Cytohistologic correlation of suspected Cervicofacial Head & Neck Extra-Pulmonary Tuberculosis in children: A retrospective case series.

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February 2022

A research report submitted to the Faculty of Health Sciences, University of Cape Town, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Anatomical Pathology .

DECLARATION

I, Dr Christopher Noel Jackson, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Date: 11 February 2022

Table of Contents

Acknowledgements

I would like to thank the following people for their meaningful contributions:

Prof. K. Pillay, Department of Anatomical Pathology, NHLS/ University of Cape Town, for her role as supervisor.

Prof. S. Peer, Division of Otorhinolaryngology, University of Cape Town, for her role as co-supervisor.

Dr A. Ramburan, Department of Anatomical Pathology, NHLS/ University of Cape Town, for her role as co-supervisor.

Dr S. Singh, Department of Anatomical Pathology, NHLS/ University of Cape Town, for her role as co-supervisor.

Trischke Watson, Department of Anatomical Pathology, NHLS/ University of Cape Town, for assisting with PCR.

The medical students from Netherlands for their initial research and for laying the foundation for mine.

The Department of Medical Microbiology, NHLS/ University of Cape Town for the TB culture and GXP results.

Mr Ray Kriel, Department of Anatomical Pathology, NHLS/ University of Cape Town for statistical assistance.

List of tables

List of figures

Abbreviations

AIDS : Acquired immunodeficiency disease syndrome

BCG : *M. bovis* bacillie Calmette-Guérin

EPTB : Extrapulmonary tuberculosis

FFPE : Formalin Fixed Paraffin Embedded

FNA : Fine needle aspiration

GXP : GeneXpert

HIV : Human immunodeficiency virus

MOTTS : Mycobacteria other than tuberculosis

MTb : Mycobacteria tuberculosis

MTC : Mycobacterium complex

ND : Not done

NTM : Nontuberculous mycobacteria

Pap : Papanicolaou

PCR : Polymerase chain reaction

PPV : Positive predictive value

PNT : Papulonecrotic tuberculid

TB : Tuberculosis

TBC : TB culture

TSC : Tissue sectioning control

WHO : World Health Organisation

Abstract

Background

Tuberculosis (TB), especially extrapulmonary TB, is a difficult diagnosis to make in children due to the paucibacillary nature of paediatric disease and difficulty in obtaining sputum and tissue samples for microbiology confirmation. Lymphadenopathy in children with suspected cervicofacial TB are amenable to FNA or surgery for further cytological and histological assessment. It is therefore important to understand how well the morphologic features (from cytology and histology) correlate with defined reference standards (TB culture and molecular evidence of MTb) for the diagnosis of TB and the reliability of these features.

Aim

The aim of the study is to determine how well the cytology and histology-made TB diagnoses in children with suspected cervicofacial EPTB correlates with TB culture and MTb PCR results.

Materials and methods

This is a descriptive retrospective study that involved a re-appraisal of all patients with suspected cervicofacial EPTB who had histology and cytology performed at Red Cross Children's Hospital identified from the National Health Laboratory Service (NHLS) Trakcare system over a 5 year period (2012-2017). Following identification of histopathology accession numbers, histopathology reports and slides were retrieved from the archive of the Division of Anatomical Pathology/ National Health Laboratory Service, Red Cross Children's Hospital, Cape Town for evaluation. In addition, results for Genexpert testing and TB culture were identified using the National Health Laboratory Service (NHLS) Trakcare system. In patients that did not have either of the above, MTb PCR testing was performed.

Results

Data from the reports of 76 children with suspected cervicofacial TB were included in this study. More biopsies were submitted for histology (48) than for cytology (22). Six children had biopsies for both cytology and histology done. Most children had suspected and confirmed TB involvement of the cervical lymph nodes. On histology, the feature that correlated the best with proven TB was necrotising granulomatous inflammation (79.5% of cases had confirmed TB). On cytology, necrotising inflammation, necrotising granulomatous and non-necrotising granulomatous inflammation correlated well with proven TB. The sensitivity of cytology was 77.3% against TB culture and 81.8% against GXP for TB diagnosis. Whilst for histology the sensitivity was 82.5% against TB culture and 90.3% against GXP as reference standards for TB diagnosis.

Conclusion

FNA for cytology is a safer procedure with less complications than biopsy for histology. Also, the use of cytology together with a GXP renders a rapid and accurate diagnosis of TB and our findings are supportive for the combined use of these modalities as first line investigations. However, every attempt should still be made to obtain a sample for TB culture (as the WHO recommended gold standard for TB confirmation).

Chapter 1: Introduction

1.1. Background

Tuberculosis (TB) is a major cause of death globally and is one of the top ten causes of death in the world. Since 2017, TB has been the leading cause of death from a single infectious agent, ranking above HIV/AIDS globally. In 2019, an estimated 10 million people were affected with 14 million TB deaths (14% among HIV-positive people) globally. Africa accounted for 25% of the global TB burden with an estimated 2.5 million cases in 2019. TB is a major public health challenge in South Africa with the country accounting for 3.6% of the global total cases (an estimated 301000 TB cases) in 2019. There was a total of 63000 TB related deaths in 2019, with two-thirds of those who died being HIV-positive (1,2).

The Western Cape, and in particular Cape Town, has one of the highest TB incidence rates in South Africa. High risk groups that are particularly vulnerable to developing TB disease include the HIV-positive, diabetics, household contacts of people with TB, pregnant women and children under the age of 5 years (3).

Children (<13 years of age) carry a high burden of TB disease. In 2019, children accounted for 12% of all cases globally (1). In 2018 children accounted for 7% of all TB case notifications in South Africa (2). A study in Cape Town found that 13.7% of cases entered into TB registers were those of children. However, this is probably not a true reflection of the childhood TB burden as cases are under reported due to the difficulties in diagnosis of paediatric TB, the application of inconsistent diagnostic criteria and inconsistencies in notification practices in endemic areas (1,4).

The paediatric population, specifically those under the age of 5 years are particularly vulnerable to developing TB infection and severe forms of TB disease (e.g.,

disseminated TB, TB meningitis). Approximately 80% of childhood TB deaths occur in this age group (5).

Factors that contribute towards the development of childhood TB infection includes an immature host immunity, poverty, malnutrition, household overcrowding and HIV coinfection (6). In HIV coinfected children, there is also a higher risk of progression to TB disease, an increased risk of developing disseminated disease and TB is the leading cause of death in these children (5). Adding to the burden of childhood TB is the poor initiation of treatment rate for children, under the age of 5 years of 10.2% nationally and 32.8% in the Western Cape (7).

Lastly, childhood TB is a marker of the ongoing and recent transmission of infection within a community and the infected children represent a source from which a large proportion of future cases will possibly arise (8).

The Western Cape and in particular Cape Town has a high burden of TB disease, and it is an important cause of morbidity and mortality in children. Therefore, an accurate and rapid diagnosis of TB is important in order to expedite the initiation of appropriate therapy.

1.2. Microbiology

Tuberculosis is a communicable disease caused by the *Mycobacterium tuberculosis* complex (MTC), a group that includes *Mycobacterium tuberculosis* (MTb)*, M. bovis* bacillie Calmette-Guérin (BCG), *M. africanum, M. canettii, M. bovis, M. microtti, M. orygis, M. caprae, M. pinnipedii, M. suricattae and M. mungi*. The complex is part of the genus *Mycobacteriaceae* which comprises four major groups: *Mycobacterium leprae*, *Mycobacerium ulcerans* and the nontuberculous mycobacteria (9). The mycobacteria species are within the order Actinomycetales, which includes other

bacteria such as *Corynecbacterium, Nocardia* and *Rhodococcus*, all of which have unique mycolic acids within the cell wall (10).

The virulence of mycobacteria is due to several factors; its ability to evade the immune system via intracellular growth, the high lipid content of the cell wall which provides resistance to both antibiotic penetration and lysosomal lysis and the expression of bacterial proteins that modulates the hosts immune response (11).

Mycobacteria are also increasingly associated with drug resistance which is mediated by mutations and rearrangements in its single, circular chromosome (12).

1.2.1. *Mycobacterium tuberculosis*

Mycobacteria tuberculosis is the most well-known member of the *Mycobacteria tuberculosis* complex and affects both humans and animals. MTb are neither Gram-positive nor Gram-negative; are nonmobile, non-spore forming rod-shaped bacilli 1 to 4μm long and are 0.3 to 0.5μm wide. The bacilli are slow-growing, obligate aerobes which survive and proliferate as intracellular parasites (10).

1.2.2. *Mycobacterium bovis*

The live, attenuated BCG strain of *Mycobacterium bovis* is present in the TB vaccine and is administered after birth as part of the national and World Health Organisation (WHO) immunisation plan to prevent disseminated disease in young children in high-prevalence areas. HIV-infected infants are at risk of developing BCG disease which causes axillary or supraclavicular lymphadenopathy which may occasionally lead to disseminated disease and death (6, 13).

However, despite these risks, the WHO still recommends that all asymptomatic HIV-exposed infants in TB-endemic areas receive the BCG vaccination with follow-up monitoring for development of BCG-related disease (4).

1.2.3. *Mycobacterium leprae* and *Mycobacterium ulcerans*

M. leprae is a slow growing Mycobacterium species, and causes leprosy that affects the skin, peripheral nervous system and the mucous membranes.

M. ulcerans is a strictly human pathogen and causes a wide range of ulcerative lesions involving the skin and subcutaneous tissues after exposure to contaminated water and soil (9).

