New tuberculosis vaccine trials in infants:
Design, diagnostics and trial site development

Nieuwe tuberculose vaccin trials in zuigelingen:
Ontwerp, diagnostiek en ontwikkeling van de trial locatie

Grace Kiringa Kaguthi

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promotor

Prof. dr. J.H. Richardus

Overige leden

Prof. dr. H.P. Endtz

Prof. dr. T.H.M Ottenhoff

Dr. M. Bakker

copromotor

Dr. J.E.M. de Steenwinkel

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Chapter 1

Introduction

In this introduction, I briefly review the natural history and pathogenesis of Tuberculosis (TB), with a focus on paediatric TB. This is followed by a summary of the global disease patterns and trends. Accordingly, I discuss the need for a new TB vaccine and the factors affecting low efficacy of the current extant vaccine. Given that there are potentially different vaccination strategies and targets, I review the selection of the target population, diagnostic considerations and practical elements for TB vaccine sites. The objectives of this thesis are to evaluate infants for suitability as a trial population, review diagnostic considerations which include evaluating the chest radiograph and the role of non-tuberculous mycobacteria. Finally, I look into the lessons learnt from the development of a TB vaccine trial site. These objectives are explained in more detail at the end of the introduction.

Pathogenesis of Tuberculosis

The causative agent of Tuberculosis is *Mycobacterium tuberculosis* complex (MTBC), mainly transmitted from person to person by droplets. Rarely, oral transmission occurs via unpasteurized milk. Thereafter, the course of the infection and disease is highly variable and to date, largely unpredictable. This is surprising, since MTBC has co-existed with man for millennia. In some individuals, certain immune mechanisms clear the infection before there is evidence of sensitization of adaptive immunity and these individuals can be resistant to TB infection (1-3). An unknown proportion of exposed persons develop latent or persistent infection (LTBI), defined as positive Tuberculin Skin Test (TST) or Interferon Gamma Release Assays (IGRAs) in the absence of clinical signs and symptoms of disease. It is estimated that about 1.7 billion individuals presently fall in this category (4). Of these, about 10% among those who are HIV uninfected will develop active disease in their lifetime. In some other individuals, infection progresses to disease, but they remain asymptomatic and capable of transmission (5). A minority develop clinically apparent disease with evidence of progressive disease in body organs plus microbiological confirmation. The incubation period varies, but it is typically thought to range from about three months to two years, and after that disease is relatively infrequent (6).

Paediatric TB

The course of paediatric TB is distinctly different from adult disease, particularly among young infants. TB is transmitted to infants through household contacts but mostly by exposures in the community (7). Studies from pre-chemotherapeutic era have attempted to quantify the natural history of the disease in children. After an infectious contact, an unknown proportion, but presumably higher than adults with similar exposure progress to primary infection. Of these, about 30-40% will progress to pulmonary disease after an incubation period of 3-8 weeks and 10-20% will develop disseminated disease one to three months after primary infection and approximately 50% of immunocompetent children will not develop disease (8). Infants therefore have a more severe course of disease with a higher proportion developing primary infection and disease within shorter time frames.

Epidemiology

Tuberculosis is the world's deadliest infectious disease, measured by the numbers of people who die each year, surpassing HIV and malaria (9). About 1.3 million people died of the disease in 2017. Those HIV infected are disproportionately affected. At least 30% of deaths among HIV infected persons are attributable to TB. There were at least 10 million people who suffered from TB in 2017, enduring social and financial distress (10, 11).

Global differences in TB burden

To optimize control strategies, it is important to map the disease and its determinants. There is considerable asymmetry in the epidemic. Of 193 nations in the world, five (3%) are responsible for more than half of the global disease burden. They are Brazil, Russia, India, China and South Africa (BRICS), characterized by steady economic growth over the last 15 years, but low commensurate reductions in TB deaths and incidence. This is attributed to economic inequalities, with certain population segments in these nations living under squalid conditions, with poor access to health care. This maldistribution nullifies the value of economic growth in driving down per capita TB, as happened in some nations.

There is considerable diversity in the age, estimated incidence and affected populations across these nations. Brazil (63/100,000), Russia (60/100,000) and China (63/100,000) have low incidence per capita, while India (204/100,000) and South Africa (567/100,000) have high incidence (12). In Brazil and Russia, TB burden is driven by transmission hotspots among vulnerable groups such as homeless persons and prisoners (12). China's disease rates are highest among the elderly who were sub-optimally treated prior to universal coverage of Directly Observed Therapy Short-course (DOTS) and set up of the National TB Program, resulting in high rates of reactivation (13). In India, patients seek care in the private sector first and frequently transition to the public sector, making it difficult for the program to track patients or assure quality of care. South Africa has the highest TB/HIV co-infection rates, and occupational hazards (mining) which drive incidence (14).

TB trends

Globally, the decline of incidence by 1.5% per year, observed over the last decade, will not achieve a world with 'zero deaths, disease and suffering due to TB by 2050'as envisioned by the STOP TB Partnership (14). The most recent targets envision a 95% reduction in TB mortality and incidence compared to the baseline period of 2015 (14). The most cost-effective long term solution for any infectious disease epidemic is effective vaccination (15, 16). This has been observed in cervical cancer (17, 18), polio (19), small pox, pneumonia (20). The discovery, development and rapid uptake of new interventions including a new TB vaccine are essential to the realization of a world free of TB (14).

The need for a new TB vaccine: Bacille Calmette-Guérin (BCG)

The only licensed TB vaccine has been administered for about a century (21). Its efficacy and cost-effectiveness against severe and disseminated forms of TB has been demonstrated (22). Protection against pulmonary TB is highly variable, between 0 to 80% (23), wanes after ten to fifteen years (24,

25) and only in one study lasted fifty years (26). The lack of consistent, durable protective efficacy against the most transmissible form of disease threatens all control efforts. The reasons for this, as well as BCG's exact mechanisms and correlates of protection are poorly understood.

Factors linked to BCG (in)efficacy

Presently, the diversity of BCG strains, age at vaccination, exposure to environmental mycobacteria and the route of BCG administration are all thought to influence BCG efficacy.

BCG strain

After development of BCG from a cow isolate in 1921, sub-cultures in various laboratories over time led to differences in BCG genotype and phenotype (27). Studies in mice have shown the strains have varying capacity to evoke delayed type hypersensitivity, T-cell cytotoxicity and proliferation (28). Strain specific differences in immune response and reactogenicity have also been shown in humans (29). However, whether strains affect vaccine efficacy in humans has not been demonstrated in head to head comparisons in clinical trials. This could suggest that apparent variances in immunogenicity do not correlate with protection (30).

Age at vaccination and revaccination

BCG is a neonatal vaccine administered intradermal. Administration at birth is supported by data showing BCG efficacy is highest when administered to unexposed (TST negative individuals), and those with low sensitivity to Non Tuberculous Mycobacteria (NTMs) (30), both conditions are assumed to be present in the early neonatal period. Additionally, BCG has beneficial off-target effects. They include promoting survival among low birth weight newborns, reducing all-cause mortality and infections in the neonatal period (31, 32) and improving heterologous Th1 responses to Tetanus Toxoid and polioviruses (31).

There have been concerns that new-born immunity is underdeveloped and could contribute to vaccine failure. When vaccination was deferred to 5-10 weeks of age, higher cytokine and T-cell responses were observed relative to neonatal vaccination (33, 34). It is not clear whether delayed vaccination translates to protective efficacy.

Delaying initial BCG vaccination to children aged 7-14 years with unknown TST status had modest (25%) efficacy against PTB (35). Boosting is a strategy to counter waning immune protection conferred by previous vaccination. Hence, the observed rise of TB incidence at the inset of adolescence ends the 'golden age' of lowest TB incidence. This led to the trial of BCG revaccination in the pre-adolescent period. However, this showed no efficacy (36). The authors conjectured that high helminth prevalence (37) may have reduced BCG efficacy in this instance. Notably, the inefficacy was present across all case definitions of TB. However, in the absence of Mantoux testing in the trial, some infected individuals may have received the vaccine, contributing to its non-efficacy (38).

Immune interference by NTMs

Contrary to long held dogma, the sterility of the lungs in normal physiology has been shown to be false (39). The lungs, like the gut, has a diverse microbiome, sources of which include micro-aspirations from upper respiratory tract, or in inhaled air (40). NTM species have been detected in human oral cavity and upper respiratory tract microbiomes in healthy subjects (41). They are likely critical for immune fitness and resistance to TB (42). Further, it has been speculated that high prevalence of NTM sensitization accounts for poor BCG efficacy in the tropics. This is mediated through cross reactive immune responses by masking (inducing an immune response to which BCG cannot improve on) or blocking (induction of a cross-reactive immune response, leading to non-replication of BCG and hence its inefficacy) (43). As such, they could be responsible for unquantified heterogeneity in vaccine responses among individuals. The species, incidence in sputum and clinical relevance of NTMs among BCG vaccinated infants in most high burden countries is unknown and will be reviewed in this thesis.

Route of administration

A large trial among South African infants found no differences in efficacy between the conventional intradermal versus sub-cutaneous BCG administration (44). Intranasal administered BCG has been tested in mice to mimic natural infection, with better protection (45, 46). No similar human trials have been conducted.

Trial design, endpoints & sample sizes

The BCG replacement vaccine, VPM1002 (www.prime-vaccine.eu) and other promising vaccine candidates (47, 48) are likely to progress to Phase III trials. In order to efficiently conduct these studies, reliable incidence estimates and cohort characterization are needed for sample size calculations, to inform trial design, selection of diagnostic tools and choice of endpoints as well as the target population.

Selection of target population

Traditionally, infants have been the natural target for new TB vaccines, since most vaccines are administered to them. BCG also has variety of beneficial target and off target effects (49, 50), which has ethical implications for the control arm in future trials. To optimize the limited resources available for TB vaccine development, numerous modelling studies have been conducted to identify the most appropriate target population, with conflicting results (51-55). Below I briefly review the arguments for neonatal vaccines. Thereafter, I also look at modelling studies which attempt to predict impact or cost-effectiveness of vaccinating neonates versus adults/adolescents versus mass vaccinations.

Neonatal TB vaccines

Conventional approaches to vaccination have failed to eliminate TB. As such the utility of neonates as a target population, must be reviewed against morbidity and mortality data as well as the potential impact of pre-infection vaccines on the End TB strategy. I also review the feasibility of a pre-infection vaccine.

Childhood TB morbidity

Challenging diagnosis

Childhood TB has been neglected by health programs. Children are considered of less public health import in disease transmission, as they frequently have pauci-bacillary disease. In addition, making the diagnosis is challenging. Signs and symptoms can be non-specific and usually associated with confounding co-morbidities such as acute pneumonia, HIV and undernutrition. Further, diagnostic aids such as sputum culture or GeneXpert, IGRAs, TSTs, expert radiograph assessment are either absent in high burden settings or lack sensitivity/specificity (56). There are also problems with under-reporting of confirmed pediatric cases to National TB Programs (57). Moreover, the lack of age disaggregated reports masks the high susceptibility of young children.

Inaccurate TB incidence estimates

The dearth of accurate TB incidence data also hampers design of interventional studies. Cohort studies deploying comprehensive diagnostic and follow up methods are few, due to the large sample sizes required (58, 59). Recently modelling studies have attempted to estimate the burden. Paediatric TB apparently contributes substantial fraction of the total global TB burden. Specifically, young children under five years bear more than half of the total incidence burden in children (60). Since modelling studies have inherent limitations (60), and program data are unreliable, cohort studies that systematically determine infant TB incidence are needed.

Childhood TB mortality

Given the paucity of incident data, non-specificity of signs and symptoms, co-existing lung pathologies and co-morbidities, it is possible an appreciable number of cases are missed. This has been confirmed by post-mortem studies (61, 62). What would be the case fatality rate of undiagnosed and therefore untreated disease? From the pre-chemotherapeutic era, at least 20% of children with untreated TB died from the disease within one year (63), with the majority of the deaths occurring among infants and toddlers. Not only is this is alarmingly high even compared to adults (64), it shows how fatal undiagnosed infant TB is and it is likely a top ten leading cause of early childhood mortality (65). Therefore, young children have a high unmet need for a new effective vaccine and constitute a top priority population.

Feasibility of a pre-infection vaccine

Neonates are presumed to be uninfected with TB. They are therefore targeted for pre-infection vaccines, which seek to prevent onset of TB infection and halt disease progression.

Simple versus complex pathogens: A question remains whether a pre-infection vaccine is even feasible? With simple pathogens, for example viruses, disease incidence is a function of infection. Hence, halving infection rates for example by vaccination, halves the disease incidence rates. This is not necessarily true for TB since MTBC has a complex pathogenic process, where infection often does not correlate with disease. There are latently infected people who don't progress to disease, but there

are also infected persons who also advance quickly to disease. Pre-infection vaccines would act on such susceptible persons who rapidly progress to disease for example infants (52).

Immunological precedence: There is some evidence BCG prevents infection in neonates, albeit from non-randomized studies (66). Recently, a trial of BCG vs H4IC31 vs placebo showed both vaccines incapable of preventing initial QFT conversion among QFT negative adolescents (48). Nevertheless, in natural infections, after MTBC exposure, it takes about six weeks to establish CD4 T cell responses, providing a window for vaccine induced immunity to combat MTBC evasion strategies, clearing the pathogen and preventing persistence of infection (67). Natural history studies show some individuals to be persistently TST/IGRA negative despite continued exposure (68). Hence, it appears that prevention of infection vaccines for neonates could have immunological precedence.

Modelling studies in selecting the target trial population

Pre-infection vaccines (Prevention of Infection/PoI)

Modelling studies show pre-infection vaccines targeting TB unexposed neonates would substantially reduce the burden of new infections. This effect would increase over time. On the other hand, post-exposure vaccines, targeting adults/adolescents would only minimally reduce the burden of new infections, and this effect would diminish over time (52).

Post-exposure vaccines (Prevention of Disease/PoD)

Modelling studies support greater and more rapid impact of adolescent/adult targeted prevention of disease vaccines in reducing the new number of cases of disease, over neonatal pre-exposure vaccines (51-53). Since infants do not transmit disease and it would be about 10-20 years before they are at increased risk of transmissible disease, a similar lag is expected before neonatal vaccination can impact the TB epidemic (54, 69). Further, economic evaluation models shows vaccinating adults to be remarkably cost effective (51). Recently released results of the phase IIb trial of M72/AS01E vs placebo in adults with LTBI showed 49.7% efficacy in preventing disease.

CONCLUSION

Modelling studies have value in conceptualizing multiple scenarios of vaccine coverage, efficacy, duration of protection and secular factors that affect disease epidemiology. They have inherent limitations which I describe below. Secondly, modelling studies are inconclusive on pre vs post-exposure vaccination. Some authors found neither pre nor post-exposure vaccines would reduce high incidence epidemics due to the complex pathogenic process of TB (52). A systematic review of 23 mathematical models exploring the potential impact of TB vaccines considered factors that would explain the inconclusiveness of pre *vs.* post-exposure vaccination (54).

Limitations of modelling:

a. External factors and geographical bias: Most models are based on an Asia like epidemic, driven by reactivation disease. Majority exclude Sub-Saharan Africa, where disease is driven by new infections (70, 71). If the rate of treatment of active disease would increase as envisioned in the present strategy (14), infections and thereby disease would decline, decreasing the relative impact of a pre-exposure vaccine in Asia like epidemics. Whereas a post-exposure vaccine would be found more useful in settings where reactivation disease drives the epidemic such as Asia (72).
b. The prevailing rates of LTBI: As few models report the LTBI rates used, it is difficult to assess comparative numbers of people most likely to benefit from post-exposure over pre-exposure vaccines (21).
c. HIV and Anti-Retroviral therapy status is largely ignored: It impacts longevity and probability of disease, vaccine efficacy (52, 54).
d. Availability of vaccination platforms and vaccine hesitance in adults is assumed in computing vaccine coverage. Vaccine hesitance is one of the top 10 global threats (73). There are few studies that have investigated the feasibility and acceptability of an adult TB vaccine. Developing a vaccination platform in most high burden countries will be costly and require complex logistical operations for health systems which are underfunded and burdened by providing care. This could undermine cost-effectiveness of an adult vaccine.

Diagnostic and endpoint considerations

Endpoints

In addition to identifying the target population, selection of endpoints also determine efficacy, as well as trial cost and duration. Non-microbiologically confirmed TB (Clinical TB) endpoints have been shown to undermine vaccine efficacy, given the vaccine is not designed to prevent non-TB respiratory ailments. The results of the M72/ASO1$_E$ vaccine trial confirmed this. Vaccine efficacy diminished proportional to the diagnostic distance from culture and molecular confirmation (47). However, when successful vaccine candidates move to efficacy trials, the relative rarity of incident TB necessitates sample sizes of tens of thousands of persons. To mitigate the risks of failure of a candidate after such a colossal investment, target vaccine profiles have been redefined to achieve lower threshold, 'proof of concept' end-points, namely: Prevention of Infection (initial or sustained IGRA conversion); Prevention of Disease (POD); Prevention of Recurrence (PoR) and therapeutic vaccines (67).

Endpoints: Prevention of infection endpoints.

Prevention of infection endpoints require significantly lower sample sizes (48, 67) and shorter durations of follow up as the incidence of latent infection is greater than disease in high burden countries. Hence efficacy in PoI acts as surrogate and potential proof of efficacy against disease and can be needed to proceed to much larger PoD efficacy studies.

Endpoints: Prevention of disease

Despite the attraction of PoI vaccines, PoD is really the most clinically meaningful end-point. Most latently infected persons have a low probability of progressing to disease, and further, prevention of initial or sustained QFT conversion, may not translate to PoD. Ultimately PoD trials are inevitable.

Chest radiograph (CXR)

Composite endpoints that include chest radiograph assessments have been used to define TB disease in trials. It is an invaluable adjunct of diagnosis. Nevertheless, it is fraught with risks. Most patients do not have classical radiological features (74), and the co-existence of multiple lung pathogens and co-morbidities among young children with TB confound the radiological diagnosis. For example, a comparison of radiological findings between severely under-nourished children with confirmed TB and those without, found no differences (75). Also a post-mortem study between the ante mortem radiographs of HIV infected and uninfected children found no difference in radiologic findings (76). Nevertheless, the specificity and reproducibility can be improved by using expert adjudication of radiographs.

Practical considerations: Site selection and development

Licensure trials for new PoI and PoD vaccines are likely to occur in high TB incidence countries. Most such settings lack the requisite clinical trial infrastructure and experience in conducting large studies. Infant mortality also tends to be high which can further undermine case ascertainment if there are numerous early deaths due to unrelated causes. Hence, large cohort studies deploying comprehensive diagnostic methods were needed to obtain incidence and mortality estimates.

Trial site description-Western Kenya

Outside of the BRICS countries, there are 25 other high burden countries, one of which is Kenya. The estimated prevalence of TB disease is 558/100,000 and about only half of those with the disease are treated (77).The estimated incidence is 319/100,000, with HIV co-infection rates of 29% (12). TB rates are highest among males and those aged between 25 and 30 years (77). The exact drivers of the epidemic have not been well characterized. The study site described in this thesis was set up following receipt of funding and co-funding from a consortium of partners to create capacity to conduct TB vaccine trials in 2007. A large complement of data and laboratory staff, nurses, pharmacists, doctors and clinical officers received training and practical experience in trial related procedures.

In 2009, a large cohort of neonates were enrolled to determine TB incidence (Infant Cohort Study). It was a landmark study that had not been done previously in the country nor has it been replicated since in Kenya. The cost investment of such studies is prohibitively high and therefore the lessons mined are to be used to optimize future vaccine trials.

AIMS OF THESIS

Overall, the aim of this thesis was to determine the suitability of infants as a trial population and inform diagnostic considerations for future TB vaccine trials. This included documenting the practical experience of site set up and trial implementation as well as the lessons for TB vaccine development. These questions are addressed in TB studies particularly among infants.

The specific objectives were:

To evaluate infants for suitability as a target population

- To assess TB incidence, post-neonatal mortality and cohort retention among infants.

Diagnostic considerations

- To evaluate the chest radiograph (CXR) for its suitability as an endpoint for infant trials where paucibacillary disease is most frequent.
- To determine the clinical relevance of non-tuberculous mycobacteria isolated in sputum, given their ubiquity and indistinguishable case presentation to TB disease.

Lessons from site development and for the TB vaccine pipeline

- To reflect on the lessons learnt in building a new TB vaccine site and review the TB vaccine development pipeline in the context of the Ebola virus outbreak.

OUTLINE OF THESIS

Chapter 2 describes the incidence of TB disease among infants, as a potential trial population. This chapter differentiates incident TB based on whether it is microbiologically confirmed or not and calculates the sensitivity of sample sizes for each case definition to determine the suitability of infants as a target population. We also examine retention, which also influences sample size. **Chapter 3.** High infant mortality is an important cohort characteristic of most high incidence countries, it undermines the ability to detect endpoints. In such settings, how much mortality can we anticipate in infant trials, and how much of it is due to background morbidity? This section determines the post-neonatal mortality in the study area, also the determinants and causes of mortality. **Chapter 4** examines the diagnostic utility of the chest radiograph for defining non-microbiologically confirmed TB endpoints. What is the inter-rater agreement of expert and non-expert readers in assessing radiographs for consistency with TB? The implications of including the expert readings of the chest radiograph on TB incidence, vaccine efficacy and sample size calculations will be reviewed. **Chapter 5** defines the species, clinical relevance, diagnostic difficulties and incidence of the ubiquitous non-tuberculous mycobacteria among infants with presumptive TB. **Chapter 6.** Phase IIb and III TB vaccine trials require large sample sizes. Therefore, multiple sites with the requisite disease burden, trial infrastructure and

expertise are needed in high incidence countries. This chapter reviews the challenges and opportunities of developing a TB vaccine site against the backdrop of the momentous breakthrough that was the demonstration of efficacy of the Ebola virus vaccine. In the discussion, I summarize our findings in relation to questions raised in this thesis, contextualize our results and provide future perspectives in light of the lessons learnt.

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Chapter 2

The incidence of tuberculosis in infants, Siaya District, Western Kenya

Grace Kaguthi[§], Videlis Nduba[§], Anna H. van't Hoog
Ellen M.H. Mitchell, Martien W. Borgdorff

[§] authors contributed equally
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Background | Infants are a target population for new tuberculosis (TB) vaccines. TB incidence estimates are needed to guide the design of trials. Objectives: To determine the TB incidence and cohort retention among young children using comprehensive diagnostic methods in a high burden area.

Methods | Infants 0-42 days were enrolled. Through four monthly follow up and unscheduled (sick) visits up to the age of two years, infants with Presumptive TB based on a history of contact, TB symptoms or pre-determined hospitalization criteria were admitted to a case verification ward. Two induced sputa and gastric aspirates were collected for culture and GeneXpert. Mantoux and HIV tests were done. Clinical management was based on the Keith Edwards Score (KE Score). Cases were classified into microbiologically confirmed or radiological**,** diagnosed by blinded expert assessment. Cox regression was used to identify risk factors for incident TB and study retention.

