or volunteered in a homeless shelter, prison jail, or drug rehabilitation unit?
☐1 Yes ☐2 No ☐9 Unknown

14. Have you ever lived, worked, or volunteered for more than one month in a hospital, or nursing home?
☐1 Yes ☐2 No ☐9 Unknown

15. Where were you born? (List the name of the city, state, and country where you were born in the appropriate box below)
a. city: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐
b. state: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐
c. country: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

16. Other than place of birth, have you ever lived or visited **outside** of the United States for more than one (1) month? ☐1 Yes ☐2 No ☐9 Unknown

b. If yes, list the most recent place by city, state, and country.
b1. city: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐
b2. state: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐
b3. country: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

c. If more than one, list second most recent place by city, state, and country.
c1. city: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐
c2. state: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐
c3. country: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Link to Mazurek et al. – *M. tuberculosis* Infection in Navy Recruits, 2007

Detection of Mycobacterium tuberculosis Infection in United States Navy Recruits Using the Tuberculin Skin Test or Whole-Blood Interferon-γ Release Assays

By

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http://cid.oxfordjournals.org/content/45/7/826.full.pdf