1.2.4. Nontuberculous Mycobacteria (NTM)

Nontuberculous mycobacteria are all the other mycobacteria other than the ones included in the MTC, *M. leprae* and *M. ulcerans*. Mycobacteria species within this group includes the Mycobacterium avium complex (MAC) which comprises *M. avium* and *M. intracellulare.* *M. kansasii* is another NTM, which causes disease predominantly in the immunocompromised (9).

1.3. Pathogenesis

1.3.1. The natural history and spectrum of TB

Typically, TB is acquired and transmitted via droplet spread of aerosolised bacilli from cough droplets. The factors that determine the probability of transmission includes the immune status of the exposed person, infectiousness of the person with TB disease, environmental factors that affect the concentration of MTb and the proximity, frequency, and duration of exposure (14). Following inhalation of the aerosolised droplets, if the bacilli are not trapped by the defence mechanisms of the upper aerodigestive tract and mucociliary apparatus of the airways, the bacilli enter the alveolar spaces. Typically, the inhaled bacilli implant in the distal airspaces of the lower part of the upper lobe or the upper part of the lower lobe in the subpleural region which is the most well aerated region of the lung. This is known as the Ghon focus

and is a 1- to 1.5 cm area of gray-white inflammation with consolidation. The center of this focus may undergo central necrosis which appears macroscopically as white, granular, or cheese-like and has classically been described as “caseous necrosis”. The associated draining tracheobronchial and mediastinal lymph nodes may also be affected, and the combined parenchymal lung nodule and nodal complex is known as the Ghon complex. The bacilli elicit an acute inflammatory response to eradicate the infection and the alveolar macrophages phagocytose the bacilli. However, the MTb inhibits the formation and maturation of the phagolysosome, which results in unabated proliferation of the bacilli (13).

About 3 weeks after infection, a Type IV delayed hypersensitivity and T-cell mediated response to the bacillus occurs which results in T-cell mediated macrophage activation and killing of the bacteria. This also results in the maturation and transformation of the alveolar macrophages into epithelioid cells and subsequent granuloma formation. This immune response controls the infection and results in latent TB infection (15).

In 95% of cases, the development of cell-mediated immunity controls the initial infection and the Ghon complex undergoes progressive fibrosis and calcification (radiologically detectable as the Ranke complex). This complex retains viable bacilli and are thus sources for long-term infection and are potential foci for reactivation (13).

1.3.2. Primary and secondary TB

Primary TB infection must be differentiated from active TB disease as the management approaches of the two conditions differ. Active forms of TB disease include primary disease and reactivation/secondary TB.

In most healthy people, primary TB infection is asymptomatic (90%) and the viable bacilli may remain dormant within the granulomas for many decades. Infection leads to the development of delayed hypersensitivity to MTb antigens which can be detected

by tuberculin skin tests. The outcome of TB infection in a previously well, immunocompetent person is dependent on the development of an anti-mycobacterial T-cell mediated immune response which controls the host response to the bacteria. When the host immunity is lowered, the infection may be reactivated, producing active disease or secondary tuberculosis. Primary TB disease occurs when there is failure of the adaptive immune system to adequately control the primary infection, which then results in unabated replication of MTb. There may be both lymphatic and haematogenous spread of bacilli throughout the body. In 90% of patients, this occurs during the first year of life after exposure and any active disease within 2 years of infection is considered as primary TB. Progression to primary disease frequently affects infants and the risk of progression decreases with age. The progression of TB infection towards disease occurs in only a small subset of patients. The risk of disease progression is due to underlying host-specific factors such as the child's age, nutritional status, immune status and any exposure to TB contacts who are mostly adults. Reactivation disease occurs after a variable period of latency, usually in the setting of immune deficiency or suppression. The granulomas fail to contain the replicating bacilli and breakdown resulting in the release and spread of bacilli through the airways. Reactivation does not generally occur until late childhood and adolescence (13, 14, 16).

Secondary pulmonary tuberculosis classically involves the apex of the lungs. Because of prior sensitisation, a prompt immune response is mounted that tends to wall off the focus of infection. Cavitation occurs readily in secondary TB and airway erosion is an important source of infection as the individual now coughs sputum that contains the bacilli (13).

1.3.3. Disease Progression

TB disease progression may extend along several different pathways:

- Progressive pulmonary tuberculosis occurs when the apical lesion expands into adjacent lung and causes erosion of blood vessels and bronchi resulting in cavitary disease.
- Miliary TB pulmonary disease is due to lympho-haematogenous spread of TB through the lung parenchyma resulting in a radiologic appearance likened to scattered millet seeds.
- Pleural TB involvement with development of pleural effusions, empyema and obliterative fibrous pleuritis with progressive pulmonary tuberculosis.
- Endobronchial, endotracheal and laryngeal tuberculosis develops via spread from lymphatic channels or from expectorated infectious material.
- Systemic miliary tuberculosis occurs when bacilli disseminate haematogenously and may involve any visceral organ or multiple organs (13).
- Cervical lymphadenitis is the most frequent extrapulmonary manifestation of tuberculosis. In HIV-negative individuals, the involved lymph nodes are unifocal and localised. HIV-positive people almost always have multifocal disease, systemic symptoms and either lung or other organ involvement by active TB (13). Of historical interest, cervical lymphadenitis was known as scrofula and as the “king’s evil” in Europe, where the royal touch was believed to cure the disease (17).
- Cutaneous manifestations are rare, but includes scrofuloderma, lupus vulgaris and tuberculid reactions. Scrofuloderma occurs when overlying skin of a tuberculous lymph node is involved by MTb and commonly affects the axillary lymph nodes. Lupus vulgaris is due to haematogenous seeding commonly

involving the face and results in a plaque with central ulceration. Tuberculid reactions are a spectrum of entities due to tuberculin hypersensitivity reactions and include papulonecrotic tuberculids, erythema induratum of Bazin, lichen scrofulosorum and nodular granulomatous phlebitis. Papulonecrotic tuberculids are papular lesions with umbilicated and occasionally necrotic centres (18).

1.3.4. Childhood TB

Childhood acquisition of MTb is almost always from adult to child transmission who typically have cavitary secondary disease. Children generally have paucibacillary and noncavitary pulmonary TB disease, along with an inadequate cough physiology to expectorate infectious sputum and thus transmission of TB from children even to close contacts is unlikely (19, 20). TB infection in childhood therefore reflects community exposure of the child to the sputum of smear-positive adults and therefore to the aerosolised TB bacilli and subsequent transmission from infected adults (21).

1.4. Microscopic Pathology

The classically described microscopic morphology of TB is necrotising granulomatous inflammation. Granulomatous inflammation is a form of chronic inflammation, and which is a tissue response to a variety of infective, autoimmune, allergic, toxic and neoplastic conditions (23). Under light microscopy a granuloma appears as a circumscribed nodular collection of mature macrophages which may or may not show an area of central necrosis, variable surrounding fibrosis, associated multinucleated giant cells and associated lymphocytic inflammation. The mature macrophages show epithelioid cytomorphology with round, oval to twisted nuclei, indistinct cell borders with a moderate to abundant amount of granular pale to eosinophilic cytoplasm and

indistinct cell borders. Multinucleated giant cells are formed by fusion of multiple macrophages and range from foreign body types (with central nuclei) to Langhans type (show a horseshoe-like distribution of nuclei). Necrosis occurs when the inciting agent is either highly toxic to the macrophages or a vigorous delayed hypersensitivity response is elicited. Necrosis also occurs in numerous conditions other than from infections including neoplasms, sarcoidosis, and autoimmune conditions.

Granulomatous inflammation encompasses a wide range of morphologic appearances, ranging from poorly formed ones composed of loosely arranged histiocytes to well-formed granulomas. The classic morphology of TB is necrotising granulomas, however non-necrotising granulomas are also commonly encountered. Necrosis is often absent in early lesions but can be so extensive in more advanced cases that the granulomatous nature of the process is masked (23, 24).

Individual granulomatous tubercles are microscopic but may be visible macroscopically when several tubercles coalesce. Immunosuppressed people often do not form the classic granulomas and instead have poorly formed granulomas, suppurative inflammation, non-specific chronic inflammation or purely necrotising inflammation (13).

There isn't usually any significant fibrosis accompanying the granulomas in active TB disease; however healed lesions can reveal dense hyaline type fibrosis often with accompanying dystrophic calcifications. The accompanying lymphocytes are predominantly CD4-positive T-cells whilst CD8-positive T-cells are fewer and confined to the periphery of the granulomas (22).

1.5. Diagnosis

TB which manifests in the lungs is known as pulmonary TB (PTB), while TB which affects other sites in the body is referred to as extrapulmonary TB (EPTB). A study from Cape Town, South Africa reported that 72/439 (16.4%) of children that were treated for active pulmonary TB had concomitant extrapulmonary disease while 65.4% of the children with extrapulmonary TB had no concurrent intrapulmonary disease manifestations (24).

The definitive diagnosis of TB in children is challenging due to the non-specific nature of the signs and symptoms, the difficulty in obtaining samples for microbiological confirmation and the paucibacillary nature of paediatric disease. The diagnosis of paediatric TB is made based on a combination of clinical history, findings from the physical examination, the Tuberculin skin test, radiographic findings, TB culture and GeneXpert results. The definitive diagnosis of TB is made by TB culture or polymerase chain reaction (PCR) demonstration of MTb in an affected organ via GeneXpert (25, 26).