Results | Of 2900 infants enrolled, 927 (32%) developed presumptive TB. Sixty-nine TB cases were diagnosed (bacteriological and radiological). All TB incidence was 2/100 person-years of observation (pyo) (95% CI 1.65, 2.65). Nine were bacteriological cases, incidence 0.3/100 pyo. Radiological TB incidence was 1.82/100 pyo. Bacteriological TB was associated with infant HIV infection and higher KE scores. Completeness of 4 month vaccinations and HIV infection were positively associated with retention.

Conclusion | TB incidence was high. An all TB endpoint would require a sample size of a few thousand children, but tens of thousands, when limited to bacteriological TB.

INTRODUCTION

Pulmonary tuberculosis (TB) is an important public health problem worldwide. With 10 million new cases of TB and 1.3 million deaths in 2017 (1). In Kenya, a recent national prevalence survey, utilizing newer tools such as the GeneXpert showed higher rates than previously reported (2). The End TB Strategy of WHO emphasizes diagnosis and treatment of TB patients, as well as the need for research on methods to prevent TB, including new vaccines for high risk populations such as adolescents (3) and infants (4, 5). Childhood tuberculosis, while not easily transmissible, has higher morbidity and mortality risk (6, 7) for both HIV infected and uninfected individuals (8). It is also harder to accurately detect (7, 9). Young children are unable to expectorate adequately, and more invasive methods are required that include nasopharyngeal and gastric aspirates, or induced sputa. The necessary skills and infrastructure are hardly available in most primary care settings (10, 11). When sputum culture or GeneXpert are available, sensitivity is somewhat low as young children often have paucibacillary disease (7).

In many settings, clinical and radiological criteria form the backbone of diagnosis. Clinical criteria include a composite score chart (12, 13) ranking for history of contact or evidence of exposure to TB by Mantoux tests or their equivalent, protracted classical TB symptoms, failure to thrive with or without a suggestive chest radiograph. The criteria have poor specificity for TB in HIV infected infants who will tend to have prolonged cough, night sweats or weight loss due to other co-morbidities. (11, 13, 14). Also, children with respiratory illnesses frequently have multiple infections, chiefly bacterial, complicating the clinical picture (8, 11, 15). Chest Computed tomography (CT), the gold standard for detection of mediastinal lymphadenopathy, the radiological hallmark of primary tuberculosis (16), is unscalable, costly and associated with high radiation exposure. Chest radiographs (CXR) are more readily available in TB endemic settings. It has been noted that CXR readings have poor specificity among non-experts or clinicians with basic training (17) and classical diagnostic features are less frequently observed than among adults (18). Having a chest radiograph compatible with TB, doubled the odds of culture positivity among children (19). The CXR therefore seems a valuable addition to the paediatric TB detection armoury.

In most low and middle income countries where TB is endemic, extensive neonatal vaccination programs using Bacille Calmette-Guérin (BCG), have reportedly reduced the incidence of severe childhood TB including TB meningitis, and miliary TB (20) (21). Nevertheless, several trials for new infant TB vaccines have advanced in the last decade (22-24) in recognition of BCG's variable efficacy against pulmonary TB (25) and evidence that protection wanes (26). We sought to conduct a large cohort study, utilizing more comprehensive case finding and diagnostic methods to determine TB incidence among infants in Western Kenya, in order to inform sample size calculations, mortality patterns and estimates ahead of a trial of new TB vaccine candidates. A large portion of the study area is covered by a Health and Demographic Surveillance System (HDSS), tracking births, deaths, and migration. The majority of the deliveries occur at home (27). The study area has a high morbidity burden from respiratory diseases (28-30), acute and chronic undernutrition (31, 32) Birth rates and infant mortality are high but declining (31).

METHODS

The Infant Cohort Study was conducted from 2009 to 2011, in the Karemo, Gem and Boro Divisions in Siaya County, Western Kenya.

Recruitment

Home births were notified via traditional birth attendants, while health facility births were notified by the respective staff to the recruitment supervisor. Due to the health and demographic surveillance system, all home births were notified within 6 weeks of birth to the study staff. Infants aged zero to 42 days, and weighing ≥1700g were eligible, if they were expected to remain in the study area for more than two years and had been in the area since birth or for at least one month. Low birth weight babies were excluded due to their higher risk of mortality (would take away potential TB disease endpoints). Families planning to out migrate from the study area would make it impossible to ascertain TB disease endpoints and increase loss to follow up. Following notification, a study nurse was dispatched to review the infant for eligibility, obtain informed consent, take anthropometric measures, and provide BCG vaccination (Danish Strain, Staten's Serum Institute).

Study follow up visits took place at health facilities closest to the parents/guardians as follows; at six weeks of age for HIV DNA PCR testing (AMPLICOR COBAS), thereafter four monthly for one to two years depending on time of enrolment. During follow up visits, parents were asked about history of TB contact, TB symptoms in their infants and history of hospitalization. Participants who were unable to come to health facilities had home visits. Loss to follow up (LTFU) was defined as unknown status after three unsuccessful tracing attempts by study close out. Free ancillary care was provided at the study clinic, with hospitalization at the Siaya County Hospital. HIV infected participants were referred for care and anti-retroviral therapy (ART) at the HIV comprehensive care clinic.

Identification of presumptive TB.

Due to the non-specific presentation of infant TB, a broad criteria for presumptive TB was defined in the protocol for study purposes. At scheduled or ancillary unscheduled visits infants meeting the following criteria were considered to have presumptive TB: parental report of household TB contact or TB symptoms (cough for two weeks or more, night sweats for two weeks or more, fever for two weeks or more or undernutrition (underweight for age)) or a history of hospitalization with severe lower respiratory tract infections, meningitis, HIV/AIDS, or malnutrition. Health record surveillance of TB registers and the laboratories in the region was conducted to identify if notified TB cases were contacts of study participants. This was operationalized by searching the HDSS database using the case name and location and matching that to our study participants' HDSS address.

TB investigations

Participants with presumptive TB were admitted to a case verification ward (CVW) for collection of two serial sputum induction and gastric lavage specimens, Mantoux (tuberculin skin test-TST) testing

using 2 Tuberculin Units PPD RT23 (Staten Serum, Denmark), DNA PCR or HIV Antibody testing, and antero-posterior and lateral digital radiographs (CXR). Clinicians evaluated the radiographs during admission. Anthropometric measurements (middle upper arm circumference and weight for age) and clinical ranking using the Keith Edward (KE) Score chart for TB was done. TB treatment was initiated if the KE score was ≥7 or if the CXR was consistent with TB or if TB was microbiologically confirmed. Participants who started TB treatment with a KE score ≥7 but a negative CXR for TB or not microbiologically confirmed were not considered cases for this study.

Sputum samples underwent liquid and solid culture by Mycobacterial Growth Indicator Tube (MGIT) and Lowenstein Jensen (LJ) media. Thereafter, speciation for positive cultures was done using Capilia (FIND and Tauns co. Ltd) or GenoType assay (Hain Diagnostika, Nehren, Germany). Later, when GeneXpert MTB/RIF became available, additional sputum testing was performed from frozen stored sputum pellets. No drug susceptibility testing was done.

Case Definitions

TB cases were classified into bacteriologically confirmed and radiographically diagnosed cases. Bacteriologically confirmed were microbiologically confirmed. An expert panel comprising a paediatric pulmonologist and radiologist performed blinded reviews of CXRs using a standard form developed for TB vaccine trials sites through consensus (17). Any radiograph classified as consistent with TB was defined as radiographically diagnosed TB. Due to subjective assessment of clinical criteria, radiological and bacteriological criteria are presented in this paper to provide a more objective assessment of actual cases that would be end-points in a future TB vaccine trial and that are more stringent. Clinical criteria are non-specific due to other prevalent co morbidities like HIV, malnutrition and failure to thrive. A positive Mantoux test was an induration of ≥ 10mm (or ≥5mm in the presence of severe acute malnutrition or HIV infection).

Statistical Methods

Electronic case report forms were used. Data was analysed using STATA 13 (STATA corp California). We defined enrolment weight as low if it was <2500g and normal at ≥2500g. Nutritional status was classified based on mid-upper arm circumference for infants older than 6 months or weight for age WHO Z scores for those less than 6 months. Person time was calculated from enrolment to the last study contact in a scheduled or unscheduled visit or death whichever was last or TB disease diagnosis. Radiographically cases diagnosed by experts were censored at the date of CXR. Incident TB was computed for bacteriologically confirmed and all TB by dividing the number of cases by the total person time. Cox proportional hazards was used to compare those who became TB cases versus the rest of the study population, identify risk factors for TB and study retention. Vaccination status at four months of follow up, when Pentavalent III has been given was tested as a potential predictor of retention.

Factors that were statistically significant during univariate analysis were further included in a multivariate Cox regression model to adjust for multiple factors to determine those remaining significant risk factors for TB and study retention. Logistic regression was used to compare the clinical characteristics

of bacteriologically confirmed and radiographically diagnosed TB. These independent variables were included: TST, KE Score, nutritional status, history of household contact, infant HIV and reason for TB investigations.

Frequency of ancillary care visits (sick visits) and hospitalization between zero and four months were considered potential risk factors for incident TB at follow up.

RESULTS

TB incidence

Of 2900 infants enrolled, 196 (6.8%), moved out of the study area upon enrolment. A total of 2704 infants were followed up for TB incidence, with 3298 person years of follow up (pyo). (Figure 1).

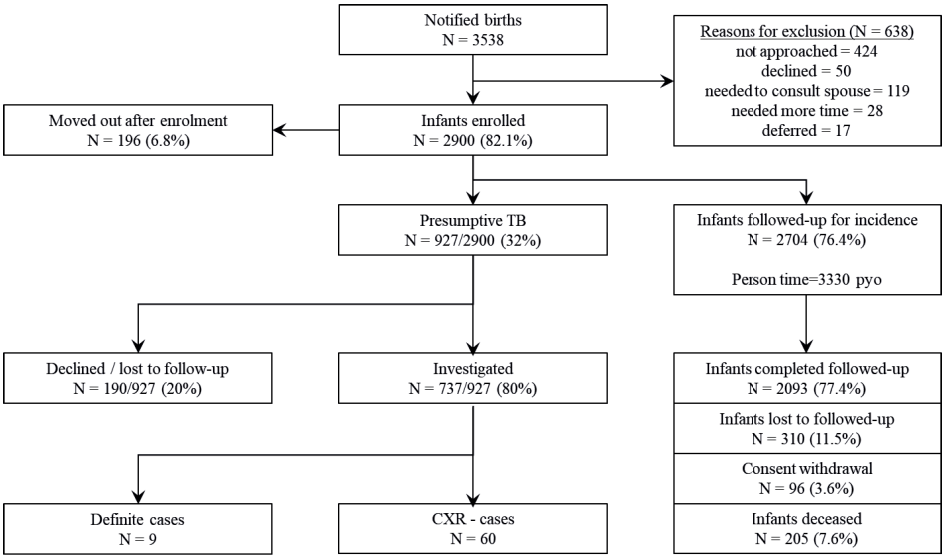

Figure 1 | Flow diagram of the selection of infants for incidence follow-up and the number, who are deceased, withdrew or were lost to follow-up.

Mean follow up time was 1.14 pyo (median 1.3 pyo). There were 205 (7%) total deaths, of whom 26/205 (13%) were known to be HIV infected. About a third of the deaths were in the neonatal period, thereafter most identifiable immediate causes of death were due to severe dehydration and acute respiratory ailments (33).

We identified 927/2900 (32%) as presumptive TB cases, of these we investigated 737/927 (80%) and found 69 individuals to have TB based on microbiological confirmation or radiological criteria. The incidence rate of all TB was 2.0/100 pyo (95% CI 1.65, 2.65). There were nine bacteriologically confirmed TB cases, incidence rate was 0.3 per 100 pyo (95% CI 0.1, 0.5) and the incidence of radiological TB was 1.82/100 pyo. In addition, sixty cases had CXRs consistent with TB. The experts

identified 64/69 all TB cases, of which they agreed on only seven. One of the experts identified 43/60 radiographically diagnosed cases and 4/9 bacteriologically confirmed on CXR. The other expert identified 21/60 radiographically diagnosed cases and 3/9 bacteriologically confirmed on CXR.

Sociodemographic predictors of TB

There were no differences in demographic (sex, maternal age) socioeconomic indicators (maternal occupation) between TB cases and the rest of the cohort at baseline. There were also no significant differences in infant enrolment weight and maternal HIV status between the groups. However, the incidence of TB was higher in those with HIV (p=0.0004) and those reporting a household contact at baseline (p=0.04). TB incidence was lower among mothers who attended antenatal care visits (p=0.04). (Table 1).

Table 1 | Baseline characteristics of TB cases versus study population and univariate HR for incident TB

Baseline Variable		TB case n=69	pyrs	HR (95% CI)
Gender	Male	38	1610	1.17 (0.73, 1.88)
	Female	31	1688	1*
Place of Birth	Home	49	2147	1.29 (0.77, 2.18)
	Health Facility	20	1131	1*
Maternal HIV	Positive	9	2766	0.87 (0.43, 1.75)
	Negative	60	474	1*
Infant HIV status	Negative	62	3228	1*
	Positive	7	69	5.81 (2.65, 12.7)
Maternal age (years)	<19	16	635	1*
	20-29	39	1722	0.86 (0.48, 1.55)
	>29	14	941	0.56 (0.27, 1.15)
ANC attendance	Yes	57	2911	0.53 (0.28, 0.98)
	No	12	328	1*
Enrolment weight (grams)	1700-<2500	4	239	0.79 (0.29, 2.16)
	>=2500	65	3058	1*
Household contact	Yes	9	203	2.11 (1.05, 4.29)
	No	60	3095	1*
Maternal occupation	Unemployed/farmer	63	2872	1*
	Business/Salaried	6	426	0.61 (0.26, 1.42)

* 1 = set as the reference

Comparative clinical characteristics of TB cases

We compared the clinical characteristics of bacteriologically confirmed and radiological cases. Compared with cases identified with radiology, bacteriologically confirmed TB patients had more often a KE score>=7 (OR 17.0, 95% CI 2.78, 104), were more often TST positive (OR 10.8, 95% CI 2.16, 54.4), and reported more frequently a history of TB contact (OR 6.13, 95% CI 1.10, 34.2). There were no other significant differences in clinical characteristics. (Table 2)

Table 2 | Comparing clinical characteristics of definite and chest radiograph TB cases

Characteristic		Definite TB N=9	CXR TB N=60	HR (95% CI)
TST	Negative	5	52	1*
	Positive	3	8	10.8 (2.16, 54.4)
	Missing	1		
KE Score⊥ category	<7	4	51	1*
	≥7	4	3	17.0 (2.78, 104)
	Missing	1	6	
Nutrition	Normal	2	7	1*
	At risk	2	10	0.70 (0.08, 6.22)
	Moderate malnutrition	4	33	0.42 (0.07, 2.79)
	Severe malnutrition	1	10	0.35 (0.03, 4.65)
History of TB contact	No	4	49	1*
	Yes	3	6	6.13 (1.10, 34.2)
	Missing	2	5	
Reason for investigation	Contact	1	4	1*
	Hospitalization	3	36	0.33 (0.03, 4.01)
	Symptoms	3	14	0.86 (0.07, 10.7)
	Missing	2	6	
Infant HIV status	Negative	6	50	1*
	Negative (exposed)	2	4	4.17 (0.63, 27.8)
	Positive	1	6	1.39 (0.14, 13.6)

* 1 = set as the reference

⊥ KE score (Keith Edward Score)

Risk factors for incident TB

In univariate comparisons, infant HIV infection, two or more hospitalisations, one or more sick visits and household TB contact at baseline were risk factors for incident TB. We adjusted for these variables and found infant HIV infection increased the risk of incident TB, adjusted Hazards Ratio (aHR) 4.71 (95% CI 2.13, 10.4)]. Two or more hospitalisations by 4 months of age also increased risk, adjusted Hazard Ratio (aHR) 2.10 (95% CI 1.09, 4.03)] as did multiple sick visits, adjusted Hazard Ratio (aHR) 2.17 (95% CI 1.12, 4.22)]. Household TB contact was not a significant predictor in the adjusted model. (Table 3)

Loss to Follow up

One year loss to follow-up was lower with increasing maternal age in both the univariate and multivariate analysis; adjusted Hazard Ratio (aHR) 0.89 (95% CI 0.79, 1.00). Complete vaccination status measured by proportion who had received all required vaccinations by four months per the Kenya Expanded Immunization Program (KEPI) was associated with lower loss to follow-up; adjusted Hazard Ratio (aHR) 0.44 (95% CI 0.39, 0.49). Employed mothers or those in business had lower loss to follow up compared to unemployed; adjusted Hazard Ratio (aHR) 0.73 (95% CI 0.64, 0.84)]. (Table 4)

Table 3 | Prospective risk factors for TB based on baseline and follow up characteristics of whole study population

Characteristic		TB cases N=69	Study Population	Person-years	HR (95% CI)	Adjusted HR (95% CI)
HIV status	Negative	56	2409	2793	1*	1*
	Negative (exposed)	6	356	436	0.68 (0.29, 1.57)	0.62 (0.27, 1.45)
	Positive	7	66	69	5.56 (2.53, 12.2)	4.71 (2.13, 10.4)
Nutrition	Normal	9	376	442	1*	
	At risk	12	656	828	0.71 (0.30, 1.68)	
	Moderate malnutrition	37	1300	1672	1.09 (0.53, 2.25)	
	Severe malnutrition	11	290	343	1.60 (0.66, 3.86)	
	Missing		209			
Hospitalization	0	52	2521	2902	1*	1*
	1	5	137	160	1.80 (0.72, 4.50)	1.43 (0.57, 3.63)
	≥2	12	173	237	2.80 (1.49, 5.24)	2.10 (1.09, 4.03)
Sick visits by 4 months	0 visits	13	1311	1269	1*	1*
	1 visits	20	663	821	2.37 (1.18, 4.76)	2.05 (1.01, 4.16)
	≥2 visits	36	926	1208	2.82 (1.49, 5.32)	2.17 (1.12, 4.22)
History of TB contact	No	60	2698	3095	1*	1*
	Yes	9	133	203	2.12 (1.05, 4.29)	1.93 (0.95, 3.94)
Maternal age	<19	16	619	635	1*	
	20-29	39	1494	1722	0.86 (0.48, 1.54)	
	>29	14	718	941	0.56 (0.27, 1.15)	
Maternal occupation	Unemployed / farmer	63	2479	2872	1*	
	Business / salaried	6	352	426	0.61 2(0.26, 1.42)	
Retained	No	2	628	170	1*	
	Yes	67	2202	3127	1.10 (0.26, 4.62)	

* 1 = set as the reference

Table 4 | One year loss to follow up (LTFU) and factors associated with LTFU of prospectively followed up infants

Baseline Variable		LTFU* (n)	Person years	HR (95% CI)	Adjusted HR (95% CI)
Gender	Male	220	1610	1*	
	Female	215	1688	1.01 (0.94, 1.11)	
Place of Birth	Home	181	1131	1*	1*
	Health Facility	252	2147	1.04 (0.96, 1.14)	0.80 (0.60, 1.09)
Infant HIV status	Negative	395	2793	1*	1*
	Negative exposed	32	436	0.98 (0.87, 1.10)	0.98 (0.86, 1.12)
	Positive	8	69	1.53 (1.20, 1.96)	1.41 (1.06, 1.89)
Maternal Age	<19	149	635	1*	1*
	20-29	232	1722	0.86 (0.77, 0.96)	0.89 (0.79, 1.00)
	>29	54	941	0.85 (0.75, 0.96)	0.89 (0.78, 1.02)
ANC attendance	Yes	43	328	1*	
	No	387	2911	1.02 (0.89, 1.17)	
Vaccination status	incomplete	78	510	1*	1*
	complete	97	2477	0.44 (0.39, 0.49)	0.44 (0.39, 0.49)
Maternal occupation	Unemployed/ Farmer	378	2872	1*	1*
	Business/Salaried	57	426	0.71 (0.62, 0.81)	0.73 (0.64, 0.84)

* 1 = set as the reference

Sample size requirements for vaccine trials in this population

We estimate based on our bacteriologically confirmed incidence rate a total of 24,321 infants in a 1:1 randomization would need to be enrolled for both arms combined at an incidence rate of 0.3/ 100 pyo to demonstrate a 50% vaccine efficacy with 91 TB cases in the placebo arm and a 20% loss to follow up over 3 years. About half that number would be required with a vaccine of 70% efficacy. Conversely a total of one thousand four hundred and four infants would be needed for both arms combined to demonstrate a 50% vaccine efficacy given an all TB rate in the order of 2/ 100 pyo. (Table 5)

Table 5 | TB vaccine sample sizes

	Presumed vaccine efficacy								
	50%			**60%**			**70%**		
Incidence rate of placebo culture confirmed	**Total size of sample***	**placebo**	**vaccine**	**Total size of sample***	**placebo**	**vaccine**	**Total size of sample***	**placebo**	**vaccine**
0.3/100	24.321	88	44	16.318	59	23	11.554	42	12
2.0/100	1.404	34	17	942	23	9	667	16	5

* Lost to follow-up percentage set to 20%

DISCUSSION

Incidence of TB and implications

We observed a high incidence of all TB among infants in Western Kenya in the order of of 2/100pyo. A much lower incidence of bacteriologically confirmed TB (0.28 per 100 pyo) was found. This is consistent with infant disease which is classically paucibacillary. It is higher than reported definite TB incidence in Mozambique, (0.14 per 100 pyo (34)) but lower than in the Western Cape, (1.2 per 100 pyo (5)). A large number of participants were identified as having presumptive TB indicating a high morbidity burden that lead to TB suspicion. TB vaccine trials will need to formulate strategies to deal with this high morbidity burden in infant vaccine trial cohorts and provide the staff capacity needed to cope with the large number of TB investigations required. In addition, 20% of participants with presumptive TB were not investigated. The requirement for a 48 hour admission for TB investigations might have led some parents to decline investigations. Future studies need to offer flexibility including exploring day admissions and discharges for infants whose parents are unwilling to have overnight admissions. The missed investigations might have underestimated the burden of TB in this cohort.

Case definitions

Our study utilized a chest radiographs (CXR) to define non-microbiologically confirmed TB. To be clear, these infants met clinical criteria for TB suspicion, therefore the CXR was a confirmatory tool, in the absence of microbiological confirmation. There are challenges related to the diagnosis of TB in children in regard to the use of CXRs which include; Identification and interpretation of CXR abnormalities are variable and often inconsistent (35), CXR parenchymal abnormalities in infants and young children with TB are not specific for TB but overlap with CXR abnormalities due to other lower respiratory tract infections (36) and CXR is less sensitive for detecting TB-related intrathoracic abnormalities than other imaging modalities (37). Despite the low agreement between the expert readers, 3 out of 7 CXRs defined as consistent with TB were also culture confirmed indicating having more than one reader is key in identifying TB. One way to improve on the poor agreement between experts would be to require an additional criteria like latent TB infection, for definition of TB in addition to a CXR consistent with TB where the experts don't agree. The study area has a high morbidity burden due to HIV, undernutrition and acute respiratory diseases (19, 27, 30, 31, 33). The limitations of a clinical score chart, namely low specificity and high sensitivity, leading to over-treatment have been documented (13, 14). The chest radiograph therefore was used for a viable case definition, in light of low sensitivity of microbiological methods. It has been shown that chest radiographs, are a significant correlate of culture confirmed TB in settings endemic for HIV and other co-morbidities (19, 38). Unfortunately, an intrinsic risk of this approach is the lack of specificity which would underestimate vaccine efficacy, given that new candidates are not geared toward prevention of non-TB respiratory ailments. As this study utilized blinded experts expected to have higher specificity compared to non-expert clinicians (17), misclassification was minimized to the extent possible. This approach also permitted evaluation of risk factors for incident TB, as they were not part of the diagnostic criteria.