1.5.1. Clinical Presentation

TB lymphadenitis is the most common cause of persistent cervical lymphadenopathy and is the most common form of EPTB in children. In a study conducted at Tygerberg Hospital, Cape Town, mycobacterial infection was diagnosed in a quarter of fine needle aspiration biopsy (FNAB) of patients who presented with cervical nodal lymphadenopathy (26). TB lymphadenitis is considered to have its origin in the lymphatic spread of bacilli from a primary pulmonary focus but which can also originate from primary foci in the mouth, tonsils, oropharynx or tissues of the head and neck. Other head and neck sites which may be involved by TB include the larynx, middle

ear, oral cavity, nose (in TB vulgaris), pharynx, maxillofacial structures, mastoid bone, neck spaces and the cervical spine (28).

The clinical manifestations of TB are variable and are dependent on the primary site of infection, the stage of disease and the immune status of the patient. Common symptoms include weight loss, failure to thrive, drenching night sweats and unusual fatigue. Older children may have respiratory symptoms like adults such as cough, dyspnoea, haemoptysis, wheeze and fever. Patients with TB lymphadenitis usually present with painless, slow enlargement of a single or group of lymph nodes. The duration of symptoms at presentation is typically 1-2 months, there is no visible local cause for lymphadenopathy and there is a lack of response to a course of antibiotics (27). The median lymph node size is 3cm but sizes up to 10cm have been described. There is usually unilateral involvement, and the lymph nodes may be soft and matted, show abscess or fistula formation (26).

1.5.1.2. Sites of involvement of head and neck TB

A thorough otolaryngologic examination is necessary to identify possible sites of involvement including the cervical lymph nodes, larynx, ear, nose, oral cavity, pharynx, skin and cervical spine. The anterior triangles, posterior triangles and submandibular areas of the head and neck are frequently involved during presentation of cervical TB lymphadenitis. The larynx is the second most involved site of cervicofacial TB, accounting for approximately 1% of cases. Patients with laryngeal involvement may present with hoarseness, cough, ulceration, odynophagia, dysphagia and weight loss. Patients with oral and pharynx TB present frequently with odynophagia, halitosis and mucosal ulceration. TB of the nasal cavity may cause nasal obstruction, epistaxis and sinonasal discharge which may be bloody or purulent. Involvement of the paranasal

sinuses may result in bony destruction and ophthalmic manifestation such as diplopia and exophthalmos (29, 30).

Cervical spine involvement occurs as a result of direct extension from infected lymph nodes or due to the haematogenous spread of bacilli. TB osteomyelitis is also known as Pott's disease and can cause destruction of the intervertebral cervical disc spaces and spondylitis. Pott's disease involving the cervical spine has potentially severe neurological complications such as hemiparesis, quadriplegia and retropharyngeal abscesses (31).

1.5.2. Ancillary diagnostic tests

Ancillary diagnostic tests are useful adjuncts which raises the suspicion of or is supportive of the diagnosis of TB.

1.5.2.1. Tuberculin skin tests

The standard screening test for TB is the tuberculin skin test (TST) or Mantoux test which involves the intradermal injection of a tuberculin purified protein derivative and the assessment of the size of induration of the cutaneous reaction after 48 to 72 hours. The cutaneous reaction is due to a delayed hypersensitivity immune response to the mycobacterial antigens and depending on the size of the induration can indicate TB infection (32).

However, the TST does not discriminate between infection and active TB disease, as a positive result only indicates that the patient has at some time, been infected with MTb. False-positive TST reactivity occurs in children who have received prior Bacille Calmette-Guerin (BCG) vaccination or recent nontuberculous mycobacterial infection. A negative TST does not definitively exclude TB disease. A false negative result may be commonly seen in HIV infection, severe malnutrition and in disseminated TB disease (26).

In addition, the TST is limited by the subjectivity of its interpretation as well as logistically by the need for a follow up visit to have the test read (25).

1.5.2.2. Imaging modalities

A proportion of patients with EPTB have concomitant pulmonary involvement and therefore radiology may be a useful test and may show both parenchymal and pleural disease. The other common chest X-ray findings include enlargement of mediastinum due to hilar lymphadenopathy which can result in lobe collapse secondary to compression of airways by enlarged lymph nodes, parenchymal opacifications including diffuse millet-sized lesions and pleural effusions. However mediastinal lymphadenopathy may not always be accurately detected on chest radiography (33).

Older children may develop reactivation disease and present with radiologic findings like those encountered in adults such as lung cavitation, apical infiltrates, and pleural effusions (32).

Other imaging modalities, such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) can provide information regarding the locality, size and number of involved lymph nodes of the head and neck and may assist in guidance of location and sampling of lesions (29).

1.5.2.3. Microbiology

The diagnosis of tuberculosis disease requires and is supported by bacteriological confirmation. This can be achieved via microscopic observation of acid-fast bacilli on stained smears and by the isolation of MTb on TB culture.

Extrapulmonary specimens includes aspirates from lymph nodes; sterile fluids; pus; body cavity fluids; tissue; stool and urine.

Young children are also usually unable to expectorate sputum so alternative methods of sputum collection are employed such as gastric aspiration, sputum induction and

bronchial washings/broncho-alveolar lavage (BAL). These methods are invasive, and the yield of bacilli is low because of the paucibacillary nature of childhood disease and the non-uniform distribution of the bacilli. These methods also require specialised training and equipment to perform the procedures. In addition, they require hospital admission of the child which incurs an increased cost to their caregivers (26, 32).

Samples that are received for microbiology are then submitted for smear microscopy, TB culture and drug sensitivity testing.

Smear microscopy

Historically the diagnosis of TB relied on the microscopic identification of acid-fast bacilli on stained smears. The two staining methods employed to highlight bacilli are the Ziehl-Neelsen stain (ZN) and fluorescent auramine staining. Although smear microscopy is useful and allows for the rapid detection of MTb, the overall sensitivity of smear microscopy for the diagnosis of childhood TB remains low due to the paucibacillary nature of disease (32).

Ziehl-Neelsen stain

The ZN stain is performed on smears in order to highlight and identify mycobacteria. The mycobacterial cell wall contains mycolic acids and other lipids which influences the penetration and resistance by acid and alcohol, hence the term acid-fast bacilli. Traditional stains used to highlight bacterial organisms such as the Gram stain fail to demonstrate the mycobacteria (34). The ZN stain contains several reagents such as carbol fuchsin, acid alcohol and methylene blue (35). The carbol fuschin dye binds to the mycolic acids in the mycobacterial wall and is resistant to acid-alcohol application. The bacilli retain the colour of carbol-fuschin which is the primary dye and stains magenta-red with a slender, slightly curved to beaded morphology (35). Thus, a

positive ZN stain will highlight bacilli as magenta-red stained rods, whilst no rods are observed on a negative ZN stain.

The ZN stain is fairly specific, fast to perform, widely available, easy to prepare and relatively affordable. However, the density of AFB in tissue needs to be >1000 per cubic centimetre for the bacilli to be seen (22). Other disadvantages of the ZN stain includes low sensitivity; the stain does not distinguish between the different species of mycobacteria; and it is time-consuming to screen the stain as the mycobacteria are often scanty and may be difficult to visualise. Because of the low sensitivity of the ZN stain (20-60%), a negative result does not exclude mycobacterial infection (15).

TB culture

TB culture is the WHO-recommended gold standard used to confirm and correctly diagnose TB (1,2). TB culture allows for the speciation of the mycobacteria; provides a semi-quantitative assessment of bacterial load and allows for drug susceptibility testing. Drug sensitivity testing is important to ensure that patients are started on the most effective treatment regimen (2). Direct inoculation of material for TB culture into a mycobacterial growth indicator tube (MGIT) or Bactec medium at the bedside gives a high yield (36).

However, TB culture has several disadvantages including slow turnaround times as cultures may take up to 6 weeks. EPTB is paucibacillary by nature and cultures from extrapulmonary sites are often negative thereby limiting the sensitivity of the test. Also, TB cultures can only be done on material submitted freshly in saline and not on Formalin Fixed Paraffin Embedded (FFPE) samples (36). In cases where TB may not have been clinically suspected, samples may have not been submitted in saline for TB culture (25, 36).

1.5.2.4. Pathology

Enlarged cervical lymph nodes and other cervicofacial masses in children who present with EPTB are easily accessible for sampling by either fine needle aspiration (FNA) or surgical biopsy. Both procedures will provide material for both cytology and/or histology evaluation, microbiology studies, drug sensitivity testing and for molecular studies (GeneXpert or inhouse PCR).

Surgical methods of biopsy includes both excisional and incisional techniques. Surgical biopsy provides material for histopathological evaluation, TB cultures, GeneXpert (GXP) and may also provide the patient with rapid symptomatic response. Material for histological evaluation needs to submitted in formalin whilst material for TB culture and GXP need to be submitted in saline. Whilst, biopsy for histology is highly sensitive for the diagnosis of TB, the procedures are invasive, requires skilled staff to perform and requires anaesthesia. Also, there are potential rare complications that may occur after biopsy including wound infection, sinus formation, nerve injury and scarring (26).

1.5.2.5. Fine needle aspiration

Fine needle aspiration (FNA) is an excellent diagnostic procedure in children with suspected TB who present with palpable masses as it is simple to perform, is inexpensive, minimally invasive, safe with limited side effects and can be performed as an outpatient procedure (28,36). FNA provides a rapid and accurate diagnosis which can be rendered immediately at the bedside. The FNA can be performed multiple times to obtain material for diagnosis to send for TB cultures, drug sensitivity testing and/or for molecular studies (36).

Aspirated material can be used to make smears and cell blocks. Two smears are usually prepared, one is air dried and stained with the Giemsa or Diff-Quick stains and

the other is alcohol fixed and stained with the Papanicolaou (Pap) stain. The air-dried Giemsa stain allows for the immediate assessment of the smear for adequacy, however the Pap stain provides good nuclear and cytoplasmic details as well as the background host's cellular response (6).

Cell blocks are created by placing aspirated material into 10% buffered formalin solution, the specimen is then centrifuged to create a cell pellet, when is then embedded in paraffin wax (37). The advantages of the cell block methodology are that it allows for multiple sections to be made and thus multiple special and immunohistochemical stains can be performed. The FFPE blocks from the cellblock are also amenable for molecular tests such as PCR (6, 37).

Most special stains can be performed on the smears. The air-dried smears are preferred for the ZN stain as the alcohol in Pap stained smears causes disruption of the mycobacterial cell walls (6). MTb are strongly acid fast positive (stains deep red), are thin and slightly curved bacilli that measure 0.3-0.6 x 1-4μm (38). Children have paucibacillary TB disease and therefore bacilli are sparse, which limits the sensitivity of the ZN stain. While the specificity of the ZN stain on cytology smears is high, the overall sensitivity has been reported as 20-60% on culture positive samples. (6,36).

The cytomorphology of the aspirate is dependent on the extent and duration of the disease as well as the patient's immunity. Patients with intact immune systems show the classic morphologic picture of epithelioid granulomatous inflammation with multinucleated giant cells and variable amounts of necrosis. When immunity is impaired, granulomas become less well-formed and eventually become infrequent. The smears instead show amorphous 'dirty' necrosis with admixed cellular debris, neutrophilic and histiocytic inflammation (6,36).

In contrast to smears from MTb infections, smears from NTM infections show abundant histiocytes with foamy cytoplasm which are packed with abundant mycobacteria and are known as pseudo-Gaucher cells (38).

Mycobacteria can also be identified on a Pap stained smear using a fluorescent microscope and which are visible as thin, slightly curved bright yellow autofluorescent bacilli 2.0-2.7nm in length. Fluorescent microscopy produces a rapid result and is inexpensive without requiring additional stains (36).

Wright et al. reported that the cytomorphology achieved a sensitivity of 78% and a specificity of 91% for the diagnosis of TB lymphadenitis on FNA (36).

1.5.2.6. Molecular Diagnosis

Recently the introduction of molecular techniques such as nucleic acid amplification (NAA) has greatly improved the diagnosis of TB as they provide rapid results as well as detection of drug resistance-associated mutations in MTb. This reduces diagnostic delay and allows for rapid initiation of appropriate drug therapy (39).

Polymerase chain reaction

Polymerase chain reaction (PCR) is a method of nucleic acid amplification (DNA and RNA). PCR involves the amplification of a specific gene target segment through cycles of enzymatic DNA synthesis, and which results in the production of multiple copies of the original segment. Several commercial and in-house PCR based assays have been developed and are currently used for the diagnosis of infectious diseases including tuberculosis. Advantages of PCR includes faster turnaround times compared with TB culture, shows comparable sensitivity with TB culture, is simple to perform and can also be used on both fresh tissue samples and on FFPE blocks (15,39).

PCR for a TB diagnosis is performed by amplifying various DNA sequences that are specific to the mycobacterial complex such as *IS6110*, devR, MTP-40, 16S rRNA, etc (39,40). The most common gene target is the insertion sequence *IS6110* which is specific to the *Mycobacterium tuberculosis* complex and which is also present on multiple locations along the *M tuberculosis* genome (39,41).

A positive PCR result indicates the presence of *Mycobacterium tuberculosis* complex DNA and thus distinguishes between MTb and NTM in the submitted sample. However, a positive PCR does not differentiate between viable and nonviable bacilli and may not be reflective of active disease. A negative PCR result does not exclude the possibility of the presence of atypical mycobacteria or mycobacteria other than tuberculosis (MOTT) (39).

Commonly used methods of PCR include real-time PCR, reverse-transcriptase (RT-PCR), multiplex PCR and nested PCR. The UCT/ Groote Schuur Hospital Anatomical Pathology Division makes use of an in-house nested PCR method for the detection of the mycobacterial complex on FFPE samples.

Nested PCR

Nested PCR is a highly sensitive and specific modification of the PCR technique which reduces nonspecific amplification of the DNA template (42,43).

This is achieved by employing two sets of primers (outer and inner primers) and two successive PCR reactions. The first reaction is performed with primers that cover the target sequence and some additional sequences flanking both ends of the target sequence. The second reaction uses the products from the first PCR with primers that bind to the target sequence within amplified sequences of the first PCR. This second run amplifies only the intended product from the first round of amplification and not non-specific products. The major disadvantage of nested PCR is contamination which

may occur during the transfer of products from the first round of amplification to the second tube for the second round of amplification and which produces false-positive results (41, 42).

Studies have shown that the use of PCR is a good alternative approach for the detection of MTb in FFPE samples with good diagnostic accuracy (39-41).

GeneXpert MTB/Rif system

There are several commercially available kits for the diagnosis of TB including the GeneXpert MTB/Rif (Cepheid, Sunnyvale, CA) system which is an automated real time nested PCR assay. The GeneXpert MTB/Rif (GXP) is a closed, self-contained platform cartridge-based system for the extraction, amplification and detection of both MTb and for screening of rifampicin resistance mutations. This test can provide a rapid diagnosis (within 2 hours) of TB with a sensitivity of 89% in smear-positive patients and in 67% of smear-negative patients (41).

Since 2013, the GXP has been recommended for diagnosis of TB meningitis and TB lymphadenitis in children (44). A meta-analysis has shown that the use of GXP for the diagnosis of TB lymphadenitis in children has a pooled sensitivity and specificity of 86% and 81% respectively (45). The WHO has recommended the use of GXP for the diagnosis of EPTB, including in affected lymph nodes (1,2). Ligthelm et al showed that the use of GXP on FNA samples to have excellent diagnostic accuracy for the diagnosis of TB lymphadenitis (46).

The GXP has also shown promising results as a diagnostic test for EPTB performed on FFPE samples (39, 47, 48).

GeneXpert MTB/RIF Ultra assay

The GXP Ultra assay was launched in 2017 and is the latest modification on the GeneXpert platform and is being recommended by the WHO as a replacement for the GXP (1,2). The Ultra has shown higher diagnostic sensitivity than the GXP for the diagnosis of EPTB. This increased sensitivity is due to the incorporation of two new gene targets (IS6110 and *IS1081*), a larger DNA reaction chamber and use of fully nested PCR reactions (49, 50)

Antel et al showed that for the diagnosis of TB lymphadenitis, the Ultra had good sensitivity and high specificity on both FNA and fresh tissue lymph node samples. In addition, Ultra had higher yield than TB culture and yielded a quicker result (51).

A limitation of both the GXP modalities is that the tests do not distinguish between live and dead bacilli thus limiting its utility in diagnosis (45-50).

1.6. Management of EPTB

Anti-TB drug therapy is the mainstay of treatment for EPTB. Current regimens are the same for both drug sensitive EPTB and PTB. The regimen comprises isoniazid and rifampicin for 6 months, together with pyrazinamide and ethambutol for the first 2 months (16, 33). The treatment is administered daily as directly observed therapy (DOT) using fixed medicine combinations. The drug dosages are dependent on the child's body weight and are adjusted according to weight changes during treatment (16, 33).

1.6.1. Drug resistant TB

Drug resistant TB is a major public health concern adding to the burden of TB in South Africa (1,2).

Drug resistant TB is defined as infection by a strain of MTb which is resistant to at least 1 of the first-line drugs. Multidrug resistant TB (MDR-TB) is infection by MTb that is resistant to both isoniazid and rifampicin. Extensively drug resistant is MTb that is resistant to both isoniazid and rifampicin plus any resistance to the fluoroquinolones or injectable anti-TB agents used in second-line treatment. Young children acquire MDR-TB mainly through transmission from close adolescent or adult contacts with MDR-TB. (5)

The diagnosis of drug-resistant TB can only be made by microbiological or molecular drug sensitivity testing, and therefore every effort should be made to obtain samples to submit for microbiologic evaluation (19).

1.6.3. Surgery

Surgery may be needed for diagnostic surgical excisional biopsy or for management of complicated cases of cervicofacial TB. This includes patients who have discomfort from tense enlarged lymph nodes that are fluctuant or who may have airway compromise secondary to enlarged lymph nodes (26).

1.7. Rationale for this study

The definitive diagnosis of EPTB in children is challenging due to the non-specific clinical presentation, the difficulty in obtaining material to submit for microbiological confirmation and the paucibacillary nature of the disease. This results in treatment delays leading to morbidity and even mortality in children. The cervical lymph nodes of the head and neck are commonly affected in EPTB and provide an opportunity for both FNA and/or surgical biopsy to be performed for cytology and histology assessment. In addition, the sampled material can also be sent for TB cultures, GXP testing and for drug sensitivity testing. Therefore, it is important to understand the

correlation of cytomorphology for TB diagnosis. In addition, it is important to determine how well cytology, histology and the ZN stain correlates with defined reference standards (i.e., TB culture, GXP and in-house PCR) and how much one can rely on these investigations for the diagnosis of TB.

1.8. Aims and Objectives

1.8.1 Aims

The primary aim of this study was to investigate the diagnostic accuracy of cytology and histology for the diagnosis of extrapulmonary TB of the cervicofacial region in children with suspected disease. The secondary aim was to determine how well morphology from cytology and histology correlates with the defined reference standards for the diagnosis of TB.