Infant TB diagnostics and alternative samples

There are currently limited options for TB diagnosis in young infants. Nasopharyngeal and stool samples could be non-invasive alternatives to induced sputum and gastric lavage. Nevertheless, when analysed using Gene Xpert, both have shown lower sensitivity in children compared to sputum (39-41). The WHO has recommended the use of Xpert MTB/RIF over conventional microscopy and culture as the initial diagnostic test in all children suspected of having TB, while acknowledging the resource implications. IGRAs and commercial serology diagnostics tests are not recommended for use in infants regardless of their HIV status (42, 43). New diagnostic tools are needed. These must then be validated in very young children.

In an autopsy study of children with lung disease, undiagnosed TB was among the top three causes of death among HIV infected and uninfected children, supplementing data showing that definite TB alone could be an insensitive indicator of disease (8). Missed TB cases are found at autopsies (44, 45), particularly among children (45, 46) in both low and high TB incidence settings. About 7% of the cohort died during the study; obtaining ethical approval and informed consent to include limited but targeted post mortem exams, would increase the number of end-points in a TB vaccine trial. One necropsy study showed an additional 25% of respiratory related deaths among hospitalized children were attributable to undiagnosed TB. This potential end-point has not been included in previous trials.

Risk factors

HIV infected infants had a two to ten-fold increased risk of TB disease. Unfortunately, most TB vaccine trials exclude this population for safety reasons. This is the population that most urgently needs a more efficacious TB vaccine. Frequent hospitalization and out-patient sick visits can be considered a marker of chronic morbidity. Patients with one or more sick visits and hospitalizations had more than one to four fold increased risk of TB. While it is possible that the sick visits increased the probability of TB detection, the higher frequency may also be an indication of underlying vulnerability to TB.

TB vaccine trial sample sizes

Our study sought to determine incidence estimates for sample size calculations of hypothetical clinical trials. Due to reliability and validity concerns around TB diagnosis in young children; relying narrowly on bacteriologic confirmation of cases would necessitate exposure of tens of thousands of trial participants to an investigational product, depending on vaccine efficacy. Unfortunately, broad criteria that include radiographic and clinical cases might mean some non-cases are included, leading to underestimates of the efficacy of a vaccine candidate.

Including HIV infected infants who are more likely to develop TB, and also most likely to benefit from the protection of an efficacious vaccine, would minimize sample sizes considerably. However, protective efficacy among HIV individuals may be harder to achieve due to challenges with achieving adequate immunological response.

Retention

Critical to the success of any vaccine trial is retention of participants for end-point determination. A considerable proportion of study participants, exited the study area after enrolment (7%). Future studies must consider cultural norms where women migrate to other areas to give birth and return to their marital homes shortly after. This should be taken into consideration on deciding on inclusion and thus reduce loss to follow-up. Thereafter, the loss to follow-up was about 12% and this would need to be factored into sample size considerations. Factors of health seeking behaviour, particularly completeness of infant vaccination predicted retention. This suggests possible selection bias in case detection, as those who are lost to follow up, may have less favourable health seeking behaviour and therefore are less likely to be diagnosed with TB.

Increasing maternal age was positively associated with retention. Vaccine trials, can possibly deploy better strategies to retain younger mothers including targeting them for phone/home follow up to increase their retention. Our study population largely comprised children of peasant farmers and unemployed women, whereas those who were employed or running businesses were more likely to be retained. In consideration of this skew, such mothers might require off-site follow up such as home visits or provisions for comprehensive tracing when they out-migrate.

Limitations

As the majority of TB is not microbiologically confirmed, alternative case definitions that use radiological criteria were used. We diagnosed TB when any of the blinded experts considered the radiograph to be consistent with TB, however most clinical trials would only consider the radiographs where experts agreed to be a TB case. Low expert inter-rater agreement has been demonstrated (17). Since experts agreed on only seven infants as having TB, the low yield would be an additional limitation to overcome. With all cohort studies, loss to follow up may occur and this may have underestimated our TB incidence estimates. In addition, sputum samples were not tested further to exclude other viral or bacterial respiratory pathogens that could have similar radiological presentation, this could potentially overestimate TB incidence.

Strengths

Our study is one of the first to reliably document TB rates among infants in Western Kenya. We deployed expert readers to adjudicate on cases, as would be the case in a vaccine trial. We deployed CXRs to estimating the TB incidence, in consideration of the disease patterns in the study area as well as the diagnostic challenges. Therefore we consider the study findings to be generalizable to similar settings.

CONCLUSIONS

High TB incidence among young children in Western Kenya particularly among the HIV infected demonstrates the need for novel infant TB vaccines, suited for these children. In light of this, this

population is suitable for new TB vaccine trials. However, large sample sizes would be needed for phase III trials, given the low definite TB incidence. Inclusion of necropsy studies and radiological TB may increase the number of study end-points and reduce the sample sizes. Retention of young women and unvaccinated infants who may have a different risk of TB profile, requires deliberate design of such studies.

Ethical Approval

The protocol and informed consent forms were reviewed and approved by the KEMRI local and national scientific steering committees and the KEMRI national ethical review committee (SSC 1465).

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Chapter 3

Predictors of post-neonatal mortality in Western Kenya: A cohort study

Grace Kaguthi, Videlis Nduba, Martien W. Borgdorff, Suzanne Verver

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Introduction: To determine the predictors of mortality in infants in Siaya, Western Kenya, ahead of novel Tuberculosis (TB) vaccine trials in the same population.

Methods: In a study to determine tuberculosis incidence, 2900 infants aged 0-45 days, weighing ≥1700g were enrolled. Four monthly follow up visits were conducted for at least 12 months. HIV testing was done at six weeks of age. Free ancillary care was provided. Deaths were reported by parents, study staff and community workers. Cox proportional Hazard analysis was used to identify risk factors. The period of analysis commenced at six weeks old and was censored at 12 months of age.

Results: Included in the analysis were 2528 infants with 2020 person years of follow up (pyo). There were 117 deaths (4.6 %). The post-neonatal mortality rate was 58 (95% confidence interval (CI): 48, 69) per 1000 pyo. In multivariate analysis, health facility births were protective against mortality (Hazard Ratio (HR) 0.54; 95% CI: 0.34, 0.84) and infant HIV infection at baseline was associated with increased mortality (HR 10.3; 95% CI: 6.40, 16.7). HIV uninfected infants born to HIV infected mothers had increased hazards of mortality (HR 1.73; 95% CI: 1.03, 2.90). Gender, weight at six weeks, maternal education and occupation were not significant predictors of mortality.

Conclusion: Infant mortality was high and was associated with being born outside a health facility, maternal HIV infection and HIV infection of the infant. Measures to decrease mother to child transmission and other HIV control measures need to be strengthened further to see incremental reductions in infant mortality.

INTRODUCTION

Strategies to reduce child deaths have been extensively studied [1-3]. Over the last two decades considerable progress has been made in averting thousands of child deaths [4]. This is attributable to improvements in socio-economic conditions and scale up of disease specific interventions such as use of insecticide- treated bed nets, oral rehydration therapy for diarrheal disease targeting the main causes of childhood deaths. In fact, Kenya has experienced reduction of infant mortality from 77/1000 in 2003 [5] to 52/1000 live births in 2008 [6] and 39/1000 live births in 2014 [7]. Despite this considerable decline from high levels of mortality, it is a forty-fold higher probability of death compared to a child born in developed countries where the chances are less than one death in every one thousand live births [8]. Several studies have revealed various determinants of child mortality. Child spacing, birth order, maternal illiteracy, income disparities, rural versus urban residence, relative distance from health facilities have all been known to account for high child mortality [9-11]. These have been identified from cross-sectional studies such as national health and demographic surveys [1, 2]. Data from cohort studies have been less frequently employed although they might provide a better assessment of relationships between possible causal factors and mortality [12]. Further, due to close follow up in cohort studies, more information on proximal health variables may be available. We conducted a cohort study between June 2009 and September 2011 to determine what factors predicted infant mortality in Western Kenya. This was a preparatory study to obtain tuberculosis (TB) incidence estimates amongst infants in preparation for trialing novel TB vaccine candidates in the same population, with infant mortality as a secondary objective. Deaths that occur in the conduct of a clinical trial may be misattributed to the investigational agent if background mortality rates and causes are not clear. Excess mortality also implies missed end-points when they occur prematurely.

METHODS

Setting

The study area was predominantly rural in Karemo, Gem and Boro Divisions of Siaya County, Western Kenya. The infant mortality rate at the time in this area was 110/1000 live births [13], more than double the national average. The area has a high prevalence of the HIV and TB syndemic, with perennial intense malarial transmission [13, 14]. About 80% of women deliver at home without the help of a skilled attendant [15, 16]. Subsistence farming is the main economic activity. Educational attainment of female population is low: 9.3% of women have some secondary education while 49.3% have some primary education. Siaya County is served by 31 dispensaries and health facilities, manned by nurses who provide vaccinations, antenatal care, contraception, and empiric treatment of basic ailments. Several of these facilities provide Anti-Retroviral Therapy at Patient Support Centres (PSCs). Hospitalizations mainly occur at the Siaya County Hospital. The proportion of low birth weight among neonates weighed at birth (53%) is 6% in the study area [17].

Study population

The target was to enrol a total of 2900 infants 0-45 days of age, between June 2009 and June 2010 who were at least 1700g at enrolment. Follow up visits were carried out every four months for at least one year, maximum of two years depending on the time of enrolment. Participants enrolled earlier had a longer duration of follow up. Visits were conducted at a health facility of the parent's choice or at home. To enrol infants, we received birth notifications from community health workers who frequently doubled as Traditional Birth Attendants (TBAs). Study nurses on motor bikes visited homes of interested potential participants. They obtained informed consent, took anthropometric measurements and administered BCG vaccine to those unvaccinated. Infants below 1700g at time of enrolment were excluded. At age six weeks, Deoxy-ribo-Nucleic Acid (DNA) Polymerase Chain Reaction (PCR) HIV testing (COBAS® HIV-1 Amplicor by ROCHE) was offered to all participants. Additional HIV testing was done as part of TB investigations for those with presumptive TB (at time of study were named TB suspects), during follow up and at study exit. Parents and participants who tested HIV positive, were referred to the Patient Support Centre for Anti-Retroviral Therapy (ART) initiation and care. A dedicated study nurse visited these participants to counsel on feeding options and encourage ART initiation.

Free ancillary care was provided at the study clinic. The study covered the cost of transport up to 5 USD and hospitalizations at the Siaya County Hospital. Supplemental feeds provided by UNICEF to the hospital were also administered by the study nutritionist for participants who met the criteria, according to the WHO guidelines for management of malnutrition. Deaths were notified by parents, study staff and community health workers. The families of participants who died at home or at facilities outside the County Hospital were visited by a study nurse to obtain ante-mortem history from the primary caregiver using a semi-structured questionnaire.

At each visit, data was entered electronically into a customized Microsoft 2009 SQL® database with edit checks and validations. The predetermined sample size was powered to identify incident tuberculosis cases, with mortality incidence as a secondary objective.

Ethical considerations

The study was approved by the Kenya Medical Research Institute Ethics Committee. (SSC Number 1465), and conducted according to International Committee for Harmonization of Clinical Trials-Good Clinical Practice (ICH-GCP). Written Informed consent was obtained from mothers prior to initiating study procedures. Study Numbers were assigned to maintain privacy and confidentiality. Access to study records was limited to study staff and monitors.

Cause of death ascertainment

To obtain the causes of death for post-neonatal mortalities, two medical officers reviewed available morbidity data from the study clinic, admission notes and investigations as well as the ante-mortem history for deaths that occurred at home. Immediate and underlying causes of deaths were independently assigned by each medical officer using the International Classification of Diseases-10 (ICD-10).

Disagreements were resolved by consensus. Anthropometric data on weight for age was available from follow up visits or unscheduled visits. Weight for age Z-Scores from the World Health Organisation Charts were used to define presence of under-nutrition [18]. Weight of two or more standard deviations from the median for age, (≥-2SD) was classified as moderate to severe malnutrition. Weight for age Z-scores of greater than one, up to three months before death, were analysed as potential underlying risk factor of death.

Statistical Analysis

To minimize survival bias introduced by variable study entry which could have been related to neonatal deaths (left truncation), the analysis time began when infants were six weeks of age and above. Follow-up time was censored at 12 months of age. Person-time was calculated from 6 weeks of age till 12 months of age, death, or loss to follow-up, whichever occurred first. Descriptive statistics were used to describe demographic and clinical characteristics, causes of death and infant mortality rate. Unadjusted and adjusted hazard ratios for mortality were computed using Cox proportional hazards analysis. Logistic regression was used in bivariate analysis to evaluate the relationship between selected predictors. Missing data was excluded.

RESULTS

In 2010, there were 3534 registered births in the study area [19]. The study received 3538 birth notifications between June 2009 and June 2010. Of these, 2900 (82%) were enrolled. There were 46/2900 (1.6%) deaths between birth and six weeks and 326/2900 (11%) were lost to follow (LTFU) up at six weeks. Therefore, 2528 infants, six weeks old and above were included in this analysis. Those included had together 2020 total person years of follow up. Average person-time was 0.8 person-years. At twelve months, cumulative LTFU was 626 (22%).

There were 117 (4.6%) deaths (table 1), between six weeks and twelve months of age. The post-neonatal mortality rate was 58 per 1000 person years of follow up (95% CI 48, 69). The post neonatal mortality ratio was 46 per 1000 live births. Four in ten deaths occurred at health facilities. Patients with sick visits at the study clinic were more likely to be hospitalized (HR 2.55; 95% CI: 1.43, 4.57). Immediate causes of death were identifiable in 88/117 (75%) cases. Of these, the most frequently identified causes of death were: hypovolemic shock/dehydration (30%), pneumonia (26%), malaria (10%) and anaemia (9%). Together these accounted for 66/88 (66%) of identifiable deaths (Figure 2a). Of the 36/117 (31%) cases for which an immediate cause of death could not be assigned, 29 cases had inconclusive information, and seven had no information at all regarding the ante-mortem circumstances.

Table 1 | Deaths at the different age groups

Age	Deaths (n)
0 to 6 weeks	46
>6 weeks to ≤ 4 months	46
>4 months to ≤ 8 months	47
8 months ≤ 12 months	24

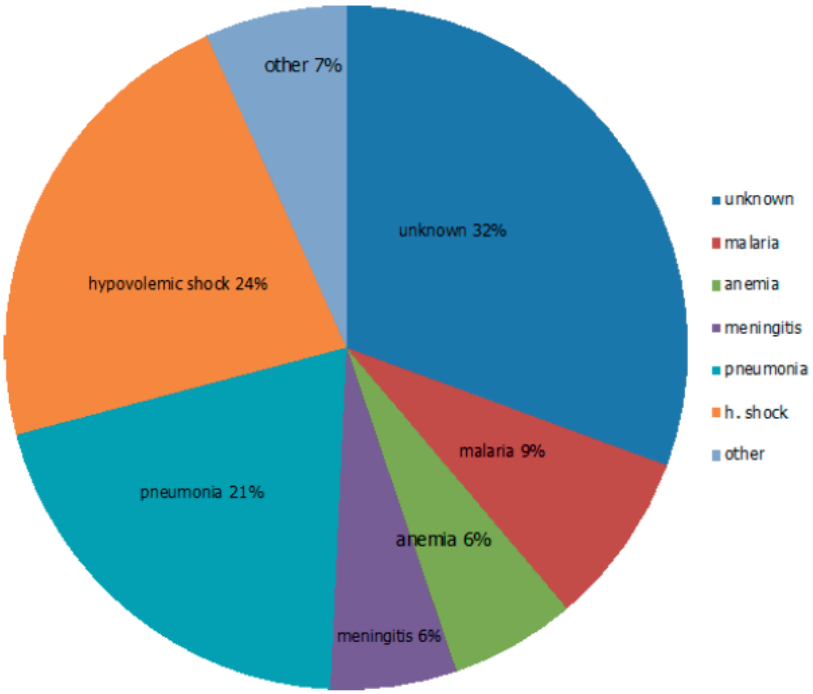

Figure 2a | Immediate Causes of Death n=88/117

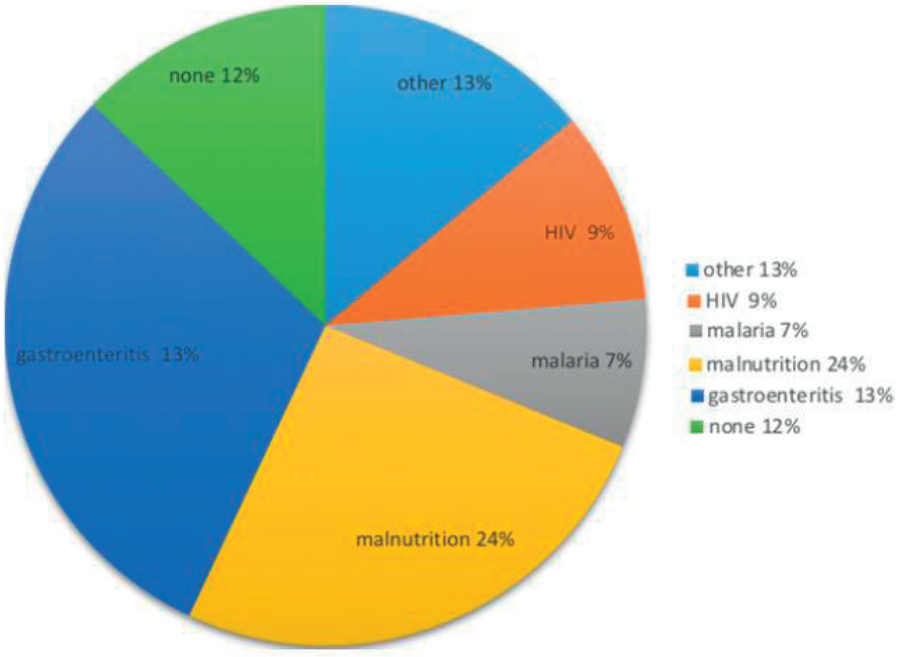

Figure 2b | Underlying Causes of Death n=110/117

Regarding the underlying causes of death, 110/117 (94%) had information derived from clinical notes on co-morbidities which were underlying causes of death. About half of the underlying causes of death were attributed to undernutrition 24/110 (22%), diarrheal disease 17/110 (16%) and anaemia 13/110 (12%). Other frequent underlying causes were infant HIV infection and malaria (Figure 2b).

In the univariate analysis of predictors of mortality, infants born in health facilities had lower hazards of mortality, compared to home deliveries (Hazard Ratio (HR) 0.53; 95% CI: 0.35, 0.82). This was also observed in the multivariate analysis; adjusted Hazard Ratio (aHR) 0.54; 95% CI: 0.34, 0.84). Mothers who had secondary education and above were three times more likely to deliver in a health facility. OR 3.02 (95% CI: 2.38, 3.84). A high proportion of HIV positive mothers (16%) transmitted the virus to their infants and tens of these infants 26/71 (36%) of the infants tested at six weeks, died before HIV results could be disclosed. Infants who were HIV infected were ten times more likely to die than uninfected infants; adjusted Hazard Ratio (aHR) 10.3; 95% CI: 6.40, 16.7). HIV uninfected infants born to HIV infected mothers were twice as likely to die compared with their counterparts born to uninfected mothers; adjusted Hazard Ratio (aHR) 1.72; HR 95% CI: 1.03, 2.90). All these effects were consistently observed in the adjusted and unadjusted models (Table 2). Infants whose weight for age category was one standard deviation or more from median (WHO growth charts 2009), had four times the rates of mortality as peers who had normal weight for age Z score. (HR 4.02; 95% CI (1.28, 12.6). However, weight for age Z score was not a statistically significant predictor in the adjusted model; adjusted Hazard Ratio (aHR) (2.81; 95% CI: 0.88, 8.96).

Maternal occupation and education and infant gender were not shown to be associated with infant survival. However, mothers with secondary education and above were 32% less likely to attend sick visits at the clinic OR (0.68 95%CI: 0.50, 0.94), and their infants were 30% less likely to be hospitalized OR (0.70 95% CI: 0.55, 0.90). Maternal occupation did not significantly impact the choice of home or health facility birth, sick visits nor hospitalisations.

DISCUSSION

The post-neonatal mortality ratio in the study area was 46 (95% CI: 39, 56) per 1000 live births and is comparable to estimates by the Health and Demographic Surveillance System in the general study area at the time of the study (52/1000 live births) [20]. Our analysis included infants who survived to six weeks of age, rather than to four weeks, hence the lower mortality ratio may be an underestimate. The post-neonatal mortality rate (58; 95% CI: 48, 69) per 1000 pyo is higher than a similar study in Eastern Uganda, which found 40 per 1000 pyo [21], Eastern Uganda has a less intense malarial transmission [19, 22] and antenatal HIV prevalence rates are less than half of those in Western Kenya [23].

After controlling for gender, maternal education and occupation, health facility births were significantly protective against mortality and infant and maternal HIV infection was associated with increased hazards of mortality.

Table 2 | Predictors of one year post-neonatal mortality

Variable	Total number of infants	Number of deaths	Person years of follow up	Mortality rate / 1000 pyrs	HR (95% CI)	Adjusted HR (95% CI)
Total	2528	117	2020	58		N=2440
Gender						
Male	1320	66	1037	64	1*	1*
Female	1224	51	983	52	0.82 (0.57, 1.18)	0.87 (0.60, 1.27)
Place of Birth						
Home	1636	91	1308	70	1*	1*
Health Facility	876	26	699	37	0.53 (0.35, 0.82)	0.54 (0.34,0.84)
Missing	16	0				
Mother's occupation						
Not working	1408	67	1110	60	1*	1*
Working	1079	48	878		0.91 (0.63, 1.32)	0.99 (0.68, 1.44)
Unknown	41	2		55		
HIV status						
Mother HIV negative/Infant HIV negative	2101	75	1681	43	1*	1*
Mother HIV positive/infant HIV negative	316	19	260	73	1.70 (1.02, 2.81)	1.72 (1.03, 2.90)
Infant HIV positive	71	23	46	506	11.1 (6.93, 17.7)	10.3 (6.40, 16.7)
Unknown	40	0				
Mother's Education						
None/Primary	2174	105	1748	60	1 (ref)	1*
≥ Secondary	326	10	251	40	0.66 (0.34, 1.25)	0.88 (0.45, 1.69)
Missing	28	2		40		
Weight for age at 6 weeks						
None to -1SD	2509	114	2007	57	1*	1*
Moderate-Severe (≥-2SD)	19	3	13	230	4.02 (1.28, 12.64)	2.81 (0.88, 8.96)

* 1 = set as the reference

Unlike previous surveys, we did not find maternal occupation or education to significantly predict mortality. Infants of mothers with secondary education attended fewer sick visits and had less frequent hospitalisations, therefore, the inability to demonstrate significant mortality benefit could be due to the small numbers of these women in the study. We found factors of morbidity such as HIV status, health access and utilization (health facility births) to predict mortality at one year of age. This could be due to the fact that the study population was relatively homogenous with respect to these socio-demographic indices. Alternatively, maternal education and occupation might be intermediate factors of mortality that drive health facility use and are less significant in the presence of more direct measures.