1.8.2. Objectives

- Describe the morphologic patterns (with/without ZN results) encountered on cytology and histology in children with suspected cervicofacial EPTB.
- Determine the number and proportion of cytology and histology cases that showed classic morphology suggestive of TB in children with suspected cervicofacial EPTB.
- Determine how well the different morphologic patterns (with/without ZN stains) encountered in cytology and histology correlate with microbiologic and/or PCR confirmation of TB.
- Determine the sensitivity and positive predictive value of cytology for the diagnosis of extrapulmonary TB against the defined reference standards (TB culture and or GXP)

- Determine the sensitivity and positive predictive value of histology for the diagnosis of extrapulmonary TB against the defined reference standards.
- Compare the performance of cytology and histology against TB culture and GXP.
- In the subset of children with clinical suspicion for TB, but without cytology or histology features suggestive of TB or without TB culture, GXP or in-house PCR confirmed proof TB- to perform a retrospective folder review to assess the clinical course of these patients.
- Determine the utility of an in-house PCR test developed for MTb detection performed on archival FFPE for a subset of patients with histology findings suggestive of TB but without bacteriological/ molecular confirmation and in subset of patients with clinical suspicion of TB but without characteristic morphology.
- Compare the performance of GXP and TB culture for the diagnosis of TB in children who had either FNA or surgical biopsy done.

Chapter 2: Materials and Methods

2.1. Background to study

Our study was a follow up to an initial study conducted by medical students from the Netherlands (Eveline Hoogendoorn & Rozemarijn Duister, based in the Division of Otolaryngology at the Red Cross Children's Hospital and which was entitled *'Paediatric Extra-Pulmonary Tuberculosis (EPTB) of the Head and Neck at Red Cross Children's Hospital, Cape Town, South Africa: A Five-year Retrospective Review'*. The study was a retrospective folder review of children seen over a 5-year time period (June 2012 to May 2017) with a diagnosis of TB in the head and neck region (cervicofacial and extracranial) at the hospital. The data extracted from the folder review included demographic information, details of the clinical presentation, diagnostic investigations performed, and the management details. Results from samples for either cytology, histology, microbiology, and molecular studies (GeneXpert) which were submitted as part of the diagnostic workup were obtained from the DISA and TRAK laboratory information systems. The reference standards used in that study for a diagnosis of confirmed TB were one/several of the following criteria:

1. Cytology/Histology: Suppurative or necrotising granulomatous inflammation and detection of acid-fast bacilli on ZN stain.
2. Microbiology: Detection of acid-fast bacilli on smear microscopy or on TB culture.
3. Molecular: GXP detection of *Mycobacteria tuberculosis*.

Findings from the study

The main findings from the study were that cervical lymphadenitis was found to be the most common site of involvement in cervicofacial TB, a substantial proportion of patients were asymptomatic, TB was confirmed in a higher proportion of children undergoing biopsy than in children undergoing FNA and that there was a significant delay in diagnosis and treatment resulting in increased morbidity.

2.2. Research Design

Our current study was a quantitatively driven investigation performed with contextual, descriptive, and retrospective components.

The study was contextual as it involved capturing and retrospectively reviewing data from the reports of cytology, histology, microbiology and GXP samples received by the National Health Laboratory Services (NHLS), Red Cross Children's Hospital taken from children with suspected cervicofacial EPTB.

The study was descriptive as the information for various parameters were recorded and extracted from the cytology and histology reports including morphologic features and results of ZN stain and other stains if available.

The study was quantitative as it involved assessing: (1) the sensitivity and (2) the positive predictive values of cytology and histology against the reference standards for the diagnosis of TB. The reference standards for the diagnosis of TB were defined as:

- TB culturing of *Mycobacteria tuberculosis*
- Molecular detection of *Mycobacteria tuberculosis* (GeneXpert and in-house PCR)

This study had a retrospective laboratory component, as a subset of children who had histological assessment had in-house PCR performed on the archival FFPE tissue blocks to detect MTb.

2.3. Ethics

This study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (HREC REF:035/2020). (Appendix 1).

2.4. Study Population and Study Setting

The study population comprised children (0-13 years of age) who had suspected cervicofacial EPTB who were seen at Red Cross Children's Hospital from June 2012 to May 2017 (5 years) and who had FNA, or biopsies taken for cytology or histology as part of the TB workup. In addition, most of these children had samples submitted for TB cultures and or GXP.

The samples obtained for cytology and histology were submitted to the Anatomical Pathology laboratory at Red Cross Children's Hospital for evaluation. Samples for TB cultures and GXP were submitted to the microbiology department.

Red Cross Children's Hospital is now one of two dedicated paediatric health institutions in the country and is the largest tertiary academic children's hospital in sub-Saharan Southern Africa. It provides care to around 250000 patients from the Western Cape, South Africa and across Africa. The Otolaryngology (Ear, nose and throat or ENT) division at Red Cross Children's Hospital is a referral-based clinical service offering specialized otolaryngology care for children.

2.5. Case acquisition

The initial search for the case cohort was performed by the aforementioned Dutch medical students, for their study and which was supervised by Professor Komala Pillay and Associate Professor Shazia Peer. This same cohort of cases was included for our study.

The initial search was on using the DISA and the Trak information database information systems. The search was done for children with suspected cervicofacial TB who had samples submitted for the one or several of the following diagnostic modalities: cytology; histology; TB cultures and/or GeneXpert testing at Red Cross Children's Hospital over a five-year period. Children with cervicofacial TB with concomitant intracranial TB (e.g., TB meningitis) or disseminated TB were excluded from the study. Some children only had results from TB culture or GXP and had no results from cytology or histology investigations; all these children were excluded.

The initial database search yielded a total of 355 cases of children, which was further narrowed down to 100 children after the exclusion criteria were applied.

A further 24 children were excluded from our study for the following reasons:

- 15 cases were excluded as no cytology or histology results were available. Only results from TB culture and GXP were available.
- 6 cases were excluded because there were no results available for cytology, histology, TB culture or GXP on the information databases
- 1 case was an autopsy case without any microbiology tests done
- 1 case was from a lymph node aspirate which was reported as suboptimal for diagnosis due to minimal material aspirated
- 1 case was of a histology sample which was assessed as suboptimal for diagnosis due to insufficient material present.

Of the 76 cases included for our study, 18 children had histology done with either negative TB cultures and GXP or these investigations was not performed. There were also children with histology that did not have 'classic' features for TB but with clinical suspicion of TB. These were the cases that were selected for an additional in-house PCR test to be performed on the respective FFPE blocks.

2.6. Data Extraction

Data was extracted from the cytology, histology, and microbiology (TB culture and GXP) reports of the 76 children. Variables recorded from the cytology and histology reports included patient age, sex, HIV status (if available), organ of involvement, the pathological diagnosis, and the results from special stains (ZN and modified ZN). From the microscopic reports the type of inflammation (suppurative, granulomatous, necrotising, chronic, non-specific) were recorded. Information extracted from the TB culture reports were origin of sample, TB culture and the drug sensitivity result. Finally results from the GXP were also recorded which included origin of sample submitted, GXP result and Rifampicin sensitivity results. All the data was recorded and stored onto a password-protected Microsoft Excel spreadsheet.

Granulomatous inflammation (necrotising and non-necrotising) and necrotising inflammation were the morphologic features considered compatible with TB. This was supported by the presence of acid-fast bacilli on the ZN stain.

2.7. Laboratory Methods

DNA extraction and reference gene PCR

The FFPE tissue blocks were sectioned at 5µm on a rotary microtome. To prevent cross-contamination during the sectioning, a negative control tissue section (blank wax block) was cut between blocks. One of these served as the tissue sectioning control (TSC) which was also included in the DNA extractions. Further, a new area of the microtome blade was used for each block and the work area was regularly decontaminated with 10% bleach. Following sectioning, the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) was used to extract DNA according to the manufacturer's recommendations. A negative control where no tissue was added assessed cross contamination during the DNA extraction (DXC). The concentration

and purity of the DNA samples were measured using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, United States of America). Thereafter, PCR for *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* gene was performed to assess the quality of extracted DNA. The PCR master mix included primers GAPDH-F1 AGGTCATCCATGACAACTTTGGTATC and GAPDH-R1 TGAGGCCCTGCAGCGTACTC using the FastStart *Taq* DNA Polymerase kit (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer instruction. Amplification was performed on a thermal cycler with an initial 5minute denaturation at 94°C, followed by 40 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57°C, and elongation for 30 seconds at 72°C, with a final extension for 7 minutes at 72°C. The PCR products were separated by agarose gel electrophoresis. An amplification product of size 220 base pairs was considered positive while the absence of a band was considered negative.