HIV infection

Infant HIV infection was negatively associated with survival. A high proportion of HIV positive mothers, (16%), in line with a contemporaneous national survey [24] transmitted the virus to their infants and tens of these infants, and (36%) of the 71 HIV infected babies, died before HIV results could be disclosed, implying that interventions after transmission has occurred appear to have limited influence on mortality. Fortunately, it appears several interventions to reduce Mother To Child Transmission (MTCT) are reversing the devastating trend of transmission and HIV mortality [25, 26].

Maternal HIV infection

More recently, these declines in MTCT have turned the focus to HIV exposed uninfected (HEU) infants who tested HIV negative but were born to HIV infected mothers who have been shown to have higher respiratory and diarrheal disease morbidity, more frequent hospitalisations, impaired immunity and growth [27, 28]. Our study tested infants at six weeks, upon suspicion of tuberculosis, and at study close out. Mothers were encouraged and supported to initiate ART, and safe feeding practices. We were able to show HEUs had higher hazards of mortality at one year, compared to HIV uninfected peers born to HIV negative mothers despite these measures. Similar outcomes have been observed in smaller studies in Malawi, Uganda [29-31]. Additional effects may be mediated by catastrophic health costs incurred in treatment of opportunistic infections which interferes with food security and health care access [32]. A sick mother is also unlikely to optimally care for her baby.

Having shown these infants to have increased hazards of mortality, assuring maternal health by motivating early initiation into ART and continued compliance could provide the most unified approach in reducing their vulnerability.

Health facility utilization

Health facility deliveries have been known to be protective against perinatal mortality [33, 34]. We could not find studies showing medium to long term effects of health facility deliveries. Our study showed that one year mortality was significantly lower for infants born in health facilities compared to those born at home. Mothers who gave birth in health facilities may have been more likely to use facilities in the future for immunization or seeking care for illnesses, leading to a longer term mortality benefit.

Causes of death

Similar to verbal autopsy reviews of under-five mortality during a similar time period in the same study area [35], the majority (60%) of identifiable causes of death were due to severe dehydration following gastro-enteritis, pneumonia, malaria and anaemia. There were slight methodological differences between that study and ours due to availability of more detailed diagnostic and clinical information to our study team. Under-nutrition was identified as underlying cause of death in a third of mortalities. Any degree of under-nutrition is known to increase mortality risk [3]. It is frequently underreported as a cause of death, and therefore this proportion may even be higher. Under-nutrition is known to be a

potentiating cause of death in up to 53% of deaths in countries in the developing world [36]. In addition to contributing to excess morbidity, hospitalization and mortality, under-nutrition has irreversible long term effects on cognitive development, individual earnings and economic growth [37].

Strengths and Limitations

Correlates of infant mortality are frequently predicted from surveys and ecological studies [38, 39]. Cohort studies are infrequently done. We accessed and followed up a large cohort of infants. Another strength is that the study recruited the large majority (82%) of the neonates born at that time in the study area. Therefore, we consider the findings largely generalizable to similar settings in Sub-Saharan Africa. The study has several limitations. By design, very low and extremely low birth weight infants were excluded, possibly under-estimating mortality. We experienced loss to follow up (LTFU) of 21%). More than half of this occurred at or before six weeks. This is cultural, closer to delivery pregnant women in the study area migrate to be closer to female relatives in proximity and return to their homes thereafter. LTFU thereafter was 10%, we therefore consider that this did not significantly impact our estimates, as our post-neonatal mortality rate is in line with separate surveys of the study area at the same time. Nevertheless it is plausible that exclusion of the early infancy period, we inadvertently selected for those with lower risk of mortality. Further, those who were lost to follow up could have different health seeking behavior and therefore higher mortality risk. This is unlikely to have shifted the results as it was less than 10% for the period under consideration.

CONCLUSIONS

We have shown that infant HIV infection and exposure significantly increased mortality among infants and that factors of health utilization such as health facility births reduced the hazards of mortality. Since then the infant mortality rate in the study area decreased from 52 to 35 deaths per 1000 [40]. There is a decline in most of the childhood death indicators in Kenya and the East African Region, possibly due to introduction of pneumococcal vaccination in 2011, a leading cause of morbidity and mortality in low income countries with high infant mortality [1, 41, 42], as well as scale up of ART for infected persons. Our study shows other areas where gains in survival can be improved, such as facilitating health facility use and access and scaling up ART among persons living with HIV. Ultimately, sustaining and improving on gains in child survival in developing countries will call for societal change [43] to address global and national economic inequities.

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Chapter 4

Chest radiographs for paediatric TB diagnosis: Inter-rater agreement and utility

Grace Kaguthi, Videlis Nduba, Jocelyn Nyokabi
Franklin Onchiri, Robert Gie, Martien W. Borgdorff

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Introduction: The chest radiograph (CXR) is considered a key diagnostic tool for paediatric Tuberculosis (TB) in clinical management and endpoint determination in TB vaccine trials.

Methods: We set out to compare inter-rater agreement for TB diagnosis in western Kenya. A paediatric pulmonologist and radiologist (experts), a medical officer (M.O) and four clinical officers (C.Os) with basic training in paediatric CXR reading, blindly assessed CXRs of infants who were TB suspects in a cohort study. C.Os had access to clinical findings for patient management. Weighted kappa scores summarized inter-rater agreement on lymphadenopathy and abnormalities consistent with TB. Sensitivity and specificity of raters was determined using microbiologically confirmed TB as the gold standard (n=8).

Results: A total of 691 radiographs were reviewed. Agreement on abnormalities consistent with TB was poor; k=0.14; (95%CI: 0.10 - 0.18), and on lymphadenopathy moderate k=0.26 (95%CI: 0.18 - 0.36), M.O [75% (95 %CI: 34.9%-96.8%)] and C.Os [63% (95%CI: 24.5%-91.5%)] had high sensitivity for culture confirmed TB.

Conclusion: TB vaccine trials utilizing expert agreement on CXR as a non-microbiologically confirmed endpoint will have reduced specificity and underestimate vaccine efficacy. C.Os detected many of the bacteriologically confirmed cases, however this must be interpreted cautiously as they were unblinded to clinical features.

INTRODUCTION

Paediatric TB remains a challenging disease to diagnose despite advances in molecular techniques in mycobacterial identification and antigen based tests for latent TB infection(1). Classical TB symptoms are nonspecific [2] and more so in settings with high HIV prevalence and malnutrition. Atypical presentation with acute severe pneumonia in young children has been observed [3]. Childhood TB is characterized by paucibacillary disease and microbiological confirmation is only possible in <50% of paediatric cases [1]. Chest imaging is therefore of great importance in identifying smear negative, culture negative TB. Among adults with suspected TB, several clearly defined chest radiograph features have been identified as having high inter rater reliability and correlation with culture positive TB [4] Unfortunately similar data among infants has been limited. Lymphadenopathy is the hallmark of primary TB [5]. However, it is frequently missed due to inadequate sensitivity of the chest radiograph [6]. Chest CT Scan (CT) has been considered the gold standard for detecting mediastinal lymphadenopathy, detecting up to 60% more lymphadenopathy in children with normal chest radiographs [7]. Despite this, use of CT has been limited in infant TB vaccine trials, which are set up to detect every TB endpoint so as to demonstrate efficacy. Reasons include; the modest agreement on lymphadenopathy on CT [7], cost limitations and the reluctance to use high dose ionizing radiation in young children. Thus, the chest radiograph is the mainstay of radiological diagnosis, and is frequently the only tool available.

There are a limited number of studies that have described inter rater agreement on chest radiograph for TB diagnosis [8, 9]. Existing studies had small sample sizes, were drawn from hospitalized children, compared agreement only among experienced and highly trained raters, and most importantly, they focused entirely on presence of lymphadenopathy as a marker of TB. While lymphadenopathy is a key feature to diagnosing childhood TB, other radiological features also contribute to the diagnosis [5, 6]

We conducted the study in Siaya County, Western Kenya, which has a high burden of both tuberculosis and HIV [10, 11]. The objective was to determine inter rater agreement on any abnormality on chest radiograph, and agreement on abnormalities consistent with TB among experienced and inexperienced raters. We also aimed to compare the raters' sensitivity and specificity against microbiologically confirmed TB in young children.

STUDY POPULATION AND METHODS

Study setting

The study was conducted in Siaya County, Western Kenya from June 2009 to December 2011. TB diagnosis is by the Keith Edward Score Chart [12] which assigns a score to suggestive pulmonary and extra-pulmonary signs/symptoms of TB. Children who score ≥7 as well as those who score <7 but have an abnormal chest radiograph are treated for TB. A total of 2900 BCG vaccinated infants, aged zero to six weeks and weighing at least 1700g were enrolled and followed up for 12-24 months to determine TB Incidence. TB suspects were identified through four monthly scheduled visits, sick visits as well as

by review of TB case records for contact tracing. Suspect criteria included, a history of contact and/or suggestive signs and symptoms of TB, and/or protocol defined hospitalization history for example for severe pneumonia.

Clinical and Laboratory Investigations

TB suspects were admitted into a case verification ward (CVW) for collection of two serial induced sputa specimens, two serial early morning gastric aspirates, DNA PCR HIV (HIV QUAL test 48 Roche Molecular Systems Inc, Switzerland) testing and Rapid HIV tests for those aged less than and greater than 18 months respectively. Tuberculin Skin Testing was also done. Digital antero-posterior (AP) and lateral chest radiographs were taken at admission and images written into CD-ROMs. The CD-ROMs had Digital Imaging and Communication in Medicine (DICOM) software (Phillips) that was used to view images. Readers could optimize the light/greyscale settings, as well as the magnification.

Definitions and Classifications

Microbiologically confirmed TB (Definite TB) was *M. tuberculosis* identified by Xpert MTB/RIF or speciated with either Capilia (FIND and Tauns co. Ltd) or GenoType assay (Hain Diagnostika, Nehren, Germany) after positive sputum culture. Probable TB was a case started on anti-TB treatment based on Keith Edward Score Chart and/or a CXR consistent with TB.

Raters and Training

There were four sets of raters: A Radiologist and a Paediatric Pulmonologist (expert readers), a Medical Officer (M.O) and four Clinical Officers (C.Os). The C.Os reviewed the images while the suspects were admitted to the CVW, all other raters read the images after close of the study and were blinded to the clinical history. All raters were trained on using the electronic interface to enter readings. The Clinical and Medical Officer(s) were trained on reading and identifying TB on paediatric radiographs prior to the start of the study. A chest radiograph reading form developed during a consensus meeting of TB vaccine sites (Cape Town December 2008) was used. An electronic version was developed to standardize reporting and minimize ambivalence in the diagnosis. The technical quality of images was assessed prior to reading. Indicators included: adequacy of collimation (visibility of the lung apices, costophrenic angles with nothing obscuring the lung fields), adequate exposure; the number of visible intervertebral spaces; adequate inspiration, by counting six anterior ribs, absence of rotation by comparing the length of the same rib on the left and right hemidiaphragms. Thereafter, the reader classified the chest radiograph quality as optimal or suboptimal or unreadable. 'Unreadable' radiographs were counted as suboptimal but were still read. Raters then systematically reviewed the images for airway narrowing, left tracheal deviation, lymphadenopathy, airway opacities, calcification and pleural effusion. The pathology items were scored individually as present, absent, and equivocal. Final assessment of the image was; normal radiograph; abnormal TB unlikely; abnormal TB likely. Radiological signs suggestive of TB were: miliary picture, airway narrowing or tracheal deviation to the left, presence of hilar, paratracheal or subcarinal or other lymphadenopathy, evidence of calcification, cavitation, pleural effusion or thickening.

Data collection and analysis

Data quality was assured by edit, logic and validation checks built onto the data entry interface. Data cleaning was also conducted, and an audit trail of changes maintained. Data was saved onto an SQL database and analysed using SAS 9.0 (SAS Institute Inc, Cary NC, USA). To quantify degree of agreement in TB diagnosis among the raters, we estimated the individual kappas for each rating category as well as a generalized (multiple rater) chance-corrected kappa statistic (a multi-rater measure of agreement), which is an extension of Cohen's kappa for assessing reliability or proportion of agreement for multiple raters. The overall/generalized kappa measures agreement across all categories.

Kappa scores were interpreted as follows: Poor 0.01-0.20, moderate 0.21-0.40, fair 0.41-0.60, good 0.61-0.80 or excellent 0.81-1.0 (13). We compared agreement on any abnormality on chest radiograph, as well as agreement on abnormalities consistent with TB. For comparability with previously conducted paediatric chest radiographs studies we examined the agreement on lymphadenopathy on AP and lateral images. Sensitivity and specificity of readers' diagnosis to definite TB was also calculated. To examine each rater's propensity to place a radiograph in a certain category, and thus elucidate patterns of agreement, McNemar Test for 2X2 tables, and Bowker's Test of symmetry were applied for tables with more than two categories. These methods compare marginal frequencies of each rater and test for statistically significant differences (Tests of marginal homogeneity). Where $p<0.05$, marginal heterogeneity exists that is, differing propensity to rate a category.

The study was reviewed and approved by the Kenya Medical Research Institute Ethics Review Committee, (KEMRI-ERC) SSC 1465. Written Informed consent was obtained from parents and Guardians before study entry and for CVW investigations.

RESULTS

TB suspects comprised 33% of those enrolled (959/2900). Of these, 767 (80%) consented to CVW admission for additional investigations and 837 chest radiographs were taken (Figure 1).

There were similar numbers of male (51.5%) and female (49.5%) suspects. Some of the prevalent co-morbid conditions among suspects included HIV exposure- nearly one-fifth (17.3%), HIV infection (4.4%) and malnutrition (46.0%). Close to half of investigated infants 355/767 (46.3%) had been hospitalized with severe lower respiratory tract infections such as bronchiolitis and pneumonia. Prevalence of acute and chronic malnutrition was as follows; wasting (WHZ < -2) n=167/767 (21.7%) stunting (HAZ <-2) 82/767 (10.2%) and underweight (WAZ <-2)104/767 (13.6%).

Agreement on CXR reading was low overall. For abnormalities consistent with TB (Table 1a and 1b), poor to moderate agreement was observed across all rater pairs: pulmonologist-radiologist (k=0.24 (95% CI: 0.15-0.34)); pulmonologist-medical officer (k=0.21(95% CI: 0.13-0.29)) and radiologist-medical officer k=0.18 (95% CI: 0.10-0.26).

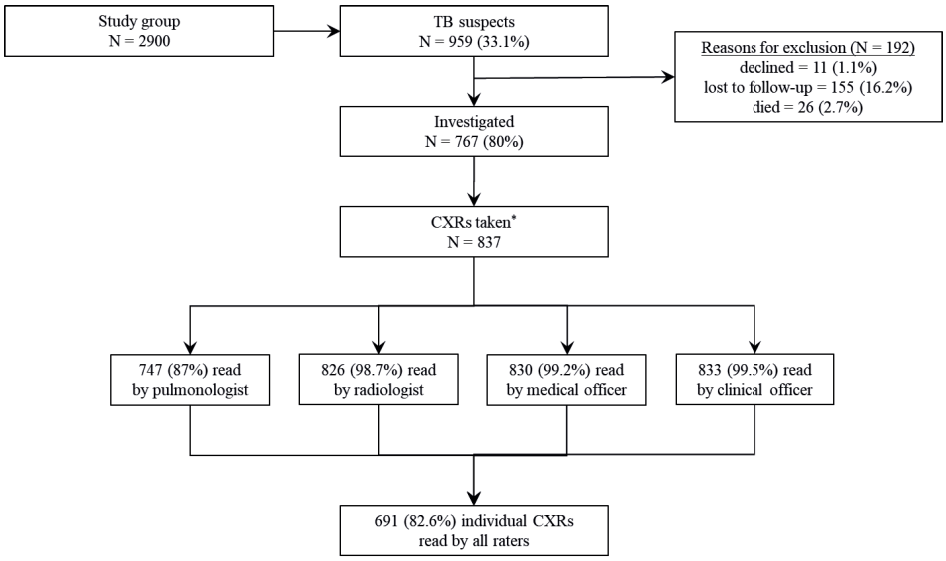

Figure 1 | Profile of presumptive TB cases and chest radiographs read per rater
* per patient more than one CXR could have been taken

Table 1a | Inter-rater agreement on abnormalities consistent with TB

Outcome	**Kappa (95% CI)**	***P* value**
TB Classification Category		
Abnormal	0.177 (0.124 - 0.237)	<0.001
Abnormal likely TB	0.193 (0.095 – 0.330)	<0.001
Abnormal unlikely TB	0.065 (0.026 – 0.125)	<0.001
Generalized/Weighted Kappa	0.136 (0.100 - 0.176)	<0.001

On any abnormality on the radiograph, agreement was moderate between expert readers [k=0.28 (95% CI: 0.19-0.37)] and between expert readers and M.O. [Pulmonologist vs medical officer (k=0.23 (95% CI: 0.15-0.31)] and [Radiologist vs Medical Officer (k= 0.22 (95% CI: 0.14-0.30)] respectively. (Table 3). Poor- agreement was registered across all other rater pairs. There was similar propensity to rate categories between the expert pair (p=0.14) and between the radiologist and the clinical officer (p=0.24). Regarding quality of radiographs, little or no agreement was registered across all rater pairs. The best kappa score was 0.07 (0.03-0.10), observed between the expert pair. (Table 4). There were eight definite and 40 probable TB cases in the study. One radiograph of a definite TB case was not reviewed by all four raters. The sensitivity and specificity of raters (Table 5) was determined using definite TB cases as the gold standard (n=8).

Table 1b | Inter-rater agreement on abnormalities consistent with TB

		RADIOLOGIST									
		Normal			Abnormal likely TB			Abnormal unlikely TB			
		PULMONOLOGIST									
Clinical Officer (C.O)	Medical Officer (M.O)	Normal	Abnormal likely TB	Abnormal unlikely TB	Normal	Abnormal likely TB	Abnormal unlikely TB	Normal	Abnormal likely TB	Abnormal unlikely TB	Total
Normal	Normal	345	5	30	12	0	3	18	0	4	417
	Abnormal likely TB	5	1	3	1	0	1	0	0	2	13
	Abnormal unlikely TB	83	1	20	3	1	5	10	1	6	130
Abnormal likely TB	Normal	12	1	1	0	0	1	0	0	0	15
	Abnormal likely TB	1	0	0	1	3	0	0	1	0	6
	Abnormal unlikely TB	4	1	0	1	1	1	2	0	0	10
Abnormal unlikely TB	Normal	51	1	4	3	0	2	4	2	1	68
	Abnormal likely TB	4	1	0	1	1	2	0	0	0	9
	Abnormal unlikely TB	10	0	2	2	1	1	4	2	1	23
Total		515	11	60	24	7	16	38	6	14	691

Agreement among all raters on the diagnosis of lymphadenopathy was moderate, with a multi-rater weighted k=0.26 (Table 2).

Table 2 | Overall agreement amongst all raters on lymphadenopathy and quality of CXR

Rating	**Kappa (95% CI)**	***P* value**
Lymphadenopathy		
Present	0.27 (0.129–0.448)	<0.001
Absent	0.29 (0.163–0.430)	<0.001
Equivocal	0.17 (0.044–0.423)	<0.001
Overall weighted kappa	0.26 (0.182–0.355)	<0.001
Quality of radiographs		
Optimal	–0.1324	0.999
Suboptimal	–0.1674	0.999
Unreadable	–0.0184	0.8822
Overall weighted kappa	–0.1452	0.998

Table 3 | Agreement on any abnormality on chest radiograph (+) abnormal/ (-) normal

N = 691						
Reader	**-/-**	**-/+**	**+/+**	**+/-**	**Kappa**	**McNemar's Test**
radiologist vs pulmonologist	515	62	43	71	0.28 (0.19-0.37)	0.44
pulmonologist vs M.O	445	132	59	55	0.23 (0.15-0.31)	<0.0001
radiologist vs M.O	450	136	55	50	0.22 (0.14-0.30)	<0.0001
pulmonologist vs CO	477	100	31	83	0.10 (0.01-0.18)	0.21
radiologist vs C.O	493	93	38	67	0.18 (0.10-0.27)	0.04
C.O vs M.O	417	143	48	83	0.10 (0.02-0.17)	<0.0001

Table 4 | Inter-rater agreement on quality of chest radiograph

N = 633						
Reader	Optimal-Optimal	Optimal-Suboptimal	Suboptimal-Suboptimal	Suboptimal-Optimal	**Kappa**	**McNemar's Test**
radiologist vs pulmonologist	29	295	301	7	0.07 (0.03-0.10)	<0.0001
pulmonologist vs M.O	10	37	271	314	-0.09 (-0.13- -0.05)	<0.0001
radiologist vs M.O	2	45	551	34	-0.02 (-0.09- 0.05)	0.22
pulmonologist vs CO	7	5	303	317	0.005 (-0.02-0.03)	<0.0001
radiologist vs C.O	2	12	586	34	0.06 (-0.05-0.16)	0.0003
C.O vs M.O	0	12	573	47	-0.03 (-0.05- -0.02)	<0.0001

Table 5 | Accuracy of TB diagnosis by the various care providers – based on culture confirmed TB

	Culture results					
CXR reader	**Positive N = 8**	**Negative N = 683**	**Sensitivity (95%CI)**	**Specificity (95%CI)**	**PPV***	**NPV***
Clinical officer						
Positive	5	126	62.5% (24.5% - 91.5%)	81.6% (78.4% - 84.4%)	3.8% (1.41%-9.13%)	99.5% (98.3%-99.9%)
Negative	3	557				
Medical officer						
Positive	6	185	75.0% (34.9% - 96.8%)	72.9% (69.4%-76.2%)	3.14% (1.28%-7.03%)	99.6% (98.4%-99.9%)
Negative	2	498				
Radiologist						
Positive	4	101	50.0% (15.7%-84.3%)	85.2% (82.3% -87.8%)	3.96% (1.23%-10.0%)	99.3% (98.1-99.8%)
Negative	4	582				
Pulmonologist						
Positive	4	110	50.0% (15.7%-84.3%)	83.9% (80.9%- 86.6%)	3.5% (1.13%-9.27%)	99.5% (98.1%-99.8%)
Negative	4	573				

*PPV = positive predictive value, NPV = negative predictive value - disease incidence 1.12% (0.54-2.36).

The clinical and medical officer(s) detected the largest proportion of definite TB, while specificity was highest for expert raters. Due to the small number of definite cases, sensitivity was imprecisely measured. The same table shows low positive predictive values (PPV) (<4.0% for all raters) and high negative predictive values (>99.0% for all raters) for the chest radiograph of 28/40 (70%) probable TB cases whose chest radiographs were read by all raters, experts agreed on only two as being consistent with TB. Such would be the stringent case definition applied in infant TB vaccine trials [14] where only radiographs in which experts agreed (Figure 2) would be admissible as non-microbiologically confirmed TB cases.