Polymerase chain reaction for mycobacterial DNA

PCRs for mycobacterial DNA were performed as nested procedures consisting of two consecutive reactions where the second reaction amplified a DNA sequence within the first amplicon. The reactions were modified from protocols previously described by Marchetti et al (1998). PCR for the repetitive insertion sequence *IS6110* for the *Mycobacteria tuberculosis complex* was performed using outer primers (IS6110 OF-CgggACCACCCgCggCAAAgCCCgCAggAC and IS6110 OR-CATCgTggAAgCgACCCgCCAgCCCAggAT) recognising a 220 base pair fragment. The FastStart *Taq* DNA Polymerase kit (Roche Molecular Biochemicals, Mannheim, Germany) was used according to manufacturer instruction. Thermal cycling was carried out with an initial 5minute denaturation at 94°C, followed by 40 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 63°C, and

elongation for 30 seconds at 72°C, with a final extension for 7 minutes at 72°C. After the first PCR was completed, 10µl of the product was separated by agarose gel electrophoresis. Cases that were negative were subject to nested PCR using inner primers (IS6110 IF-CCTgCgAgCgTAggCgTCgg and IS6110 IR-CTCgTCCAgCgCCgCTTCgg) to amplify a 123 base pair product. The PCR master mix for both PCRs was the same except for the primers and template (2µl of the first PCR product). Thermal cycling parameters were the same. Cases that remained negative were subject to PCR for the *65-kD heat shock protein (HSP65)* to determine the presence of MOTTS. A double PCR approach was adopted using primers (HSP65 OF-gAgATCgAgCTggAggATCCgTAC and HSP65 R-gCCCTCgTTgCCCACCTTg) that amplified a 342 base pair product. The FastStart *Taq* DNA Polymerase kit (Roche Molecular Biochemicals, Mannheim, Germany) was used according to manufacturer instruction. Thermal cycling parameters were the same as for the IS6110 PCR. After the first PCR cycling was completed, 10µl of the PCR product was separated by agarose gel electrophoresis. Cases that were negative was subject to double PCR using the same primers. The reaction mixes for both PCRs was the same except 2µl of the first reaction product was transferred to the second PCR mixture. All PCR reactions included positive and no template controls.

An amplification product of size 220 or 123 base pairs for the IS6110 PCR was indicative of the presence of *Mycobacteria tuberculosis complex* while an amplification product of size of 342 base pairs for the HSP65 PCR was indicative of the presence of MOTTS. The absence of bands was considered negative. The amplified products were all analysed in an ethidium bromide stained 2% agarose gel in Tris-Borate-EDTA buffer. The gels were visualised and documented in a gel documentation system.

2.8. Data Analysis

Descriptive statistics were used to describe the data, i.e. non-normal continuous variables such as age were described using the median and interquartile range. Categorical variables were described using frequencies and percentages. The performance of histology and cytology was evaluated against the reference standards of TB culture, GXP and in-house PCR. The calculations and summary statistics for cytology and histology (sensitivity and positive predictive value) were performed using the STATA 15 software package (manufacturer details).

Chapter 3: Results

3.1. Case characteristics

A total of 76 children were included in the study:

- 22 children had cytology results available (28.9%)
- 48 children had histology results available (63.2%)
- 6 children had results from both cytology and histology available (7.9%).

The median age was 4 years with the interquartile range being 2.0 to 7.5 years. The youngest was 1 month old, and the oldest was 11 years old.

There were more female (56.6%) than male (43.4%) children included in the study.

The lymph nodes of the head and neck were the most frequently sampled site of biopsy, with the cervical lymph nodes the most frequently sampled (74.6%) followed by the submandibular lymph nodes (9.3%). Other sampled sites were the soft tissues of the head and neck (6.7%), skin (6.7%), cervical vertebrae (including bone) (4%), larynx (2.7%), mastoid bone (2.7%), retro-pharynx (1.3%) and the tonsil (1.3%).

Additional clinical information and the HIV status of the children were not available from the reports on the database systems and therefore were not included as variables in this study.

There were in total 73 ZN stains performed on both the cytology and histology cases from the 76 children (96.1%).

Sixty eight of the 76 children had material submitted for TB cultures (89.5%). However 3 of the 60 samples submitted for TB culture were contaminated.

Fifty six of the 76 children had samples submitted for GXP (74.7%) (table 1).

Not every child with a TB culture had a GXP done.

Eighteen of the 76 children who had biopsies for histology had their FFPE blocks sent for an in-house PCR test to detect MTb; however, one case was inadequate to proceed with the test.

Table 1: Summary of the case characteristics of children with suspected head and neck TB

Characteristic	Number of patients (N=76)	% of patients
Age (years)		
<1	3	3.9
1 to 4	41	53.9
5 to 9	23	30.3
≥ 10	9	11.8
Gender		
Male	33	43.4
Female	43	56.6
Origin of sample		
Cervical lymph node	49	64.5
Submandibular lymph node	7	9.3
Soft tissue	6	8
Skin	5	6.7
Cervical vertebrae	3	4
Larynx	2	2.7
Mastoid	2	2.7
Retro-pharynx	1	1.3
Tonsil	1	1.3
Study modality		
Histology	54	71
Cytology	28	36.8
Histology + Cytology	6	7.9
TB culture	68	89.5
GXP	56	74.7
In-house PCR	18	23.7
Special investigations		
Number of ZN stains performed	73	96.1

TB confirmed cases

Of the 76 children with suspected TB included in the study, there were 56 children with cases of confirmed TB (73.7%).

TB was confirmed by one or several of the following methods; TB culture, GXP or by the in-house PCR test. Sixty eight children had samples submitted for TB cultures, and of which 47 were positive for MTb (63.5%). Fifty six children had samples submitted for GXP, and of which 51 were positive for MTb (87.9%). Fifty four children had samples submitted for both GXP and TB cultures (72%), and of which 42 were positive for MTb (77.8%). There were 4 cases of MTb identified by GXP whilst the TB culture was negative.

An additional 4 cases of TB were confirmed by in-house PCR of the 18 cases submitted for testing (22.2%) (table 2).

Table 2: Method by which TB was confirmed

Investigation	No. of + results	% of cases
TBC (N= 68)	46	67.6
GXP (N= 56)	47	83.9
TBC & GXP: (N= 54)		
TBC+/GXP+	42	77.8
TBC-/GXP+	5	9.3
TBC+/GXP-	1	1.9
In-house PCR (N=18)	4	22.2

TBC: TB culture; GXP: GeneXpert; PCR: Polymerase chain reaction; + : Positive; - : Negative; N: Number

Sites with confirmed TB

There was TB confirmed in 36 of the 49 cervical lymph node samples (73.5%), 5 of the 7 submandibular lymph nodes (71.4%), 5 of the 6 soft tissue samples (83.3%), 3 of the 5 skin samples (60%), all 3 cervical vertebral samples (100%), all 2 mastoid samples (100%) and in the retropharynx sample (100%).

TB was not proven on the laryngeal and the tonsil samples (table 3).

Table 3: Sites with confirmed TB

Site of origin of TB sample	No. of samples submitted	No. of samples positive for TB (%)
Cervical lymph node	49	36 (73.5)
Submandibular lymph node	7	5 (71.4)
Soft tissues of head and neck	6	5 (83.3)
Skin	5	3 (60)
Cervical vertebrae	3	3 (100)
Mastoid bone	2	2 (100)
Retropharynx	1	1 (100)
Larynx	2	0
Tonsil	1	0

No: Number; TB: Tuberculosis

TB drug resistance

There were TB drug sensitivity results available for 52 children. These results were obtained from either the TB culture and/or GXP reports.

Of the 52 results, 49 were reported with sensitivity to both rifampicin and isoniazid (94.2%) .

There were 3 cases with reported drug resistance (5.8%).

There were 2 cases that were reported with resistance to both isoniazid and rifampicin (3.8%) and 1 case was reported to rifampicin only (1.9%).

Of the 52 TB drug sensitivity results there were 36 results available from children who had histology samples submitted (69.2%) :

- 30 cases were of drug sensitive TB
- 1 case reported both isoniazid and rifampicin resistance
- 1 case reported rifampicin resistance only.

There were 22 drug sensitivity results available from the children who had cytology samples submitted (42.3%):

- 21 cases were of drug sensitive TB
- 1 case was reported with both isoniazid and rifampicin resistance.

The children that had TB confirmed by the in-house PCR did not have TB drug resistance information available (4 cases).

3.2. Histology cases

3.2.1. Characteristics of the histology cases

Histology features

Fifty-four children had results from histology and of which 39 were reported as necrotising granulomatous inflammation (72%), 9 (17%) were reported as acute inflammation (features ranging from acute suppurative inflammation to organising granulation tissue formation), 4 were reported as necrotising inflammation without granulomas (7%) and 2 were reported as non-necrotising granulomatous inflammation (4%) (table 4).

Table 4: Summary of histology features of cases of suspected TB

Histology features	No. of patients (n=54)	% of patients
Necrotising granulomatous inflammation	39	72
Non-necrotising granulomatous inflammation	2	4
Necrotising inflammation	4	7
Acute inflammation	9	17

No: number, n: total number

Of the 54 histology cases, there were 45 cases that were reported with features consistent with TB (83.3%).

These features were necrotising granulomatous inflammation (39 cases, 86.7%), non-necrotising granulomatous inflammation (2 cases, 4%) and necrotising inflammation (4 cases, 9%) (table 5).

Table 5: Histology cases with features consistent with TB

	Number of cases	% of cases
Histology (n= 54)	45	83.3
Necrotising granulomatous inflammation	39	86.7
Non-necrotising granulomatous inflammation	2	4
Necrotising inflammation	4	9

n: total number; %: percentage

Of the 45 children with histology features compatible with TB, 34 had TB proven by either TB culture, GXP or in-house PCR (75.6%).

There were 9 patients with clinically suspected TB who had biopsies showing features of acute inflammation and of which 4 patients had TB proven (44.4%).

ZN stain

There was a ZN done in 53 of the 54 histology cases (98%). Only 1 case reported as acute inflammation did not have a ZN stain done and the subsequent in-house PCR was negative for MTb.

Of the 53 ZN stains done, 32 were positive for acid fast bacilli (60.3%). The ZN stain was positive in 28 cases reported as necrotising granulomatous inflammation (72%), Two cases of necrotising inflammation (50%) and all 2 cases of non-necrotising granulomatous inflammation were ZN positive (100%). All 8 cases that were reported with features of acute inflammation were ZN negative (table 6).