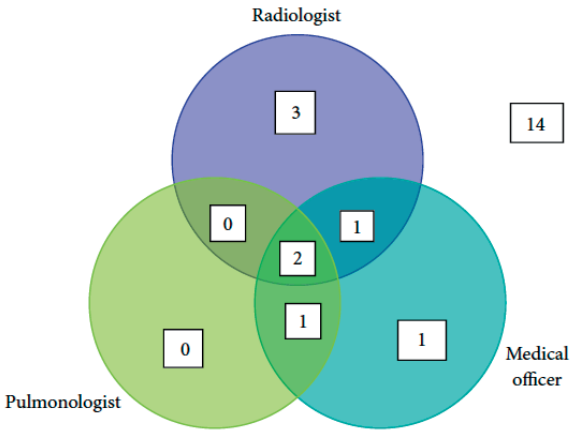

Figure 2 | Venn diagram showing radiographs of probable TB cases reviewed by all raters (n=28) and classified as 'abnormal likely TB'.

DISCUSSION

Overall, we observed poor to moderate agreement between experts and between expert, non-expert pairs. This was consistent across the rater's summary opinion of radiograph:-as well as on specific pathology such as lymphadenopathy. Our findings fit in with other studies comparing inter-rater agreement on lymphadenopathy for TB diagnosis [9]. Additionally, we demonstrate that agreement is also poor for the composite assessment of the radiograph, beyond individual radiographic abnormalities. This undermines the reliability of the chest radiograph for TB diagnosis in infant vaccine trials, as well as possibly for clinical diagnosis and patient care. We suggest several contributing factors. In an infant cohort study with active case finding, suspects are likely to be picked up and investigated before advanced disease with well-defined radiographic features sets in. Early tuberculosis is likely associated with greater diagnostic uncertainty and risk of misclassification [15]. Inter rater agreement studies in a clinical setting where TB suspects may present with more advanced disease are needed to confirm this.

A study of two large infant cohorts in South Africa showed frequent discordance between radiological and microbiological features of TB [2, 16]. The absence of clear, defined radiological abnormalities

that correlate well with microbiologically confirmed disease contributes to lack of a reproducible, standardized criteria that raters can use with certainty to evaluate radiographs.

In radiographs of young children, mediastinal abnormalities are difficult to assess and interpret [17] particularly in inexperienced readers; in this case the C.O and M.O. However, even experienced readers have been found to have poor agreement on lymphadenopathy on chest radiographs [9].

Previous studies had smaller sample sizes for assessment of inter rater variability[8, 9] One of the strengths of this study is the inclusion of a large sample of radiographs for evaluation, from young children with a broad range of respiratory illness and co morbidities. The varying levels of raters' expertise represents the general clinical care structure from primary level to specialized referral hospitals. The findings are therefore applicable to a broad range of settings.

The study had limitations. It was not possible to obtain consensus from expert readers on radiographs on which they differed. This would have increased agreement scores and validity of the chest radiograph as a diagnostic tool. It would also have elucidated patterns of disagreement, in order to refine criteria for identifying pathologies consistent with TB on chest radiographs as has been previously recommended [9]. Obtaining high inter rater agreement for paediatric chest radiographs in acute paediatric respiratory illness is difficult. Pneumococcal vaccine trials have succeeded in this for opacities consistent with pneumonia on paediatric chest radiographs[18] Unfortunately, it has been difficult to replicate this success in TB vaccine trials.

Conventionally, TB vaccine trial efficacy sample sizes are calculated based on composite endpoints. These include bacteriological and non-bacteriological criteria as it is expected microbiologically confirmed cases will contribute a limited number of endpoints. To increase the number of endpoints, and thus reduce sample size requirements, non-bacteriologically confirmed endpoints that rely on chest radiograph findings are included. The latter is defined as radiographic findings compatible with tuberculosis identified independently by two experts [15, 19]. This approach has some limitations. We found that experts agreed only on five of 35 radiographs, as being consistent with TB. Of these, three were bacteriologically confirmed, therefore the chest radiograph contributed only two additional endpoints. Poor agreement and high variability in interpreting paediatric CXRs for TB diagnosis among experts increases the probability of misclassifying true disease status and thus underestimating vaccine efficacy [20]. C.Os and M.Os, picked up majority of the cases, that were later bacteriologically confirmed. This would seem like a positive outcome, given that they work as primary health care providers and would therefore accelerate diagnosis and treatment of infants with TB. However, the limited number of definite cases results in imprecise estimates of sensitivity and should be cautiously interpreted. The high sensitivity also trades off on specificity and could result in unnecessary TB treatment.

Prevailing disease rates influence predictive values. Among infants, the TB incidence of 1.12% (95% CI 0.54%-2.36%) would be considered high, however low PPV relative to sensitivity is attributed to low disease rates. While the NPV was high, where prevalence is not much above 1%, even a non-informative test may have a NPV close to 100%.

CONCLUSION

Poor agreement and high variability in classifying paediatric radiographs underscores need for caution in diagnosing TB in clinical settings where bacteriological confirmation is unavailable; as in most resource limited settings. It further demonstrates that addition of radiographic, non-bacteriologically confirmed endpoints will be of low benefit in decreasing sample sizes for TB vaccine trials.

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Chapter 5

The Incidence of Non-Tuberculous Mycobacteria in infants in Kenya

Grace Kaguthi, Videlis Nduba, Wilfred Murithi, Suzanne Verver

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Introduction: There is inadequate understanding of the epidemiology of Non Tuberculous Mycobacteria (NTMs) among infants in high tuberculosis burden countries. The objective of this study was to document the incidence and diversity of NTM disease or colonisation in sputum specimens from infants with presumptive TB, the risk factors and clinical characteristics, in a high TB & HIV burden setting in Western Kenya.

Methods: A cohort of 2900 newborns was followed for 1–2 years to assess TB incidence. TB investigations included collection of induced sputa and gastric aspirates for culture and speciation by HAIN®, Tuberculin Skin Testing (TST), HIV testing and chest radiography. The American Thoracic Society Criteria (ATS) were applied to identify NTM disease.

Results: Among 927 (32% of 2900) with presumptive TB, 742 (80%) were investigated. NTMs were isolated from 19/742 (2.6%) infants. *M. fortuitum* was most frequently speciated (32%). Total person-time was 3330 years. NTM incidence was 5.7/1,000 person-years; 95% CI (3.5, 8.7). Infants diagnosed with TB were more likely to have NTM isolation (odds ratio 11.5; 95% CI 3.25, 41.0). None of the infants with NTM isolated met the criteria for NTM disease.

Conclusion: The incidence of NTM isolation was comparable to similar studies in Africa. NTM isolation did not meet ATS criteria for disease, and could represent colonisation. TB disease appears to be structural lung disease predisposing to NTM colonisation.

INTRODUCTION

Non Tuberculous Mycobacteria (NTMs) are environmental saprophytes widely distributed in water and soil (1). They are the genetic progenitors of *M. tuberculosis* Complex (MTBC), after a series of gene deletions and gene acquisitions (2) with MTBC evolving to a more virulent pathogen. NTMs rarely cause disease except when immune function is impaired (3), elderly patients and chronic lung disease. However, some NTMs are pathogenic, and recently there has been a reported increase in NTM lymphadenitis (4, 5) and Buruli ulcers (6, 7).

The shared ancestry of NTM and MTBC is responsible for immune interference in BCG vaccination, via cross reactive immune responses (2). This could be one of the reasons for low BCG efficacy where NTMs are prevalent (2). Absence of NTM sensitization was associated with higher efficacy of BCG against pulmonary and severe forms of tuberculosis in a systematic review (8). Surprisingly, the discontinuation of universal BCG vaccination in these countries has seen an increase of NTM lymphadenitis in children, suggesting BCG was also protecting against NTMs in that setting (4). NTMs appear to be immune modulators influencing host interactions in BCG efficacy, TB burden and NTM disease. The antigen homologues (2) further decrease accuracy of biomarkers distinguishing latent TB infection (LTBI) and NTM exposure.

Pulmonary NTM disease is clinically and radiologically identical to TB, and is so diagnosed, in the absence of microbiological confirmation in high TB burden settings. It is a relevant distinction to make as almost all NTMs do not respond to anti-tuberculous therapy (9). Isolation of NTM in sputum is not necessarily disease (9). Data on NTM disease and prevalent subtypes is limited particularly in countries with a high TB burden.. Most studies report on adults (10-12). Few studies on NTM in children have been published on the continent (13-15). Most document the proportion of NTM among those with presumptive TB. There is also a dearth of knowledge on risk and exposure factors. As infants are the target age group for TB vaccines in the pipeline, it is useful to describe the epidemiological landscape of NTMs, given their role in tuberculosis incidence and possibly vaccine efficacy.

The objective of this study was to document the incidence and diversity of NTM disease or colonisation in sputum specimens from infants with presumptive TB, the risk factors and clinical characteristics, in a high TB & HIV burden setting.

Study population and methods

The study took place in Siaya, Western Kenya, a predominantly rural community north of Lake Victoria. The area has a high prevalence of HIV, TB and malaria. Most women delivered at home (16). The NTM sub-study was part of a prospective cohort study to document the incidence of TB ahead of TB vaccine trials in the same population. Presumably, infants are born uninfected we present the incidence of NTM in this cohort.

Briefly, parents or guardians of 2900 infants aged zero to six weeks gave written permission for enrolment of their newborns between June 2009 and June 2010. Patients were followed up for at least one year and a maximum of two years. Through four monthly scheduled visits and ancillary care visits,

infants were identified as having presumptive TB if they had history of TB contact, symptoms or signs of pulmonary TB (failure to thrive, cough or night sweats or fever for more than two weeks, a history of hospitalization for HIV/AIDS related illness, lower respiratory tract infections, meningitis or TB). Consequently, they were admitted into a case verification ward for three days. Two fasted sputum induction specimens and two gastric aspirates were collected on subsequent mornings. Tuberculin Skin Testing (TST) was done with two Tuberculin Units (2TU) from Statens Serum Institute (SSI). TST readings of 10mm and more, or 5mm or more among HIV infected children were considered to be positive readings. Further, DNA PCR HIV (COBAS® HIV-1 Amplicor by ROCHE) tests and digital chest radiography were performed.

Patients received anti-tuberculous therapy if they had microbiological confirmation (definite TB) or clinically, based on the Keith Edward TB Score (KE Score) Chart of >7, or <7 if the chest radiograph was suggestive (probable TB). Mid upper Arm Circumference (MUAC) was used to determine nutritional status for children older than 6 months old at time of TB investigations. Weight for Age Z Score was used for those less than 6 months. HIV infected infants were referred for anti-retroviral treatment initiation and care. Patients vital status at last study contact was documented.

Chest radiographs were read systematically and classified as Abnormal probable TB, Abnormal not TB or Normal (17). The study was approved by Kenya Medical Research Institute Independent Ethics Committee (KEMRI-IEC) SSC 1465. The data used to support the findings of this study are available from the corresponding author upon request. We applied the American Thoracic Society's (18) criteria to establish clinical significance of positive NTM cultures.

Laboratory Methods & Sample Decontamination

Induced sputum and gastric aspirates were transported to the laboratory at 2 - 8^{0}C, processed using freshly prepared N-acetyl L-cysteine (NALC)-4% Sodium Hydroxide (NaOH)-2.9% Sodium citrate at a final concentration of 1%. Gastric aspirates with >5ml volume, were concentrated by centrifugation and pellet re-suspended with 5ml phosphate buffer saline (PBS). Digestion was stopped using pH 6.8 PBS after 20 minutes. Centrifugation was done at 3,000 x g for 15 minutes at 4^{0}C. Supernatant was discarded and the pellet re-suspended with 2ml PBS. This was used for inoculation of Lowenstein Jensen (LJ) [BD] media (0,2ml), fluorescent microscopy and mycobacteria growth indicator tube (MGIT) [BD] (0.5ml). LJ were incubated in 37^{0}C CO_2 incubators for 8 weeks, MGIT was incubated in automated BACTEC ™ MGIT ™ 960 [BD] for 42 days. Artificial sputum was used as a negative control sample to check for cross-contamination with each batch processed.

MGIT cultures that turned positive were stained for acid fast bacilli (AFB) using Ziehl Neelsen (ZN). Contamination was checked by inoculation and incubation of blood agar plates at 37^{0}C and read after 48 hours. Samples that tested ZN negative but Blood Agar Plate (BAP) positive ≥7 days later were discarded as contaminated. Those <7 days were re-digested using 4% NaOH as described in MGIT™ procedure manual (19). AFB positive cultures were tested by immunochromatographic assay (ICA) such as Capilia™ TB-Neo (TAUNS Laboratories, Numazu, Japan) or BD MGIT™ MTBC identification kit ((BD, Franklin Lakes, NJ, USA) to identify whether NTM or MTBC. For

LJ cultures with visible growth, we assessed colony morphology. Those suggestive of mycobacteria were identified using ZN smear, those AFB positive were tested with ICA. NTM culture isolates were genetically identified to the species level using Genotype Mycobacterium Common Mycobacterium (CM) or Additional Species (AS) kits (HAIN Lifescience, Nehren, Germany). The procedure was done according to manufacturer's instructions.

Statistical Methods

Frequency methods were used to describe the baseline characteristics. Odds ratios were used to analyze whether differences between those with and without NTM were due to chance. T-tests was used to compare the mean age at TB investigations. To evaluate differences in clinical characteristics, known and potential risk factors, logistic regression was performed. NTM cases that had microbiologically confirmed or clinical TB were analysed as TB cases. A-priori risk factors included infant and maternal HIV infection, nutritional status, housing and number of siblings.

RESULTS

Of 2900 infants enrolled, 927 (32%) were suspected to have TB (presumptive TB) during their 1-2 year follow-up. Of these 742 (80%) were admitted for investigations (Figure 1).

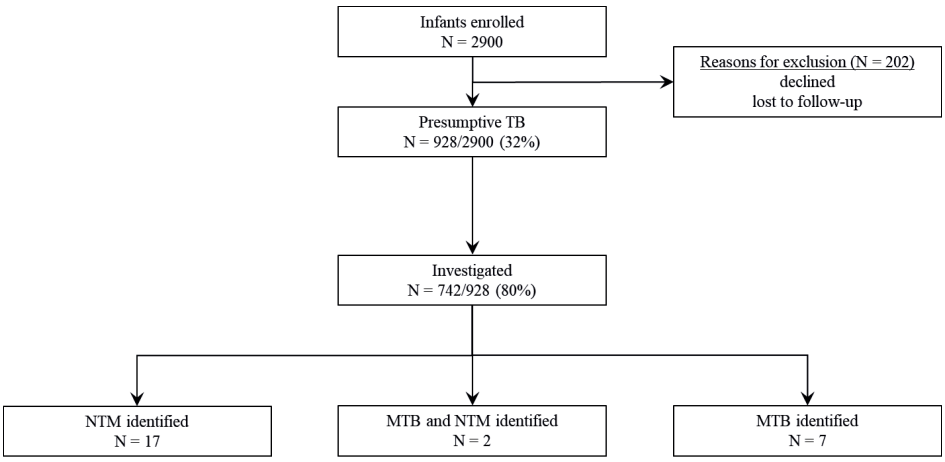

Figure 1 | Study flow chart

There were 19 NTMs identified following culture (2.6% of 742). Total person-time of follow-up was 3330.3 years. The incidence of NTM was 5.7 per 1,000 person years (pyo) of follow up (95% CI 3.5, 8.7), while all TB incidence (49 cases) was 15/1000 pyo (95% CI:11-20) and microbiologically confirmed TB incidence was 2.7/1,000 pyo. At baseline there were no statistically significant differences between those who had NTMs identified versus all other infants (Table 1).

Table 1 | Baseline characteristics of infants with NTM isolated

	Study sample (N = 2900)	**Investigated for TB** (N = 742) (N, column %)	**NTM positive** (N = 19) (N, row %)	**OR** (95%CI) **
Gender				
female	1412	358 (48%)	10 (2.8%)	1*
male	1488	384 (52%)	9 (2.3%)	0.85 (0.35, 2.11)
Enrolment weight				
normal	2674	667 (90%)	16 (2.4%)	1*
low	226	75 (10%)	3 (4.1%)	2.24 (0.65, 7.73)
Place of birth				
health facility	1038	229 (31%)	8 (3.5%)	1*
home	1840	510 (69%)	11 (2.2%)	0.77 (0.31, 1.93)
missing	22	3 (<1%)		
Maternal HIV status				
negative	2451	598 (81%)	16 (2.7%)	1*
positive	401	127 (17%)	3 (2.4%)	0.88 (0.25, 3.08)
unknown	48	17 (2%)		
Infant HIV status				
negative	2827	708 (95%)	18 (2.5%)	1*
positive	73	34 (5%)	1 (2.9%)	2.17 (0.29, 16.5)
Maternal age category				
<19	635	152 (21%)	1 (0.7%)	1*
20 – 29	1533	384 (52%)	16 (4.2%)	6.69 (0.89, 50.5)
>29	732	206 (28%)	2 (1.0%)	1.74 (0.16, 19.2)
Maternal Occupation				
unemployed	1676	409 (55%)	11 (2.7%)	1*
farmer	864	250 (34%)	5 (2.0%)	0.88 (0.31, 2.54)
business	260	61 (8%)	2 (3.3%)	1.17 (0.26, 5.32)
salaried	71	13 (2%)	1 (7.7%)	2.16 (0.28, 17.0)
unknown	29	9 (1%)		
Housing type				
mud house	1912	523 (71%)	11 (2.1%)	1*
semi-permanent	527	125 (17%)	4 (3.2%)	1.32 (0.42, 4.17)
permanent	426	84 (11%)	4 (4.8%)	1.64 (0.52, 5.17)
other	6	1 (0.1%)		
unknown	29	9 (1.2%)		
Number of siblings				
none	649	129 (17%)	3 (2.3%)	1*
one to three	1497	391 (53%)	14 (3.6%)	2.03 (0.58, 7.10)
>3	754	222 (30%)	2 (0.9%)	0.57 (0.10, 3.44)
Vaccination status at 6 weeks				
incomplete	133	29 (4%)	2(7.0%)	1*
complete	2205	682 (92%)	16(2.4%)	0.48 (0.11, 2.10)
unknown	562	31 (4%)	1(3.2%)	

* 1 = set as the reference

** Odds of being NTM case among those investigated for presumptive TB.

Table 2a | Clinical characteristics of infants investigated for TB and with NTM isolated

Clinical Characteristics	**Investigated for TB** (N = 742) (N, column %)	**NTM positive** (N = 19) (N, row %)	**OR** (95%CI) **
TB case (clinical / confirmed)			
no	694 (94%)	16 (2.3%)	1*
yes	48 (6.5%)	3 (6.3%)	11.6 (3.25, 41.0)
MTB culture positive			
no	733 (99%)	17 (2.3%)	1*
yes	9 (1%)	2 (22.2%)	48.3 (9.34, 249)
Chest radiograph			
normal	590 (80%)	13 (2.2%)	1*
abnormal – not TB	110 (15%)	4 (3.6%)	1.71 (0.55, 5.35)
TB	35 (5.0%)	2 (5.7%)	2.80 (0.61, 12.9)
missing	7 (0.9%)		
Keith Edward TB score			
< 7	675 (90%)	17 (2.5%)	1*
≥ 7	32 (4.3%)	2 (6.3%)	2.62 (0.58, 11.9)
Missing	35 (4.7%)		
History of hospitalization			
no	283 (38%)	8 (3.4%)	1*
yes	426 (57%)	11 (2.6%)	0.78 (0.31, 1.97)
missing	33 (5.0%)	8 (3.4%)	
TB contact history			
no	579 (78%)	13 (2.3%)	1*
yes	131 (18%)	6 (4.6%)	2.57 (0.96, 6.88)
missing	32 (4.0%)	13 (2.3%)	
TST result			
negative	555 (76%)	14 (74%)	1*
positive	172 (24%)	5 (26%)	1.15 (0.41, 3.25)

* 1 = set as the reference

** Odds of being NTM case among those investigated for presumptive TB

Upon bivariate comparison of clinical characteristics between presumptive TB patients and NTM cases, there were no statistically significant differences (Table 2a).

However, odds of a positive NTM among infants with TB was eleven fold that of infants with no TB (OR 11.6 (95% CI 3.25, 41.0). NTM cases had forty eight fold higher odds of having microbiologically confirmed TB compared to all presumptive TB (OR 48.3 95% CI 9.3, 249) (Table 2a). There were no differences between NTM cases and other presumptive TB cases in mean age at time of TB investigations (Table 2b).

Table 2a | Clinical characteristics of infants investigated for TB and with NTM isolated

	Investigated for TB (N = 742) (N, column %)	**NTM positive** (N = 19) (N, row %)	**OR** (95%CI) **
TB symptoms			
no	530 (71%)	15 (2.8%)	1*
yes	180 (24%)	4 (2.2%)	0.85 (0.28, 2.58)
missing	32 (4.0%)		
Nutritional status at admission			
healthy	379 (51%)	8 (2.1%)	1*
at risk	195 (27%)	6 (3.0%)	1.48 (0.51, 4.32)
MAM	113 (15%)	2 (1.8%)	0.84 (0.18, 4.03)
SAM	40 (5%)	3 (7.5%)	3.73 (0.95, 14.7)
missing	15 (2%)	8 (2.1%)	

* 1 = set as the reference

** Odds of being NTM case among those investigated for presumptive TB

MAM = moderate acute malnutrition, SAM = severe acute malnutrition

Table 2b | Comparative clinical characteristics for those investigated for presumptive TB and infants with NTM isolated (continuous variable)

Categories	**Investigated for TB** (N = 742) (N, column %)	**Mean age** (95% CI)	**Rank sum p-value / t-test p value**
NTM negative	718	9.34 (8.95, 9.74)	0.20
NTM case	19	11.0 (8.02, 13.9)	
Unknown	5		

Table 3 shows the NTMs identified and the individual's clinical characteristics. *M. fortuitum* (6/19 32%) and (*M. scrofulaceum* 2/19 11%) were most frequently isolated.

Two of the 19 (11%) were unidentifiable. Two patients had MTB and NTM co-infection. Applying the ATS criteria for diagnosis of NTM disease, none of the NTM cases qualified as having NTM disease. Only 1/19 (5.3%) NTM case was HIV infected (Table 3b) while 3/19 (16%) were born to mothers who tested HIV positive but were themselves uninfected (HUE).

In our study, rapidly growing mycobacteria (RGM) which form colonies in less than seven days, were isolated most frequently (10/19) (Table 3a). The most frequently isolated NTM in paediatric studies are shown in Table 4. *M. fortuitum* was the most frequently isolated NTM among the identified studies.