Table 6: ZN positivity vs histology feature

Histology feature	No. of ZN stains done (N=53)	No. of cases with ZN+	% of cases with ZN+
Necrotising granulomatous inflammation	39	28	72
Non-necrotising granulomatous inflammation	2	2	100
Necrotising inflammation	4	2	50
Acute inflammation	8	0	0
Total number of ZN stains done	53	32	60.3

No: number, n: total number; ZN: Ziehl Neelsen; +: positive

In addition to the ZN stains that were performed, 3 modified ZN stains were performed on cases with necrotising granulomatous inflammation involving the lymph nodes:

- Two of the three cases (with ZN+ modified ZN stains performed) were positive for both ZN and the modified ZN stains, both cases were TB culture and GXP positive for MTb.
- One case was only positive with the modified ZN stain whilst the ZN was negative. The follow up culture from this case showed non-tuberculous mycobacterium and the GXP was negative for MTb.

TB culture

There were TB culture results available for 46 of the 54 histology cases (85%). However, 2 of the TB cultures were contaminated (from 1 tissue sample and 1 pus swab).

Of the 44 TB cultures, there were 25 that were positive for MTb (56.8%), 1 was positive for *Mycobacterium bovis* (2.2%) and 1 was positive for non-tuberculous mycobacterium (2.2%).

There were 41 tissue samples (93.2%), 13 sputum samples (29.5%), 2 gastric washings (4.5%), 1 pus swabs (2.3%) and 2 tracheal aspirates (4.5%) submitted for TB culture. Some patients had more than one type of sample sent for cultures.

TB was cultured in 25 of 41 tissue samples (61%), 3 of 13 sputum samples (23.1%), One of the 2 gastric washings (50%) and in 1 of the 2 tracheal aspirates (50%). The pus swab was culture negative for MTb (table 7).

Table 7: Origin and results of samples submitted for TB culture

Cases submitted for TB culture (N=44)	No. (% of total TB cultures)	No. of + TB cultures (%)
Tissue	41 (93.2)	25 (61)
Sputum	13 (29.5)	3 (23.1)
Gastric washing	2 (4.5)	1 (50)
Pus swab	1 (2.3)	0
Tracheal aspirate	2 (4.5)	1 (50)

N: Total number of TB cultures performed; No: Number; +: Positive

GXP

There were GXP results available for 38 cases of the 54 histology cases (70.3%).

Of these 38 cases, the GXP was positive for the MTb complex in 30 cases (78.9%).

There were 32 tissue samples (84.2%), 6 sputum samples (15.8%), 2 tracheal aspirates (5.3%) and 1 gastric washing (2.6%) submitted for GXP.

The MTb complex was detected in 28 of the 32 tissue samples (87.5%), in 4 of the 6 sputum samples (66.7%), in both tracheal aspirates (100%) and in the single gastric washing submitted (100%) (table 8).

The nasopharyngeal aspirate was negative for the MTb complex on GXP.

Table 8: Summary of origin of samples submitted for GXP and breakdown of positive GXP results according to these sites.

Origin of sample submitted for GXP (No.=38)	No. (% of total GXP)	No. of + GXP (%)
Tissue	32 (84.2)	28 (87.5)
Sputum	6 (15.8)	4 (66.7)
Tracheal aspirate	2 (5.3)	2 (100)
Gastric washing	1 (2.6)	1 (100)

No: Number; +: Positive; GXP: GeneXpert

TB culture and GXP

Material for both GXP and TB culture were submitted in 36 out of the 54 histology cases (66.7%). Of these 36 cases, 25 were positive on both TB cultures and GXP (69.4%).

There were 33 tissue samples (91.7%), 6 sputum samples (16.7%), 1 gastric washing (2.8%) and 2 tracheal aspirates (5.6%) submitted for both GXP and TB culture.

Mycobacteria tuberculosis was proven by TB culture and GXP in 23 of the 33 tissue samples (69.7%), in 4 of the 8 sputum samples (50%) and in the single gastric washing submitted (100%) (table 9).

Table 9: Summary of origin of samples submitted for both TB culture and GXP and breakdown of positive results according to these sites.

Origin of material submitted for GXP & TBC (No=36)	No. of cases with GXP & TBC (% of total)	No. of + GXP & +TBC (%)
Tissue	33 (91.7)	23 (69.7)
Sputum	8 (42%)	4 (50%)
Gastric washing	1 (5%)	1 (100%)

No: Number; +: Positive; GXP: GeneXpert; TBC: TB culture

In-house PCR

A total of 18 cases had the in-house PCR performed on their respective FFPE blocks (33.3%) retrospectively. Of the 18 cases, one FFPE block was not suitable for the in-house PCR. Of the remaining 17 cases with in-house PCR, 4 cases were positive for the MTb complex (23.5%). There were 9 cases in which no material was submitted for either GXP or TB culture. All 9 of these cases had in-house PCR done, of which 2 were positive for MTb. The rest of the PCR tests were performed on cases with histology features compatible with TB but with negative TB culture or GXP results.

Of the 18 cases sent for in-house PCR:

- 10 in-house PCR tests were performed on cases showing necrotising granulomatous inflammation; 1 case had a FFPE block that was not suitable for PCR; of the remaining 17 cases, 4 cases were positive for the MTb complex
- 6 in-house PCR tests were performed from cases showing acute inflammation; all were 6 were negative for the MTb complex
- 2 in-house PCR tests were performed from cases showing non-necrotising granulomatous inflammation; all were negative for the MTb complex (table 10).

Table 10: Summary of the origin of samples submitted for in-house PCR

Histology morphology of cases submitted for in-house PCR (No.=17)	No. (% of total in-house PCR)	No. of + in-house PCR (%)
Necrotising granulomatous inflammation	10 (58.9)	4 (40)
Acute inflammation	6 (35.3)	0
Non-necrotising granulomatous inflammation	2 (5.3)	0

No: Number; +: Positive; GXP: GeneXpert

3.2.2. Correlation of cases with necrotising granulomatous inflammation

This section is a summary of all the histology cases that were reported as necrotising granulomatous inflammation and the correlation with the defined reference standards. In addition, the results from the ZN stain, cytology, TB cultures, GXP and inhouse PCR are discussed. Some cases had modified ZN performed. Not every case had cytology, GXP and an inhouse PCR done. Some of the cases had samples submitted from sputum, pus and tracheal aspirates only (table 11).

Table 11: Histology cases of necrotising granulomatous inflammation and results from ancilliary studies

No.	Site of Biopsy	Histology	ZN	Cytology	TBC	GXP	Inhouse PCR
1	Skin	necrotising granulomatous inflammation ; PNT	-	ND	Sputum -	Sputum +	ND
2	ln	necrotising granulomatous inflammation	+	necrotising inflammation, zn-	Sputum, tissue +	Sputum, tissue +	ND
3	Skin	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
4	ln	necrotising granulomatous inflammation	+	ND	Pus -	ND	-
5	neck soft tissues	necrotising granulomatous inflammation	-	ND	Tissue +	ND	ND
6	ln	necrotising granulomatous inflammation	+	ND	Trach aspr -	Trach aspr +	ND
7	ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
8	larynx	necrotising granulomatous inflammation	+	ND	Tissue -	Tissue -	ND
9	ln	necrotising granulomatous inflammation	+	ND	ND	ND	+
10	retropharyngeal	necrotising granulomatous inflammation	zn+, mod zn+	ND	Tissue, trach aspr +	Tissue +	ND
11	ln	necrotising granulomatous inflammation	-	ND	Tissue +; sputum -	Tissue +	ND
12	ln	necrotising granulomatous inflammation	-	ND	Tissue, sputum -	Tissue +, sputum -	ND
13	ln	necrotising granulomatous inflammation	zn+, mod zn+	necrotising inflammation, zn-	Tissue +	Tissue +	ND
14	ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
15	ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
16	ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
17	cervical vertebrae	necrotising granulomatous inflammation	-	necrotising inflammation, zn+	Tissue, sputum -	Tissue +	ND
18	ln	necrotising granulomatous inflammation	+	necrotising inflammation, zn-	Tissue +	Tissue +	ND
19	Submandibular ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
20	ln	necrotising granulomatous inflammation	+	ND	Tissue +; sputum -	Tissue +	ND
21	mastoid	necrotising granulomatous inflammation	-	ND	Tissue -	Tissue +	ND
22	ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
23	ln	necrotising granulomatous inflammation	+	ND	Tissue -	ND	+
24	skin	necrotising granulomatous inflammation; PNT	-	ND	ND	ND	-
25	ln	necrotising granulomatous inflammation	+	ND	ND	Tissue +	ND
26	bone and soft tissue of scalp	necrotising granulomatous inflammation	-	ND	Tissue +	Tissue +	ND

27	ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
28	mastoid	necrotising granulomatous inflammation	-	ND	Tissue +	Tissue +	ND
29	cervical vertebrae	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
30	ln	necrotising granulomatous inflammation	+	ND	ND	ND	Inadequate
31	ln	necrotising granulomatous inflammation	+	necrotising inflammation, zn-	Tissue -	ND	+
32	ln	necrotising granulomatous inflammation	+	ND	Tissue -	ND	-
33	ln	necrotising granulomatous inflammation	+	ND	Tissue +, sputum -	Tissue +	ND
34	submandibular ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
35	ln	necrotising granulomatous inflammation	+	ND	Tissue +	Tissue +	ND
36	submandibular ln	necrotising granulomatous inflammation	+	ND	ND	ND	-
37	ln	necrotising granulomatous inflammation	zn-, mod zn +	Nd	Tissue -, sputum +(NTBM)	ND	ND
38	ln	necrotising granulomatous inflammation	-	ND	Tissue -	Tissue -	-
39	ln	necrotising granulomatous inflammation	+	Atypical spjndle cells	ND	ND	+

No.: Number; LN: cervical lymph node unless otherwise specified; ZN: Ziehl Neelsen; ND: Not done; +: positive; -: negative; NTMB: non-tuberculous mycobacteria; trach aspr: tracheal aspirate; GXP: GeneXpert; TBC: TB culture

Summary of correlation of cases with necrotising granulomatous inflammation

There were 39 histology cases that were reported as necrotising granulomatous inflammation (see figures 1A & figure 1B).