Table 3a | NTMs identified; clinical and radiological profile of cases (Rapidly growing mycobacteria)

Number	Age (months)	NTM	MTB co-infected	HIV status	Nutritional status at admission	TST (mm)	Keith Edward TB score	Vital status at admission	CXR	Siblings	Housing
Rapidly growing mycobacteria (RGM)											
52452	14	*M. peregrinum*	no	negative	at risk	0	0	alive	normal	4	mud
50170	14	*M. smegmatis*	no	negative	at risk	0	0	alive	normal	4	mud
50220	5	*M. smegmatis*	no	negative	healthy	4	0	alive	normal	1	semi
51388	11	*M. chelonae*	no	negative	at risk	3	1	alive	normal	unknown	semi
52696	5	*M. fortuitum*	no	negative	healthy	0	0	alive	normal	2	mud
52727	5	*M. fortuitum*	no	negative	healthy	0	0	alive	abnormal no TB	6	mud
50206	13	*M. fortuitum*	no	negative	SAM	10	6	alive	normal	3	mud
50523	6	*M. fortuitum*	no	negative	healthy	12	3	alive	normal	1	stone
51104	22	*M. fortuitum*	no	negative	at risk	0	0	alive	normal	1	stone
52024	19	*M. fortuitum*	no	HUE	healthy	1	1	alive	normal	1	mud

HUE = HIV uninfected newborns, born to mothers who tested HIV positive, MAM = moderate acute malnutrition

Table 3b | NTMs identified; clinical and radiological profile of cases (Slowly growing mycobacteria)

Number	Age (months)	NTM	MTB co-infected	HIV status	Nutritional status at admission	TST (mm)	Keith Edward TB score	Vital status at admission	CXR	Siblings	Housing
Slowly growing mycobacteria (SGM)											
51599	9	*M. asiaticum*	yes	positive	SAM	7	10	died	abnormal no TB	unknown	stone
50049	4	*M. celatum*	no	negative	MAM	0	0	alive	normal	3	mud
51598	7	*M. gordonae*	no	negative	at risk	1	1	alive	abnormal no TB	3	mud
52683	9	*M. intracellulare*	no	negative	healthy	0	0	alive	abnormal no TB	3	Semi-
51119	12	*M. malmoense*	no	negative	at risk	2	1	alive	normal	2	Semi-
50380	23	*M. scrofulaceum*	no	HUE	at risk	12	4	alive	abnormal TB likely	3	mud
50108	20	*M. scrofulaceum*	no	negative	healthy	3	3	alive	normal	1	mud
Unidentified mycobacteria											
50178	11	Unidentified	no	HUE	healthy	0	0	alive	normal	unknown	mud
51706	6	Unidentified	yes	negative	SAM	12	13	alive	abnormal TB likely	2	stone

HUE = HIV uninfected newborns, born to mothers who tested HIV positive, MAM = moderate acute malnutrition, SAM = severe acute malnutrition

Table 4 | Paediatric NTM studies in Africa between years 2000 and 2018

Country Year (reference)	Study Type	Study population	NTM proportion of presumptive TB	Most frequently isolated NTM	Clinical relevance*	MTB-NTM co-infection	Proportion of participants with TB	TB prevalence per 100.000 at time of study
Uganda 2013 (14)	Prospective cohort study	< 1 year	3.7%	*M. fortuitum* (64%)	not specified	0	not specified	193 [32]
South Africa 2006 (13)	Prospective cohort study	< 2 years	6 %	*M. intracellulare* (41 %)	7/109-NTM disease	5/109	11%	960 [33]
Mozambique 2017 (15)	Prospective cohort study	< 2 years	26 %	*M. intracellulare* (68 %)	colonisation	0	> 1.4%	> 544 [34]
Ethiopia 2013 (20)	Cross sectional hospital survey	< 15 years	9.9 %	*M. fortuitum* (29 %)	not specified	0	15%	237 [20]
Kenya Present Study	Prospective cohort study	< 2 years	2.6 %	*M. fortuitum* (32 %)	colonisation	2/19	1.5%	600 [31]

*based on authors' description of suggestive clinical and radiological features

DISCUSSION

Burden of NTM

The proportion of NTM in pulmonary samples of presumptive TB cases in this infant cohort was relatively low (2.6%; 95% CI 1.5, 3.8). Standard sputum decontamination procedures were judiciously applied, hence it is unlikely that NTM yield was affected by this. A similar study among infants in Uganda and South Africa found 3.7% (14) and 6% (13) respectively. The epidemiology of exposure in this region could be non-linear, where exposure in early childhood is minimal but increases rapidly in adolescents. A significantly higher proportion of NTMs were identified among presumptive TB cases in an adolescents in the study area (37.5%), at the time of the study (V. Nduba, Personal communication). Nevertheless, the Mozambique cohort and a survey in Ethiopia had more NTMs (15, 20), and the average prevalence in African adult pulmonary samples was 7.5% in a systematic review (10). It is possible that BCG is protective against NTM colonisation. A twenty year retrospective study of NTM notifications in children demonstrated increased odds of NTM disease when universal BCG vaccination was halted in Finland (4). Therefore, BCG could also protect against colonisation. This can be evaluated conclusively in head to head comparisons of BCG and recombinant BCG vaccines presently in phase III clinical trials (21).

Colonisation or NTM disease / Clinical relevance

We did not find statistically significant differences in baseline characteristics between NTM cases and other presumptive TB patients suggesting widespread exposure across the study population. There were no differences in the clinical or radiological characteristics between presumptive TB and NTMs cases.

NTM disease is clinically and radiologically indistinguishable from TB (9). Two NTM cases were symptomatic with a suggestive radiological picture and would have qualified as NTM disease, but MTBC was also isolated from their sputum. The remainder had no combination of suggestive clinical or radiological features. We therefore conclude the NTM cases represent colonization. There is a possibility that these are laboratory contaminants, however this is unlikely since we checked for contaminants by having negative controls.

Risk factors - Environmental exposure

We did not identify any environmental risk factors for NTM incidence. Unlike MTBC which is transmitted from person to person, NTM transmission occurs via repeated environmental exposure. In infants this would be through handling by parents and siblings. The study area is rural. Risk for acquiring NTMs is significantly higher in communities engaged in occupations that generate aerosols and are exposed to soil for prolonged periods such as agriculture (22). It is not clear what the environmental source of these NTMs is.

Risk factors - Host factors

Host factors predisposing to NTM isolation were inter-current MTBC disease and severe undernutrition, although the latter did not reach statistical significance. Past history of TB has been known to be a risk factor for NTM disease (23, 24); since we studied infants that could not be confirmed. Interestingly, in this study, MTBC isolation increased the odds of NTM isolation almost fifty fold. NTM-MTBC co-infection in the same infant host has been observed (13, 15), and in adults in high TB burden countries (25). TB appears to be a pre-existing lung condition predisposing to NTM colonisation (9).

Low Body Mass Index and poor nutrition are other possible host factors, even predicting risk of disseminated NTM disease in other studies (26, 27), our study seemed to show the same trend.

Only in one case was the NTM case HIV infected, indicating among infants in this region, immunodeficiency is not a factor in NTM isolation in sputum.

NTMs isolated

The spectrum of organisms identified in this NTM study are in type and frequency to those reported in Uganda (14), Ethiopia (20), and Saudi Arabia (24). *M. fortuitum* was most frequently isolated in these studies. There could be geographic and climatic factors in the distribution. All the regions have warm climates. Increase in latitude and polarity has been shown to be associated with higher isolation rates of more pathogenic, slow growing mycobacteria (4, 13, 15).

TB diagnostics

There was no detectable difference in TST positivity between NTM cases and other patients whereas NTM sensitization is known to be responsible for false positive TST readings. Indeed false positive TSTs due to NTMs are infrequent and mainly relevant in areas with low TB endemicity (28).

BCG efficacy

NTMs influence the relative efficacy of BCG vaccines (29). The nature and type of NTM isolated in TB endemic countries are critical to an efficient vaccination campaign (2). The relative frequency of isolated species may correlate with the prevalence of skin sensitivity to their antigens, as was shown in Malawi (11). RGMs have been shown to be protective against leprosy and TB (11). This could not be confirmed in the current study due to the low numbers of NTM isolated. As there was no unvaccinated control group, it is not possible to assess efficacy of BCG. Thus, it appears that the risk of exposure to NTM as a covariate of vaccine efficacy as has been previously suggested, is quite low in the target age group.

Limitations

Our analysis was limited due to the small proportion of NTMs isolated in this age group. Nevertheless, it forms a baseline assessment for future studies including future vaccine trials. Also, not all infants could be tested for NTM, this was not the primary objective, and it is challenging to obtain samples

from children without presumptive TB. Therefore, the NTM incidence may be an underestimate of the NTM burden in the population.

CONCLUSION

This study has attempted to document the incidence of NTM among infants thought to have TB. The clinical relevance of NTMs isolated points to colonisation and not disease, as all the infants from whom NTMs were isolated did not meet the ATS criteria for disease. Our data shows that a patient presenting with features of TB, is less likely to have NTM disease, in similar settings.

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Chapter 6

Development of a tuberculosis vaccine trial site in Africa and lessons from the Ebola experience

Grace Kaguthi, Videlis Nduba, Prisca. Rabuogi, Douglas Okelloh, Samuel.G Ouma, Gretta Blatner, Sebastian Gelderbloem, Ellen Mitchell, Cherise P. Scott, Suzanne Verver, Tony Hawkridge, Jurriaan. E.M. de Steenwinkel, Kayla. F. Laserson, Jan Hendrik Richardus

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Tuberculosis is the deadliest infection of our time, with about 1.3 million deaths in 2017. In contrast, about 11,000 people died of Ebola between 2014 and 2016. Despite this manifest difference in mortality, there is now a licensed vaccine with up to 100% efficacy against Ebola. The developments that led to the trialing and licensure of the Ebola vaccine were historic and unprecedented. The single licensed TB vaccine (BCG) has limited efficacy. There is a dire need for a new, more efficacious TB vaccine. In order to license and deploy such vaccines, trials are needed in a limited number of sites that combine high disease incidence, research infrastructure and expertise. We first chronologically describe our twelve-year experience in building a TB vaccine trial site, an arduous and incremental process in contrast to the recent Ebola outbreak, which illustrates that sites can be set up under great adversity. Due to the striking differences in the vaccine development of the two diseases, we also compare the number of respective human trials between the two diseases, using publicly available data. Relative to the Ebola pipeline, TB vaccines have fewer trials and a paucity of government and industry led trials. While acknowledging pathogens have varying levels of difficulty in the development of new vaccine candidates, there yet appears to be greater interest in funding and coordinating Ebola interventions. We are convinced that TB is a global threat that requires similar concerted effort for elimination.

INTRODUCTION

Tuberculosis (TB) is a blight to the technological advances of the 21st century. A global blueprint to combat it has been outlined in the end TB Strategy (1). New drugs, diagnostics, and vaccines are in development with the goal of elimination. For external validity and generalizability, trial sites in endemic areas are needed to advance the various candidates through phase III licensure trials. Many countries with a high burden of TB are infrequently set up to conduct clinical research. These challenges hinder advancement of TB vaccine candidates.

From 2014 to 2016, there was an outbreak of the Ebola virus in West Africa. Outbreaks had been documented periodically from 1976, with case fatalities of 90%. However, in the recent outbreak, the risk of widespread contagion from global travel and its potential for bioterrorism, prompted strong responses. In a highly entropic environment, 13 prophylactic vaccines were in clinical studies with at least 40% evaluated on the African continent between 2014 and 2015 (2). This demonstrated that under sufficient threat, the global community can mobilize resources and overcome adverse circumstances. Determining vaccine efficacy during an unpredictable outbreak, in low resourced settings for an acutely lethal disease is singularly difficult. However, there is now a vaccine, with up to 100% efficacy. (2).

In light of these unusual events, we compare the TB and Ebola vaccine development in two ways. Firstly, we chronologically narrate our experience and learning in building a TB vaccine trial site from the ground up. Thereafter, we compare this with the site set-up process for Ebola vaccine trials. Finally, we review the number of clinical vaccine trials for both diseases, by phase and funding, from 2014 when the Ebola outbreak began, and Phase I trials initiated. This review incorporates our unique site

experiences in relation to current and future TB vaccine trial sites and places our perspectives on TB vaccine development in the context of the Ebola outbreak.

TB vaccine trial site development

Study area and study population

Our reflections are based on studies conducted from 2007 to 2019 at a site in Siaya county, Western Kenya (Figure 1). The area is rural, 400km west of the capital Nairobi. The population mostly comprises peasant farmers. There is a high but declining prevalence of TB, HIV and malaria (3). Part of the study area was under health and demographic surveillance (HDSS), tracking births, deaths, and migrations.

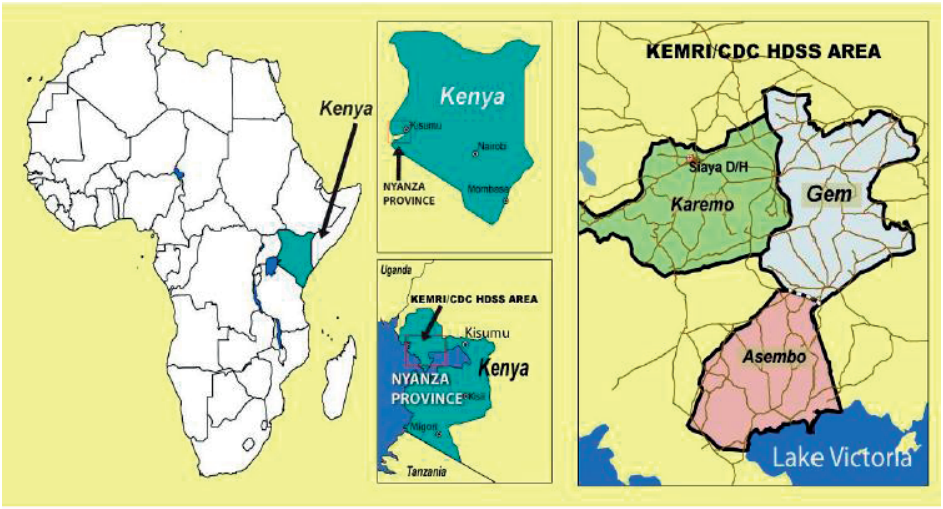

Figure 1 | Study area

Site history and development

In 2007, a consortium received a European and Developing Countries Clinical Trials' Partnership (EDCTP) grant to create capacity necessary for TB vaccine trials. Two epidemiological TB studies in adolescents and infants were conceived. At the time, there was little research infrastructure. A collaboration with the then Siaya Hospital was set up. Additional training for nurses on TST administration and reading, collection of induced sputa and gastric aspirates was provided by a team from a sister site. Study staff were extensively trained in collaboration with the Vienna School of Clinical Research on (among others) biostatistics, research methods, ethics, Good Clinical Practice (GCP), and trial site management. Also, several site staff received concurrent sponsorship for MSc's and PhD's.

Tuberculosis studies

We describe five TB studies conducted serially at our site and the lessons learnt in each.

1. Adolescent Cohort Study (ACS)

The ACS began in 2007, with the objective of recruiting adolescents to establish the optimal way to access them in the study area and determine TB prevalence and incidence in preparation for TB vaccine trials. A mobile field site (MFS) was used to enroll adolescents. The MFS was a collection of tents, a mobile radiography unit and mobile generator pitched in a grassy open field in a school, church compound or other open public arena (Figure 2).

Figure 2 | Mobile field site

Potential MFS sites were identified by study staff, and the site assembled every week. The tents housed study staff, parents and adolescents who moved serially from consenting, TB symptom screening, TST administration or reading, to chest radiography and then to dispatch. Data entry was on Personal Digital Assistants and laptops, using a local area network powered by the mobile generator. Upon exhausting all potentials at that catchment area, the site would demobilize and set up in a contiguous site and the process would start over again. Follow-up visits were conducted in similar fashion. The challenges of operating in an open field were particularly apparent during the rainy seasons. The site would get flooded or barely accessible, disrupting study processes. In that case it would be disassembled and relocated. A total of 5004 adolescents were recruited on target over 12 months and 83% retained (4). Most of this team rolled over into the infant study.

2. Infant Cohort Study (ICS)

Paediatric TB estimates from program data were not age-disaggregated, masking the disproportionate affliction of young infants with TB. In 2008, the thinking of the TB vaccine community leaned heavily towards infants as a target population. Therefore, the ICS was conceived to provide estimates of TB

incidence using comprehensive diagnostic methods (Nduba V, 2019-submitted), and to demonstrate capacity to deliver BCG within 96 hours of birth. This was to anticipate the administration of BCG replacement candidate vaccines.

At the time, it was reported that 80% of mothers delivered at home, with the help of a traditional birth attendant (TBA). We engaged TBAs to assist in notifying births to the study staff. The recruitment coordinator would then dispatch a nurse on a motorbike to perform enrolment procedures. Thereafter, a nurse would deliver BCG to enrolled infants. The median age of enrolees was 10 days, therefore in many cases it was not possible to deliver BCG within 96 hours of birth due to late notifications and problems locating mothers who were highly mobile in the study area. This was accentuated when due to insufficient notifications, the study area was extended to Boro, a nearby sub-county, which was not under the HDSS. In addition, the terrain was frequently challenging. Nurses were inexperienced motorbike riders and predominantly female. There were several falls that injured their morale more than anything. Nevertheless, the team rallied, and 2900 infants were enrolled on target within 12 months.

Fortunately, the number of home births in the study area continued to decline. By 2011, at least 40% occurred at health facilities (5). Future trials of BCG replacement vaccine trials can therefore enrol infants at health facilities.

The ICS operated out of two tents in a grassy field of the Siaya Hospital, seeing scheduled and unscheduled visitors. The hospital allocated a store in the paediatric ward for creation of a negative pressure room for specialized sputum collection procedures. We renovated a defunct, dilapidated amenity wing designed for private patients, and converted into a case verification ward for overnight admissions for TB investigation procedures. The existing hodgepodge of facilities did not meet trial requirements, as there was no GCP compliant pharmacy, archival facilities, or laboratories. Therefore, in 2009, construction of a state-of-the-art research annex (Figure 3) with all the requisite infrastructure at the hospital grounds began, funded by several partners. It was completed in 2012 and auspiciously launched.

Figure 3 | Siaya clinical research annex

The study also created a previously non-existent capacity to diagnose paediatric TB using induced sputa and gastric aspirates as well as structured chest radiograph review. There were thousands of ancillary care visits that contributed the largest number of presumptive TB cases, given the non-specific presentation of TB in infants.

3. Aeras-402 (Nochak)

The first TB vaccine trial at the site followed closely on the heels of the ICS. It was a phase IIb trial of AERAS 402 in healthy, HIV uninfected, BCG vaccinated infants (6). The site was the first to be initiated into the trial in 2010. The study team was now proficient in recruitment of infants and requisite community links had been forged. There was widespread enthusiasm for study participation due to intense community mobilization by site staff. Locals had branded the study 'NoChaK' (Nonro mar Chanjo mar Kahera), translated 'a new TB vaccine' in Luo, the local language (Figure 4).

Figure 4 | Community engagement

Disappointment ensued when there was an inordinate number of screen failures due to abnormal biochemistries, particularly elevated total bilirubin in infants who were in every other sense healthy. To determine eligibility, the National Institutes of Health (NIH) reference ranges were used, which, in hindsight, were clearly derived from a distinct population. It would have been unethical to continue screening, and therefore enrolment was paused to investigate the issue. To exclude invalid laboratory results, the local laboratory that was also being used for the RTS,S malaria vaccine trial, was scrutinized to determine the reproducibility and repeatability of affected parameters, as well as External Quality

Assurance (EQA) program results. The laboratory cleared. We found no antecedent literature describing what we were observing. Following wide consultations, we developed a new set of reference ranges. This allowed the study to proceed with enrolment. The area was hyper endemic for malaria and that was considered the prime reason for the elevations in healthy infants. Years later, two publications emerged on the subject, but for an adult population proximal to the study area, which mirrored our observations (7, 8).

There were significant problems with collection and processing of Peripheral Blood Mononuclear Cells (PBMCs). The cells needed to be separated and frozen within 4 hours of collection. The study site was about 80 km away (about 1.5-hour drive) from the immunology laboratory, with predictable inaccessibility in wet weather. The process of separation took at least two hours. In addition, eight ml of blood were collected in several tall vials. Like many African communities, there were strong sensitivities to blood sampling, despite our best efforts to explain that the relative blood volumes sampled would not adversely impact the babies given their age and weight. Furthermore, as infants were significantly dark skinned and healthy, if more than one attempt was needed to collect these samples, parents would get distressed and the time taken also derailed the tight separation timelines. In response, tight orchestration of the visits with PBMC collection was done. An experienced phlebotomist, efficient in cutting down the collection time was added to the team. Technicians were alerted once the sample left and were on stand-by ready to immediately begin to process the samples.

Unfortunately, the laboratory team had no prior immunology experience. They failed proficiency testing, some on more than one occasion and this skill was only gradually acquired. In addition, on one occasion the dry ice shipper containing EQA samples arrived at the destination laboratory dry, compromising cell viability. The laboratory teams were retrained, and this capacity was then available for the future trial among adults.

The stability of AERAS 402 vaccine at 2-8 degrees was short-lived. It was stored at -80 ^{0}C in a research pharmacy in Kisumu (Figure 5).

DBS: Dry Blood Spot, PBMC: Peripheral Blood Mononuclear Cells

Twenty-four hours prior to dosing, it would be transferred from the freezer to the refrigerator and could keep for only six weeks. There were frequent power outages at the clinic, therefore it was not possible to safely keep vaccine on site. Therefore, an alternative thaw protocol was used. Vials would be removed from the freezer, thawed at room temperature for one to two hours, and then shipped to site under rigorous temperature monitoring. After review of potential participants, the study doctor would call up the vaccine from the central pharmacy, and vaccinations would happen upon arrival two hours later. As there were mandatory post-vaccination observation procedures, many mothers were dropped off at their homes late in the night, which created friction in families. Mothers agreed to assemble for early pick-ups on days of randomization, and thus there was only one vaccine run to Kisumu.

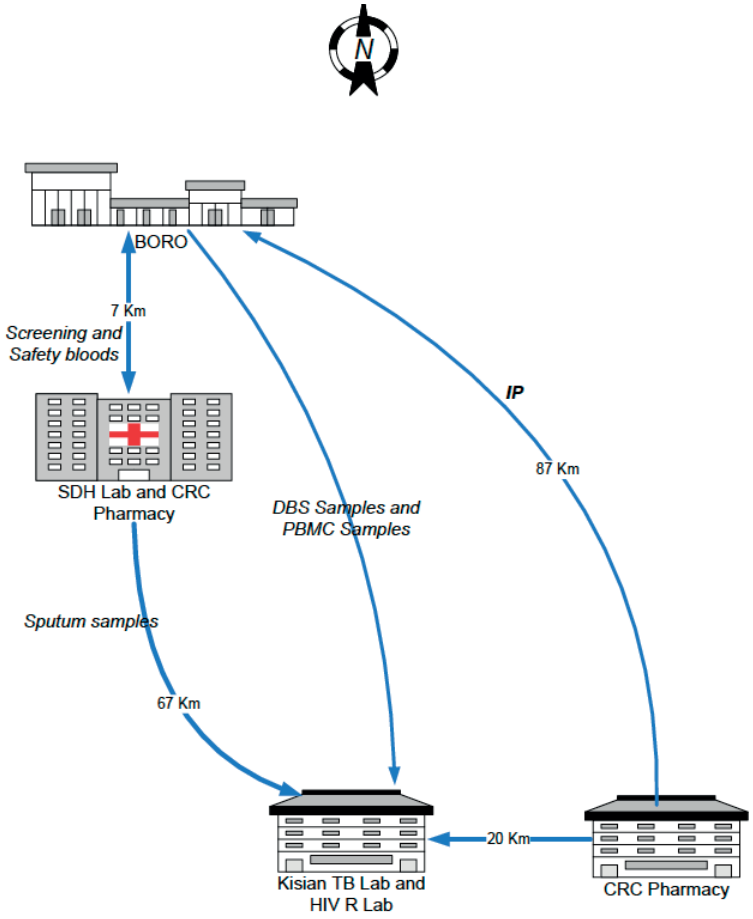

Figure 5 | Logistics

4. TB018 (M72)

The second vaccine trial in this site was a phase IIb trial of M72/AS01E against placebo (9). The site's successful experience with the MFS made it a compelling recruitment strategy for this trial. A total of 538 individuals were randomized over a period of eight months. The laboratory was now experienced in handling immunology samples and there were few hitches.

5. VPM1002

In 2018, towards the end of the M72 study, our site was selected to participate in a phase III trial of VPM1002 against BCG among infants. The cumulative site experience in immunology, infant recruitment, TB case detection, and adverse event surveillance continues to be applied for this trial. Recruitment is expected to end by the second quarter of 2022, with a minimum follow-up time of 12 months.

Lessons from site development

1. Importance of epidemiological studies

The epidemiological studies seriously enhanced our capacity to perform future trials with increasing ease and excellence. Increasingly, funding pressures have constrained the space for epidemiological studies, as they offer knowledge with no prospects of 'return on investment'. The placebo arm is sometimes viewed as the epidemiological understudy. The trial population, however, is usually a selected sub-group that are healthier, HIV uninfected, and probably more accessible to the research team based on their different health seeking behaviour. Being subjects of intense health monitoring, they likely have lower risks of TB acquisition. The placebo arm therefore provides little context for the interpretation of trial results, which are rarely linear or straight-forward. Therefore, epidemiological studies should not be considered optional.

2. Biobanking inclusivity

Health research capacity building is not without power relations (11). Biobanking is a part of virtually every TB vaccine trial. However, once samples are shipped to central laboratories in Western Europe or North America, research teams have no say in the deployment of those assays, even while they made great efforts to collect them. It is intellectually impoverishing; research teams having the derogatory epithet 'sample traffickers'. In hindsight, a biobanking capacity-building component should have been included in the initial studies, creating the legal and scientific framework to ensure democratization and inclusivity.

Ebola site development

Having reflected on the development of our TB vaccine site, we turn our attention to the development of sites during the Ebola outbreak. As Ebola mortality increased during the 2014 outbreak, promising vaccine candidates stuck in preclinical phases for years (2, 3) due to lack of funding or interest, were fast-tracked into human trials (12-14). Clinical trial protocols were ready within weeks. Ethical and regulatory reviews were turned around in two weeks (15). Preliminary efficacy results were availed in unprecedented time for rapid decision making (16). The transition to Phase II/III studies was unmatched, at eight months, instead of ordinarily one to three years (17, 18) after initiation of the phase I study (2).

To have any hope of demonstrating efficacy, the studies needed to be initiated before the unpredictable outbreak ended. However, most study areas were unmapped, and lacked electricity and basic amenities. In some places years of civil war had destroyed health infrastructure. Scores of health workers had lost their lives in the outbreak (19), and those who remained were involved in emergency response to victims. Thus, local trained research teams were virtually non-existent (20), as were any 'clinical research sites' (15, 20). Despite all this, the necessary facilities were put up on the go, and teams adapted other trial aspects to the less ideal realities in the field (18).

The World Health Organization (WHO) was pivotal in overcoming all the challenges by fostering interactions with scientific, ethics, regulatory, industry and funder groups from every continent (16). Evidently, with high stakes, absent infrastructure or research teams are not obstacles to trial implementation or success. Further, most countries with high TB burden have significantly fewer barriers to trial set-up compared to those setting up Ebola trials.

Lessons for TB vaccine trials from Ebola

The trialing and availability of a highly efficacious vaccine for Ebola in an extraordinarily short amount of time, led us to compare the vaccine pipelines, the relative numbers of clinical trials, vaccination strategies as well as the theoretical probability of success for each disease.

1. Vaccine trial pipeline

Table 1 shows the trials initiated for Ebola versus TB vaccines in the last five years when Ebola vaccine trials entered the clinical phase.

We assume that a disease's vaccine pipeline is a proxy for intensity of research and that a diverse, robust pipeline is more likely to produce a candidate that shows efficacy (21, 22). The TB vaccine pipeline has fewer studies than doe Ebola and there is also under-representation of government and industry sponsored trials. The economic cost of each epidemic makes a compelling case for both industry and government involvement. Economic losses, in West Africa and globally, were estimated at USD 18 billion as a result of the 2014-2016 outbreak (23). The annual societal cost alone of TB is USD 19 billion (24). The WHO has supported TB vaccine initiatives (22), but the Ebola outbreak showed us that much more is possible, especially in solving macro-level problems and mobilizing resources, probably even in sponsoring trials (16).

As of September 2019, there were only eight preclinical vaccine candidates for TB (www.tbvi.eu/what-we-do/pipeline-of-vaccines). A main reason for this, other than minimal public interest in accelerating a new TB vaccine, is funding restrictions (25). As a result, stage-gate criteria have been developed to constrict the movement of candidates from one phase to the next, with the idea of advancing only the most promising candidates, defined largely by available immunogenicity, efficacy, and safety data (21, 26). At most of the early stage gate points, particularly pre-clinical to clinical, and Phase I to Phase IIb, the candidate has the least possible 'value chain' defined as the accumulation of value due to demonstrated safety or efficacy or both (27).

Furthermore, immunogenicity criteria (26) that quantify vaccine-induced immune responses may not represent protection against disease. These criteria may be flawed, leading to false negatives or positives. While clearly some criteria are needed to advance candidates, the way out seems to be through actual clinical studies.

Table 1 | Ebola versus TB vaccine candidate pipeline from 2014 (WHO Vaccine Pipeline Tracker (www.who.int) accessed 12 Oct 2019). (Some trials have more than one sponsor)

	Ebola vaccine trials	TB vaccine trials
Total clinical trials	60	30
Phase 1	34	13
Phase 2	16	10
Phase 3	8	6
Phase 4	2	1
Sponsors		
Industry	21	7
Non-profit	0	6
Academia	14	16
Government sponsors	17	3
Russia (MoH)	3	1
China (Jiangsu Province)	3	0
USA (CDC, NIAID)	11	2
France	0	0
Australia	0	0

2. Probability of success of a vaccine trial

The difficulty in vaccine design differs from pathogen to pathogen. The average vaccine requires a clinical development timeline of 10.7 years and has a market entry probability of 6% (27). Table 2 shows selected requirements thought to impact the feasibility of accelerated vaccine development. Mycobacterium tuberculosis is considerably more complex, with great capacity to evade host immunity (28). Also, animal models that closely mimic human disease are better for Ebola virus than TB. Hence a new TB vaccine could be termed 'unprecedented' and the odds of success are pegged considerably lower than for most other diseases (29). These estimates are valid but are based on past experience with limited government and industry involvement and a minimal global effort.

3. Vaccination strategies and phase II/III trial sites

Ring vaccination in the Guinea Ebola vaccine trial provided preliminary efficacy data in a highly dynamic outbreak (2). The most at risk persons were singled out for vaccination, as opposed to vaccinating an entire community. There are irrefutable data on asymmetries of TB risk ('TB hotspots') (40) based on residence (urban vs. rural) (41, 42), certain geographic locations including prison (43) and mines (44, 45), social class interactions (46), and age (47, 48). Such epidemiological data have not been applied in selecting trial populations for phase IIb-IV trials, yet they would critically minimize sample sizes, guide site selection and provide rapid answers as to a vaccine's efficacy. This was one of the pathways explored in the Ebola outbreak.

Table 2 | Feasibility of accelerated vaccine development (Institute of Medicine (US) committee on issues and priorities for new vaccine development (30)

Requirement	Ebola (ref)	Tuberculosis (ref)
Knowledge of clinical signs and symptoms of the disease to allow differentiation from similar syndromes	+	++
Knowledge of pathogen characteristics: strains / serotypes, infectivity / virulence, antigenicity, immunogens	++ (31)	++ (32, 33)
Ability to cultivate pathogen	+ (34)	++
Identified non-human models of infection, closely mimicking human disease	+++ (35)	++ (36)
Knowledge of human immune response to the pathogen (duration, type of response)	+ (37)	+ (38)
Definition of the target population	+ (39)	++ (22)

CONCLUSIONS

We have described our incremental 12-year process of site development, in contrast to an acute, chaotic and very productive process in the Ebola virus outbreak. Since the ACS in 2007, about a dozen other phase II and III trials have been conducted at our site. Subsequent studies have found better infrastructure, more highly experienced teams, and the complexity of protocols implemented at the site has also increased. Nevertheless, the end goal is a new efficacious TB vaccine brought to bear on the epidemic.

TB unlike Ebola has a lower case fatality, slower epidemic, and lower potential for bioterrorism. But its total mortality is hundreds to thousands of times higher. It is our view that developing a new TB vaccine will also require centralized coordination of efforts.

The TB vaccine pipeline is plagued not simply by funding limitations, but by an inadequate number of 'hands on deck'. The Ebola crisis calls for a sense of urgency towards the TB epidemic, and for a narrowed focus. For example, the lack of knowledge of correlates of protection has not prevented the advancement of a new malaria, polio (50), or Ebola vaccine (51, 52), neither the eradication of small pox (49). Funds can be prioritized for pre-clinical development and field testing of candidates in order to stop the scourge, else the goal of TB elimination will remain elusive (1). Given an Ebola vaccine was evaluated and licensed under extreme conditions, it is a rallying call for the TB vaccine community to champion the cause.

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Chapter 7

Discussion

The overall aim of this thesis was to determine the suitability of infants as a trial population and inform diagnostic considerations for future TB vaccine trials. This thesis also documents the practical experience of site set-up and trial implementation in Kenya as well as the lessons for TB vaccine development following the successful trial of a new vaccine against Ebola virus. These questions have been addressed in TB cohort studies particularly among infants.

In the discussion, I review the principles for selecting a trial population, grouped into trial related factors and post-trial considerations using an infant population. Thereafter, I review epidemiological principles that can guide selection of other possible target populations considering disease patterns in endemic countries. This is followed by an appraisal of the chest radiograph as a diagnostic tool in trials. I discuss the role of Non-Tuberculous Mycobacteria in TB vaccine trials, as concerns TB diagnosis. Furthermore, I chronicle the lessons learnt in site development and examine the current pipeline of candidate vaccines targeting infants. Finally, I provide possible future perspectives, and provide a conclusion to the thesis.

The specific research questions addressed in this thesis are grouped thematically as follows:
The suitability of infants as a target population.

1. To assess TB incidence, post-neonatal mortality and cohort retention among infants.
2. To evaluate the chest radiograph (CXR) for its suitability as an endpoint for infant trials where paucibacillary disease is most frequent.
3. To determine the clinical relevance of non-tuberculous mycobacteria (NTM) isolated in sputum, given their ubiquity and their indistinguishable case presentation to TB disease.
4. To reflect on the lessons learnt in building a new TB vaccine site and review the TB vaccine development pipeline in the context of the Ebola virus outbreak.

Ad 1: Selecting a target population: Infants as a trial population. The selection of a target population requires consideration of multiple factors. I classify them into those related to trial implementation and those that concern post-trial roll-out.

1. Trial Implementation Related Factors

1a. Sample size calculations: Incidence, recruitment, retention, mortality

First, I compare trial efficiency of infant studies with adult/adolescent studies, computed using estimates of incidence, retention and mortality from an infant cohort study. The incidence of microbiologically confirmed TB among young children in our cohort study was high, at 0.28 per 100 person years of follow up. About 80% of the cohort was retained. We showed that in a superiority parallel arm TB vaccine trial, assuming definite TB incidence of 0.28 per 100 pyo (Nduba V, submitted), retention of 80%, vaccine efficacy (VE) of 50% (1, 2), 24,300 infants would need be recruited and followed up for three years.

In the recent M72 trial, TB incidence was 0.3% among LTBI positive adults who have higher risks of disease progression (1). A phase III licensure trial would need to include LTBI negative individuals

for validity, who have lower TB incidence, about 0.15% (3), with 80% power and 50 % VE. Based on these estimates about 50,000 healthy adults would be required. Further, larger sample sizes often require longer patient accrual times, multiple sites in different locations and complex logistics. All these inflate the trial costs (4). By recruiting 2900 infants in twelve months at a single site, we demonstrated the feasibility of accruing thousands of infants within a reasonable time frame. Therefore, we conclude that the feasibility and greater efficiency of infant trials has been shown.

1b. Mortality

About 7% of the infant cohort died over twenty-four months. The most frequent immediate cause of death was hypovolemic shock, while undernutrition was most frequently the underlying cause of death. Background mortality provides context for interpretation of vaccine efficacy. For example, Mosquirix®, in its phase III trial reduced the number and severity of malarial episodes but surprisingly had no impact on mortality (5). This has been attributed to local factors such as co-morbidities which drive mortality (6). In addition, as BCG has mortality benefits, all-cause mortality is an important secondary endpoint for infant TB vaccine trials. We conclude that baseline infant mortality estimates guide the comparisons of vaccine and control (BCG) arms in every site.

1c. Immunological factors: Vaccine design

MTBC is a complex pathogen, extensively evolved to evade and manipulate host immunity (7, 8). On the other hand, neonatal immune systems are underdeveloped, lack antigenic experience as a result of protected in-utero conditions, have poor immunological memory and heightened immunoregulatory responses to facilitate maternal co-existence (9, 10). Therefore, vaccinating adults has been more attractive. In addition, in high burden countries, maternal infections such as HIV, Malaria and helminths influence the development of fetal and neonatal immunity decreasing vaccine responsiveness (11).

2. Post-trial roll-out related factors: Vaccination platforms and impact on the epidemic

2a. Ethics

The high TB incidence among infants is further evidence of their higher susceptibility to infection after exposure, higher risks of progression to disease given infection and propensity to severe or disseminated disease (12). From an ethical perspective, infants are particularly vulnerable and need protection through an efficacious vaccine.

2b. Vaccination platforms

Vaccination of children is routine and largely accepted. BCG is the most widely administered vaccine (13). There are established cold chain infrastructure and implementation mechanisms even in poor countries. This infrastructure is currently not available for other target populations. They would likely require costly platforms to maximize coverage.

2c. Impact

As infants do not transmit disease, the public health impact of a neonatal vaccine would be delayed by about a decade (14), when they would become vulnerable. They are therefore secondary in TB control efforts. This has been viewed as a major limitation of infant vaccination. A long-term view is needed, as has been shown in some modeling studies, the rapid impact on TB burden of vaccinating adults with a post-exposure vaccine would plateau, while that of infants would continue to increase (15).

In summary, choosing infants is beneficial for trial efficiency, ethics and post -trial roll out; but has limitations of underdeveloped infant immunity and delayed impact on TB case load.

Additional considerations for selecting an adult target population. As infants do not transmit disease, adults and possibly adolescents must be included as 'immunisable' populations. I discuss some principles for selecting other target populations based on epidemiological principles and available data.

3. Trial implementation related factors

A way to minimize sample sizes, increase trial efficiency and feasibility, is to target high risk populations, contrary to the current paradigm which considers all persons living in high burden countries to have similar risks of disease acquisition. I discuss the rationale for this, provide real world examples and suggestions based on epidemiological data from high burden countries.

3a. 20/80 rule

The 20/80 rule in epidemiology states that 20% of the host population are responsible for at least 80% of the net transmission potential, as measured by the basic reproduction number R_0 (16). The consequence of heterogeneity in transmission of infectious agents is the commonly observed clustered distribution of infections and disease within the host population, such that few hosts are rapidly, frequently or heavily infected while the majority either evade the infection or rarely suffer infection. For example, one prisoner was responsible for 63% of incident TB cases in a Brazilian prison (17). These have been termed 'super-transmitters'. They combine high infectiousness with access to many casual contacts in congregant settings. It has been observed in different settings that household transmission accounts for <20% of cases (15). Surveys have shown areas of preferential transmission (18). Conversely, there are persons who despite repeated MTBC exposure remain IGRA/TST negative, termed 'resistors' (19, 20). This phenomenon has been observed in several diseases such as Ebola and HIV and the underlying factors are not always well characterized (21, 22). The reverse could also be true, where 20% of the population are particularly susceptible to acquiring infection and progressing to disease following casual contact. As correlates of risk have not yet been identified, using susceptibles as a target population has not yet been envisioned.

3b. Heterogeneity of disease burden

The 20/80 principle is exemplified in heterogeneity in TB burden based on spatial and social settings and interactions. They are discussed below.

3b1. Spatial heterogeneity

In high burden countries, 80% or more of cases can be confined to a particular geographical location. In a prospective cohort study of Multi-Drug Resistant (MDR-TB) cases and their household contacts in Peru, there was increased risk in specific neighborhoods proximal to a certain health centre and the authors argued targeted interventions at areas of concentrated risk would be more useful than a blanket approach. (23). Likewise, modeling transmission in 3rd most populated city in all of South America, Rio de Janeiro, showed only 6% of cases accounted for 35% of city-wide transmission (24).

3b2. Social heterogeneity

In the same vein, prisons, slums, public transport and health facilities have also been found to be hotspots of transmission (25-28). The prevalence rates in these areas are significantly higher than the general population regardless of economic status or local TB burden (29). In a recent vaccine modeling study using South African data, targeting miners and delivering the vaccine to them at the mines averted more TB cases compared to community targeted vaccination (30).

3b3. Wealth assortative mixing

A concept related to social hotspots is that of wealth assortative mixing (31) and is the basis of targeting the poor for TB vaccines and other control activities. It is hypothesized that the poor are more likely to contact the poor who in turn are more likely to develop disease and transmit it to other poor people. Such dynamics increase the R_0 and make it more challenging to control disease using a blanket, 'one size fits all' approach. In India, the poorest quintile of the population had a five-fold disease prevalence of that of the wealthiest (1% vs 0.2%). After accounting for poverty associated risk factors (alcohol and tobacco use, HIV, diabetes, low BMI), residual variance in TB rates was thought to be due to wealth assortative mixing. The authors recommended for equity reasons but also to improve efficiency to target the poor for interventions in order to optimize limited resources. It follows that the same should be selected as a trial population. There is precedence for targeted interventions in HIV (32-34), cholera (35, 36), and malaria (37).

3c. Post-trial access

Adult vaccination would likely struggle to achieve the high coverage observed with neonatal BCG vaccination. Vaccination platforms for adults would need to be created. Many high incidence countries have poorly funded health systems, overburdened by providing care and treatment. They may not be able to develop or sustain the logistical operation of delivering mass vaccination campaigns. Further, the acceptability and feasibility of adult vaccination has been taken for granted. As at publication, we could not find data on perceptions and acceptability of adult TB vaccination. Vaccine hesitance has now been recognized as one of the top ten global threats (17) and could undermine decades of efforts. This shows the difficulties that would need to be overcome with adult vaccination as a strategy for TB disease control.

In summary; there is heterogeneity in susceptibility to TB as well as transmission patterns. This should be taken into consideration when selecting the target population for efficient vaccine trials and consequently for disease control efforts.

Ad 2: The Chest Radiograph as a diagnostic tool for TB endpoints

We found low inter-rater agreement on abnormalities consistent with TB on the chest radiograph poor; kappa =0.14; (95% CI: 0.10 - 0.18). This and the frequently atypical picture in infants are limitations to its use. In another setting with high TB/HIV burden, abnormalities consistent with TB on CXR were predictive of culture confirmed TB among hospitalized children (38). One critical difference is that hospitalized children would be presenting with advanced disease and likely to have more classical features on which most readers would agree. Trials deploy active follow up strategies to detect every potential endpoint. This blunts the sensitivity of the CXR, as early disease is detected which causes non-specific and subtle radiological changes. Nevertheless, in the trial of MVA85A, odds of microbiologically confirmed TB increased two-fold when at least two experts agreed there was radiographic TB defined by classical features (39). Therefore, the CXR remains useful for endpoint detection when two or more experts agree on radiological TB. Diagnostics deploying minimally invasive samples, such as stool and urine, with high sensitivity and specificity, are needed for young children.

Automated reading of chest radiographs has been used to screen adult patients with presumptive TB before further diagnostic evaluation. It has been shown to have similar sensitivity but albeit lower specificity compared to expert readers (40, 41). It has not been evaluated in diagnosis in young children who have a different radiological pattern of TB. Therefore, its use in trials is presently not recommended.

Ad 3: Non-Tuberculous Mycobacteria (NTM): Clinical Relevance and Differential diagnosis for TB

NTM disease is clinically and radiologically indistinguishable from MTB disease (42). NTM disease does not respond to anti-tuberculous therapy. In paediatric studies, composite endpoints are used in the absence of microbiological confirmation. Unidentified NTM disease can therefore be a source of false positives, given the similar case presentation. However, we found that all NTMs isolated in sputum represented colonisation and not disease. There was also no association between NTM isolation in sputum and TST positivity. This could have been due to the small proportion of NTMs isolated. It was not a study objective to evaluate the immunological pathways of interaction between TB vaccines and NTMs. Their ubiquity in the environment and subsequent presence in the lung microbiome implies an immunomodulatory role. This should be evaluated in epidemiological studies and in future trials of both infants and adults with a view to define how they interface with host immunity. Induced sputum samples should be aliquoted and stored for ribosomal RNA sequencing to explore the role of flora in infection, susceptibility and disease. NTM based vaccine of *M. obuense* showed efficacy in HIV infected persons against TB (43). This is further proof of their immunomodulatory role.

Ad 4: Site development and the TB vaccine pipeline: Lessons learnt

We chronicled the history of the predominant TB vaccine trial site in Western Kenya. Some of the challenges encountered with respect to logistics and operations have been observed in other TB vaccine trial sites (44, 45). Many were unique to our specific settings such as the lack of reference ranges relevant to our population and immunology inexperience.

The TB vaccine pipeline calls for governments, particularly in high burden countries, to prioritize contributing to the research and development process, as happened with the Ebola virus outbreak of 2014-2016.

TB vaccine pipeline targeting infants: The review below shows candidate TB vaccines in which infant trials are planned, ongoing or have been completed.

Phase I trials

MTBVAC: MTBVAC is a live attenuated MTBC strain, first of its kind. Genes coding (*phoP*) for transcriptional factors associated with virulence have been deleted (46). A phase IIa dose finding, safety and reactogenicity study is underway in infants in South Africa, to be completed by December 2020. It could have potential for immunocompromised patients, as it showed efficacy despite hyper-attenuation (47).

HYBRID 56/IC31: Hybrid 56/IC31, is an adjuvanted vaccine currently in Phase I trials in South Africa for safety and immunogenicity in BCG primed infants. It contains Rv2660c, an antigen strongly recognized by cells of LTBI individuals. The adjuvant is IC31 which is a strong Toll Like Receptor 9 agonist.

Phase II trials

Phase II trials are the graveyard of new molecular entities particularly vaccines (www.bio.org/clinical-development-success-rates-2006-2015 accessed Dec 2019) (48-50). Only 31% of candidates progress, more frequently for lack of efficacy rather than safety. This contributes significantly to high research and development costs (48, 50). I examine the current status for infant vaccines.

MVA85A: MVA85A a modified vaccinia Ankara virus expressing antigen 85A (MVA85A), intended as a boost after BCG priming. The vaccine was safe and tolerable intradermal, in preclinical studies, and in the phase IIb trial in infants. However, it showed no efficacy against TB (51). The result was termed surprising based on preceding animal studies. It was later claimed that the animal efficacy results from these studies downplayed negative results. Apparently, macaques vaccinated with BCG and boosted with MVA85A had poorer outcomes comparable to those that received no vaccine (52). Additionally, there were doubts on the rigor of the pre-clinical studies (51, 53), which are said to be geared towards establishing safety for marketing authorization rather than providing evidence of efficacy. The authors refuted this (51). The vaccine has recently been reformulated as an aerosol. It was safe and immunogenic in a phase I study, for possible further investigation in phase II trials (51).

AERAS 402: Aeras 402 was a replication deficient serotype 35 adenovirus vectored vaccine containing DNA expressing a fusion protein of three MTBC Antigens, TB10.4, Antigen 85A, and 85B. It was

intended as a boost after BCG priming. Phase IIb trials were initiated in 2009 in Kenya, Mozambique and South Africa. After the dose-finding phase, the predefined immunogenicity target was not met, and the trial was terminated for futility (54). The vaccine was broadly immunogenic, however some of the reasons for failure were the immunodominance hierarchy elicited by adenoviral vaccination may not adequately mimic natural infection, leading to a non-protective immune response (55). Further development has stopped.

M72/AS01E: This is an adjuvanted vaccine expressing two Mtb 39A and Mtb 32A present in MTBC and BCG, combined with AS01E adjuvant. It has successfully completed Phase IIb trial in adults in Kenya, South Africa and Zambia, demonstrating 50% efficacy in preventing disease in LTBI individuals (Tait D, 2019). A phase II safety and immunogenicity study among infants was completed that demonstrated non-interference with the Expanded Program of Immunization (EPI) schedule, safety and satisfactory induction of CD4 T-cell responses. It is not clear whether phase IIb studies in infants are planned.

Phase III trials

VPM1002: VPM1002 is a urease C-deficient Listeriolysin expressing BCG vaccine strain. Listeriolysin facilitates the perforation of the phagosome membrane, allowing the release of recombinant BCG antigens into the cytosol of host cells, and activate appropriate Th1 and Th17 immune responses. The deletion of the Urease C gene acts synergistically with the Listeriolysin expression by creating an acidic milieu for its action. Its safety and immunogenicity have been assessed in a Phase II trial in South African infants (56). A phase III trial with the endpoint of preventing TB infection is underway in Kenya, Uganda and South Africa.

FUTURE PERSPECTIVES

Immunomics

The most suited trial population would take into account heterogeneity in individual susceptibility, and be well characterized by genetic, age and geographic factors. Immune responses would likewise accurately predict probability of progression to infection or to disease status after MTBC exposure or propensity to an inflammatory immune response which is more damaging to host tissues (7). In addition, a-priori knowledge of vaccine induced immunity would then be used to identify the target population most likely to benefit from vaccination. This would minimize sample sizes, minimize potentially harmful exposure to the investigational vaccine and maximize benefit, thereby increasing trial efficiency. After licensure, it would be cheaper and easier to create tailored platforms for vaccinating a small proportion of susceptibles, assuming that outside of neonates and adolescents, vaccination platforms would need to be created in the developing world.

This information is largely unavailable, and this remains an aspirational goal that could be achieved with the help of technology such as artificial intelligence and immunomics. Immunomics is a 21st

century approach that combines immunology, genetics, proteomics, transcriptomics and bioinformatics to characterize host -pathogen interactions that is particularly useful for vaccine development for complex pathogens such as TB (57).

Such an approach supports a rational approach to vaccine development based on identifying antigenic targets, Correlates of Protection and can unlock the keys to developing vaccines that confer lifelong protection.

CONCLUSION

We sought to determine the most appropriate population for vaccination, review the chest radiograph as a diagnostic tool for TB end-points in vaccine trials, the species and clinical relevance of NTMs in TB trials and the lessons learnt from a TB vaccine field site in the context of the Ebola outbreak. We found that selecting the trial population requires consideration of pre- and post-trial factors, and our studies provided data for such decisions. We found the CXR remains a useful diagnostic tool in the hands of two or more experts. In addition, we found NTM isolation in sputum of presumptive TB patients to represent colonisation and not disease, hence there is apparently no diagnostic dilemma. Finally, we described our twelve year experience developing a TB vaccine site and lessons learnt and concluded global efforts can be intensified and harmonized if a global coordinator such as the WHO were to take lead in the process of bringing a new TB vaccine to the market.

RECOMMENDATIONS

1. The most appropriate target population

Under a scenario of unlimited resources, vaccines targeting every high-risk population should be developed with the relevant endpoints. These include post-exposure vaccine for adults and adolescents (Prevention of Disease or Prevention of Recurrence), or Prevention of Infection for neonates. HIV infected persons would also benefit from a safe and effective vaccine as outside of infants, TB mortality is highest in this group. In the reality of the current funding scenario, vaccines targeting infants should be prioritized, due to the high incidence, the difficulty of diagnosing TB using current tools and the subsequent high case fatality of undetected and untreated disease. The second most urgent priority is adults in slums or other impoverished rural settings and prisons. From a bioethical standpoint, where the benefits outweigh risks, as with TB vaccine trials, and with proper oversight as is the current standard practice, research participation of prisoners can be informed, deliberate and voluntary (58-60). Further, experts have considered such that there is a tremendous need for scientifically sound research among prisoners (58). Vaccine acceptability studies should be done among adults in high burden countries to ensure high coverage post-approval. As was observed with the Ebola virus vaccine, funds should be committed to developing vaccination platforms for adults which are self-sustaining.

2. Greater investment in Research and Development by governments

Under the coordination of a global health entity such as the WHO, governments of high burden countries can be mobilized to contribute financially or in kind to vaccine research and development process. Many of these have reported unprecedented economic growth over the last decade. In kind or indirect contributions will also expedite the development process. For example, by removing barriers to research including costly tariffs on research equipment, including mandatory research training in the curriculum for health workers, thereby enhancing local capacity to conduct trials or by facilitating speedy ethical and regulatory review of research protocols.

3. The chest radiograph is still a useful diagnostic tool in trials

The chest radiograph should be included in infant trials with centralized, blinded, expert adjudication. New diagnostics, including point of care tests with high sensitivity and specificity that utilize minimally invasive techniques are needed. In addition to increasing end-point detection for trials, it will likely reduce TB mortality.

4. Limited autopsies to increase end-point yield in paediatric trials

Techniques for limited autopsies should be part of infant trials, as the causes of death were only identifiable in 31% of cases in our study. It is likely that there were TB deaths among these based on other autopsy studies in similar settings (61).

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Chapter 8

English Summary

Tuberculosis is a major public health problem, with particularly high morbidity and mortality among infants. The only licensed TB vaccine, BCG confers only partial protection against pulmonary disease, the most common and most transmissible form of the disease. Novel TB vaccines with better efficacy are urgently needed as part of the armoury of tools to eliminate TB by 2050. To properly design and implement these trials, several factors need to be borne in mind. These include, the most suited trial population, estimates of disease incidence, cohort retention and mortality. There are also diagnostic considerations, such as the utility of the chest radiograph and the possible role of the more prevalent non-tuberculous mycobacteria in confounding TB diagnosis. Finally, there are practical factors related to selection and development of research sites with the required trial infrastructure and expertise in high burden countries. This thesis explored the factors of design of new tuberculosis vaccine trials, diagnostic considerations and process of site development.

In **Chapter 1** the status of TB control and the need for new tools including a novel TB vaccine is examined. I explored the strengths and limitations of BCG vaccination, and reviewed the arguments around infant versus adult vaccination. In **Chapter 2**, we found a high culture confirmed TB incidence among infants (0.2/100 pyo) in Western Kenya showing recent on-going transmission. Definite TB was associated with infant HIV infection, higher Keith Edward Scores. Modeling study data of incidence and retention, we presented sample size estimates for future vaccine trials. In addition, cohort retention was positively associated with infant HIV infection and complete vaccination status at the four-month follow up visit. Thus, cohort characterization is useful in guiding future trial design and sample size estimates.

We examined post-neonatal mortality, its predictors, immediate and underlying causes of death in infants in **Chapter 3.** Post-neonatal mortality was high in 2009 to 2011 (58/1000 pyo) but lower than preceding years. Infant HIV status and being born in a health facility were positive (HR 10.3; 95% CI: 6.40, 16.7) and negative predictors (Hazard Ratio (HR) 0.54; 95% CI: 0.34, 0.84) respectively, of mortality at 12 months. Mothers delivering in a health facility may have different health consciousness, behaviour and access to health facilities that lead to longer term mortality benefits. Pneumonia and dehydration were the most frequently identified immediate causes of death. Since the study was conducted, pneumococcal and rotavirus vaccines were added to the national immunization schedule, therefore a decline in mortality is likely to be sustained. These gains are also positive for end-point detection in vaccine trials. In **Chapter 4** we examined the utility and inter-rater agreement of chest radiographs, a key diagnostic of composite TB endpoints in infant trials. Agreement on abnormalities consistent with TB was slight at best (k=0.14; (95%CI: 0.10 - 0.18) but was significantly better than agreement on any abnormality or normal radiographs. Hence the chest radiograph still has some utility as a diagnostic tool for vaccine trial endpoints despite its noted limitations. In **Chapter 5**, the clinical relevance, incidence and types of Non-Tuberculous Mycobacteria were described. NTM incidence was 5.7/1,000 person-years; 95% CI (3.5, 8.7). We found no association between TST positivity and NTM isolation OR 1.15 (0.41, 3.25). Isolation in all cases represented colonisation and not disease, based on the American Thoracic Society Criteria. Infants diagnosed with TB were more likely to have NTM isolation (odds ratio 11.5; 95% CI 3.25, 41.0). Therefore, NTMs are unlikely to

confound TB diagnosis in future trials. In **Chapter 6** the process of TB vaccine trial site set-up from 2007, infrastructure development and growth in expertise to conduct TB vaccine trials is described. Site development was gradual and incremental. Challenges encountered in each study were solved and turned into learning points, improving the implementation of future vaccine studies. Key lessons in site development include, the importance of conducting thorough epidemiological studies to provide context for clinical trials, including investigators in discussions of assays to be run on bio-banked samples. In context of the momentous breakthrough of an Ebola vaccine, I compare the TB vaccine development pipeline and suggest a shift to greater industry and government involvement, as well as greater global coordination of efforts by the WHO, will accelerate the development and approval of a novel TB vaccine. In **Chapter 7,** I reviewed trial and post-trial considerations in selecting the most appropriate target population for future TB vaccine trials. Trial related factors included the incidence of disease and the impact on trial sample sizes, complexity of vaccine design for a particular high risk group. Post-trial factors included the availability of vaccination platforms, the quantity and speed of impact on the TB epidemic, among others. I also reviewed the TB vaccine candidates targeting infants in phase I and II trials.

CONCLUSIONS

The high incidence of TB among infants makes them a suitable trial population. On the other hand, they hardly transmit disease and therefore vaccinating them will have a much slower impact on the TB epidemic, adults could be a more urgent priority. Nevertheless, developing vaccination platforms to reach adults will be costly and logistically challenging.

The chest radiograph is still an important diagnostic tool in infant TB vaccine trials. NTMs among presumptive TB patients are either colonizing organisms or super-imposed on pulmonary TB. The development of TB vaccine trial sites requires multi-national collaborations similar to what was observed during the Ebola outbreak of 2014-2016.

Recommendations

1. Selection of high risk populations will improve the speed and feasibility of TB vaccine trials. This may require inclusion of ignored sub-groups such as prisoners who suffer disproportionately high TB burden compared to the rest of the population.
2. Greater involvement of governments in both high and low burden countries will expedite the possibility of a new, more highly efficacious TB vaccine in our lifetimes.
3. Limited autopsies in infant trials may enhance our understanding of the disease process and increase trial end-point yield.

Chapter 9

Nederlandse samenvatting

Tuberculose (TBC) is een groot probleem voor de volksgezondheid, waarbij de hoge morbiditeit en mortaliteit bij zuigelingen het probleem alleen maar groter maakt. Het enige erkende TBC-vaccin, BCG, biedt slechts gedeeltelijke bescherming tegen de pulmonale (long) vorm van de ziekte. Nieuwe TBC-vaccins met een betere werkzaamheid zijn dringend nodig als onderdeel van het arsenaal om TBC rond 2050 te kunnen elimineren. Om hier goed onderzoek naar te kunnen doen, is het essentieel om inzicht te hebben in de meest geschikte onderzoekspopulatie, schattingen van ziekte-incidentie, cohortretentie en mortaliteit van TBC in de te onderzoeken patiëntenpopulatie. Daarnaast zijn ook diagnostische overwegingen, zoals het nut van de thoraxfoto en de mogelijke rol van de niet-tuberculeuze mycobacteriën. Ten slotte zijn er praktische factoren in verband met de selectie en ontwikkeling van onderzoek sites met de vereiste studie-infrastructuur en expertise in landen waar TBC veel voorkomt. In dit proefschrift worden de ontwerp-factoren van nieuwe TBC-vaccintrials, diagnostische overwegingen en het proces van ontwikkeling van de onderzoek locatie besproken.

In **hoofdstuk 1** wordt de status van TBC-bestrijding beschreven en onderzochten we de sterke punten en beperkingen van BCG-vaccinatie. Daarbij brachten we ook de argumenten rond vaccinatie van zuigelingen versus volwassenen in kaart. In **hoofdstuk 2** beschrijven we onze bevinding van de hoge TBC-incidentie bij zuigelingen in West-Kenia, wijzend op recente en doorgaande transmissie. Met het modelleren van onderzoeksgegevens over incidentie en retentie, geven we schattingen voor de omvang van steekproeven voor toekomstige vaccinonderzoek. Bovendien was cohortretentie positief geassocieerd met hiv-infectie bij kinderen en volledige vaccinatiestatus bij het follow-up bezoek van vier maanden. Daarom is cohort karakterisering nuttig bij het opstellen van toekomstig trial ontwerpen en het schatten van de omvang van steekproeven. Het onderzoek naar post-neonatale mortaliteit, de voorspellers ervan, directe en onderliggende doodsoorzaken bij zuigelingen staat beschreven in **hoofdstuk 3**. De post-neonatale mortaliteit was hoog in 2009 tot 2011 maar lager dan in voorgaande jaren. De hiv-status van baby's en het geboren worden in een gezondheidsinstelling waren respectievelijk negatieve en positieve voorspellers van mortaliteit na 12 maanden. Moeders die in een gezondheidsinstelling bevielen, kunnen een ander gezondheidsbewustzijn, gedrag en toegang tot gezondheidsfaciliteiten hebben, die leiden tot voordelen op de langere termijn. Longontsteking en uitdroging waren de meest frequent geïdentificeerde directe doodsoorzaken bij de zuigelingen. Sinds het onderzoek werd uitgevoerd, werden pneumokokken- en rotavirusvaccins aan het nationale immunisatieschema toegevoegd, waardoor een daling van de mortaliteit waarschijnlijk zal aanhouden. In **hoofdstuk 4** beschrijven we in welke mate er sprake is van verschil in beoordeling van thoraxfoto's door verschillende artsen. De X-thorax is namelijk een belangrijk diagnostisch onderdeel van de samengestelde TBC-eindpunten in onderzoek van zuigelingen. Het bleek dat in de beoordeling van TBC-afwijkingen er een goede overeenstemming was tussen de verschillende beoordelaars, maar dat deze overeenstemming minder sterk was als het andere afwijkingen betrof. Toch kon gesteld worden dat dat de thoraxfoto nog steeds nut heeft als een diagnosticum bij Tbc-patiënten en dus in TBC (vaccinatie) studies, ondanks de geconstateerde beperkingen. In **hoofdstuk 5** werden de klinische relevantie, incidentie en soorten van niet-tuberculeuze mycobacteriën (NTM) beschreven. We hebben geen verband gevonden tussen Mantoux (TST)-positiviteit en NTM-isolatie. Gebaseerd op de criteria

van de American Thoracic Society, konden we bij geen van de patiënten waarbij een NTM-bacterie geïsoleerd werd, ziekte aantonen. Daarom is het onwaarschijnlijk dat NTM's de diagnose van TBC in toekomstige studies zullen verstoren. In **hoofdstuk 6** wordt het proces beschreven van het opzetten van een TBC-vaccintrial locatie, de ontwikkeling van infrastructuur en de groei van expertise voor het uitvoeren van trials met TBC-vaccinatie. De ontwikkeling van de site verliep geleidelijk en stapsgewijs. Uitdagingen in elk onderzoek werden opgelost en omgezet in leerpunten, waardoor de uitvoering van toekomstige vaccintrials mogelijk kan worden verbeterd. In de context van de gedenkwaardige doorbraak van een ebolavaccin staat in hoofdstuk 6 ook de vergelijking van de ontwikkelingspijplijn voor TBC-vaccins. Daarbij valt op dat er een mogelijk meerwaarde is als er een grotere betrokkenheid van de industrie en de overheid zou zijn in de TBC-vaccinatiestudies, evenals een grotere wereldwijde coördinatie van inspanningen door de WHO.

CONCLUSIES

De (triest) hoge incidentie van TBC bij zuigelingen maakt hen tot een geschikte populatie om TBC-vaccinonderzoek in te doen. Aan de andere kant is de transmissie van TBC vanuit zuigelingen zeer beperkt, waardoor ook een goed vaccin slechts heel langzaam impact zal hebben op de TBC-epidemie. De röntgenfoto van de longen is nog steeds een belangrijk diagnosticum en bruikbaar als uitkomst parameter in het onderzoek naar de effectiviteit van TBC-vaccins in zuigelingen. NTM-infecties bij patiënten die verdacht worden van TBC zijn ofwel slechts koloniserende organismen of zijn additioneel aan de een *M. tuberculosis* infectie. De ontwikkeling van nieuwe triallocaties voor TBC-vaccinstudies vereist multinationale samenwerkingsverbanden.

Aanbevelingen

1. Selectie van patiënten-populaties met een hoge kans op TBC zal de snelheid en haalbaarheid van vaccintrials verbeteren. Hierbij kan gedacht worden aan het opnemen van bijvoorbeeld gevangenen (bekend vanwege een hoge TBC-incidentie) in toekomstige studies.
2. Een grotere betrokkenheid van overheden in zowel landen met een hoge als met een lage TBC-belasting zal de mogelijkheid van een nieuw, zeer doeltreffend TBC-vaccin binnen onze generatie versnellen.
3. Beperkte autopsies in kindertrials kunnen ons begrip van het ziekteproces verbeteren en de eindpuntopbrengst van de trial verhogen.

Chapter 10

Epilogue

ABOUT THE AUTHOR

Grace Kaguthi was born in Nairobi, Kenya on 21st September 1982. She completed her undergraduate degrees in Medicine and Surgery at the University of Nairobi in 2006 and her MSc in Clinical Trials from the University of London in 2012. In 2008, having worked for the Ministry of Health, she joined the TBVAC research project as a study coordinator, conducting the studies described in this thesis. Since then she has led more than a dozen phase II and III trials of TB vaccines among adults and infants, as well as malaria, tuberculosis and sickle cell disease drug therapies. In 2014, she began her PhD. She has been supervised and mentored by Prof Jan Hendrik Richardus, Dr Jurriaan E.M. de Steenwinkel and Dr Suzanne Verver.

PHD PORTFOLIO: SUMMARY OF PHD TRAINING AND TEACHING

Name PhD Candidate: **Grace K. Kaguthi**	Promoter**:** **Prof. dr. J.H. Richardus**
Erasmus MC Department: **Department of Public Health**	Co-promoter: **dr. J.E.M. de Steenwinkel**

1. PhD training

Courses	**Year**	**ECTS**
Good clinical practice (online)	2008 - 2020	1
Erasmus summer program	2012	4
Erasmus summer program	2013	4
Advanced vaccinology (Pasteur institute, Annecy)	2013	3
Publications masterclass (Vienna school of clinical research)	2014	1
Advanced epidemiological analysis (University of London - LSHTM)	2015	4
Inferential statistics (Centre for statistical analysis and research - CESAR)	2014	1.5
International course on clinical management of drug resistant TB (Bangkok)	2014	1.5
Presentations		
3rd Global TB vaccine forum (Estonia)	2010	1
6th EDCTP Forum (Addis Ababa)	2011	1
42nd World Union Conference on Lung Health (Lille)	2011	1
61st ASTMH (Atlanta)	2012	1
45th World Conference on Lung Health (Barcelona)	2014	1
5th Global TB Vaccine Forum (Cape Town)	2014	1
4th Global TB Vaccine Forum (Shanghai)	2015	1
68th ASTMH (Washington DC)	2019	1

2. Teaching

KEMRI Statistical Analysis and Publications workshop	2017	1
KEMRI Systematic Reviews and Meta-analysis workshop	2018	1

PUBLICATIONS

Chest Radiographs for Pediatric TB Diagnosis: Inter-rater Agreement and Utility. Interdisciplinary Perspectives on Infectious Diseases Volume 2014, Article ID 291841, **G. Kaguthi**, V. Nduba, J. Nyokabi, F. Onchiri, R. Gie and M. Borgdorff

A double-blind, randomised, placebo-controlled, dose-finding trial of the novel tuberculosis vaccine AERAS-402, an adenovirus-vectored fusion protein, in healthy, BCG-vaccinated infants (Vaccine. 2015 Jun 9;33(25):2944-54. doi: 10.1016/j.vaccine.2015.03.070. Epub 2015 Apr 28.) Tameris M, Hokey DA, Nduba V, Sacarlal J, Laher F, **Kiringa G**, Gondo K, et al

Predictors of post neonatal mortality in Western Kenya: a cohort study. **Grace Kaguthi**, Videlis Nduba, Martien W. Borgdorff, Suzanne Verver. The Pan African Medical Journal 2018;31:114. doi:10.11604/pamj.2018.31.114.16725

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The incidence of tuberculosis in infants, Siaya District, western Kenya. Videlis Nduba[§], **Grace Kaguthi**[§], AH van 't Hoog, E.M.H.Mitchell, M. Borgdorff. Accepted, The Pediatric Infectious Disease Journal, Jul 2020. *([§]contributed equally)*

The impact of the nurses', doctors' and clinical officer strikes on mortality in four health facilities in Kenya. **Grace Kaguthi**, Videlis Nduba, Mary Beth Adam. Submitted, BMC Health Services Research

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I especially thank my parents. There is no doubt, your names should be appended on the cover page. This belongs as much to me as it does to you. Your encouragement and grit are irreplaceable. I dedicate this to you.