Of these 39 cases:

- 33 cases had samples sent for TB cultures (85%)
- 29 cases had samples sent for GXP (74%)
- 10 cases had samples sent for inhouse PCR (25.6%).

There was evidence of MTb in 31 (79.5%) of the 39 cases:

- 21 of the 31 cases were TB culture positive (65.6%)
- 26 of the 31 cases were GXP positive (81.2%)
- 4 of the 31 cases were inhouse PCR positive (12.9%).

There were a total of 39 ZN stains done or which 28 were positive.

There were 28 cases of necrotising granulomatous inflammation with a positive ZN stain and there was evidence of MTb in 23 of these 28 cases (82%):

- 17 cases were TB culture positive (73.9%)

- 19 cases were GXP positive (82.6%)
- 4 cases were in-house PCR positive (17.4%).

There were 11 cases of necrotising granulomatous inflammation with a negative ZN stain; however, there was evidence of MTb in 8 of the cases (72.7%):

- 3 cases were TB culture positive (37.5%)
- 7 cases were GXP positive (87.5%) (table 12).

Table 12: Summary of the correlation of histology cases with necrotising granulomatous inflammation and the ZN stain with TB cultures, GXP and the in-house PCR for the diagnosis of TB

Morphologic features (n)	No. of TB proven cases (%)	No. of TBC +	No. of GXP +	In house PCR
Necrotising granulomatous inflammation (39)	32 (82.1)	21 (65.6)	26 (81.2)	4 (12.5)
Necrotising granulomatous inflammation and ZN+ (28)	23 (82)	17 (73.9)	19 (82.6)	4(17.4)
Necrotising granulomatous inflammation and ZN- (11)	8 (72.7)	3 (37.5)	7 (87.5)	0

N, no: number; ZN: Ziehl Neelsen; +:positive; -: negative; GXP: GeneXpert; TBC: TB culture

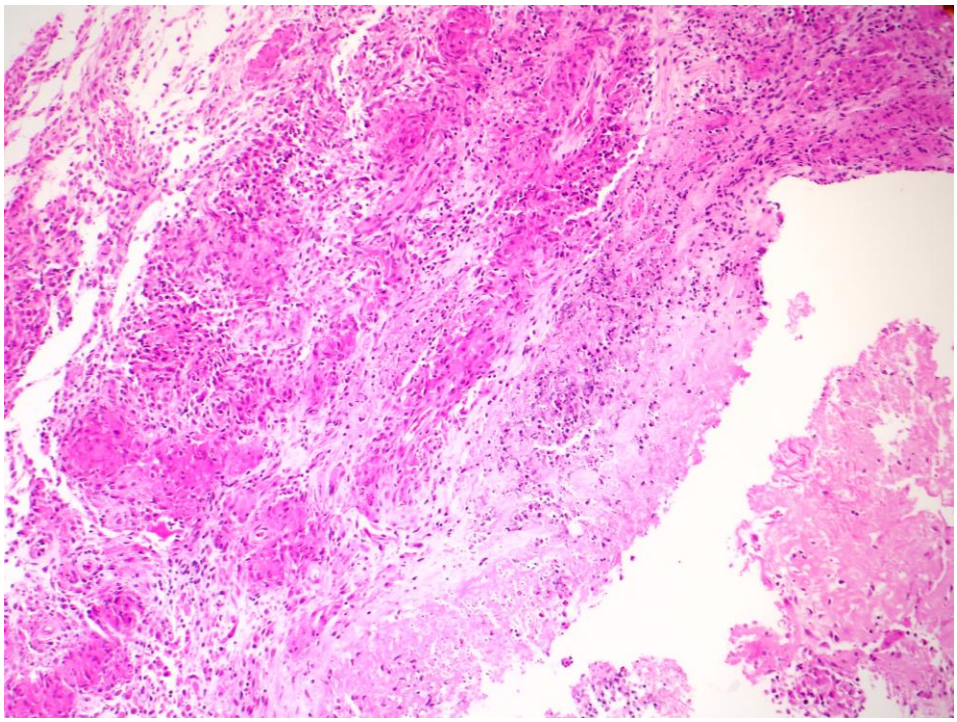

Figure 1A: HE photo micrographs of granulomatous inflammation with central necrosis (200x magnification)

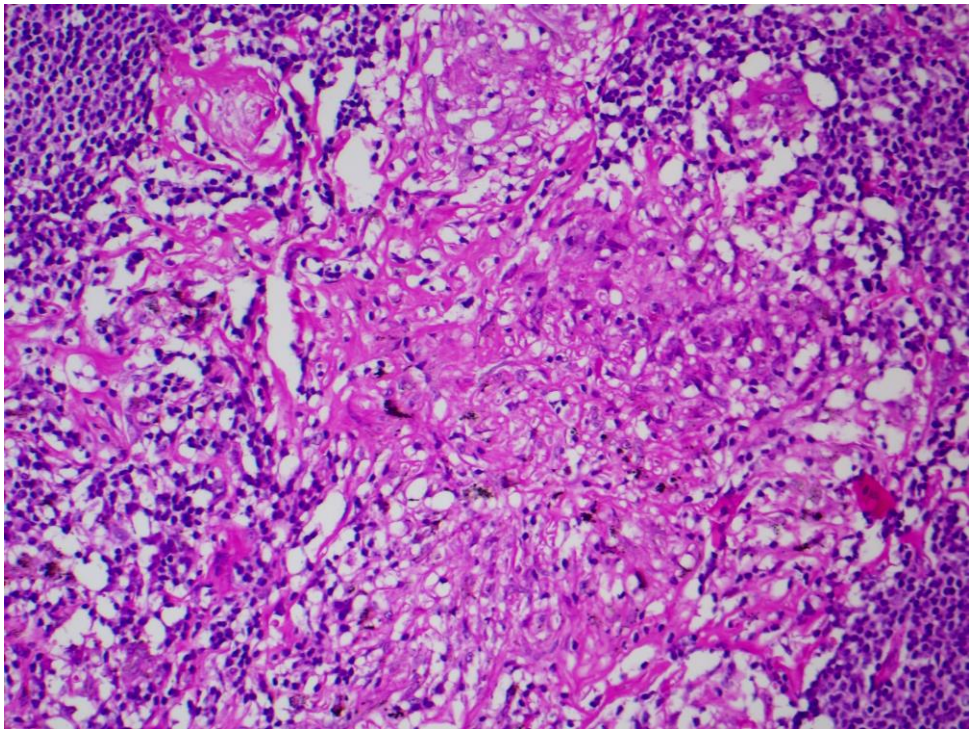

Figure 1B: HE photo micrographs of granulomatous inflammation with central necrosis (400x magnification)

Folder review of patients without proven TB

A folder review was done on 7 children in which there was no evidence of MTb via TB culture, GXP or inhouse PCR.

Four children were initiated on TB therapy and were referred back to peripheral clinics for continuation of management and thus no further clinical details were available.

One child with suspected laryngeal TB was initiated on TB therapy and was referred to their local clinic for continuation of management. The child was referred back to the dermatology department at Red Cross with suspected PNT lesions, and were due to have skin biopsies but were lost to follow up.

In 2 children clinical improvement was noted on initiation of TB therapy.

One of these children had a skin biopsy showing features consistent with PNT was noted to have suspected bilateral TB keratitis and had follow up at the ophthalmology clinic. No further biopsies were done and there was clinical improvement on therapy.

Necrotising granulomatous inflammation vs TB culture

Of the 39 cases reported as necrotising granulomatous inflammation there was material submitted from 33 cases for TB cultures (85%). *Mycobacterium tuberculosis* was cultured in 21 out of the 33 cases reported as necrotising granulomatous inflammation (72%).

Material was sent from 30 tissue samples (91%), 8 sputum samples (24%), 2 tracheal aspirates (6%) and 1 pus swab (3%) for TB culture.

Mycobacterium tuberculosis was cultured in 21 out of the 30 (70%) tissue samples, 1 of the 8 sputum samples (12.5%) and in 1 of the 2 (50%) tracheal aspirates. The pus swab was TB culture negative (table 13).

Table 13: Types of samples submitted for TB culture in cases of necrotising granulomatous inflammation

Sample type submitted (Total no: of samples submitted=33)	No. of samples submitted for TB culture (%)	No. of TB culture + (%)
Tissue	30 (91)	21 (70)
Sputum	8 (33.3)	1 (12.5)
Tracheal aspirate	2 (6)	1 (50%)
Pus swab	1 (3)	0

No.: Number; +: positive

There were 33 cases that was reported as necrotising granulomatous inflammation that had samples submitted for TB culture; the following samples were submitted for culture:

- 22 tissue samples (66.7%); 16 samples were culture positive (72.7%)
- 7 cases where both sputum and tissue were both submitted for TB culture (21%)
- 1 sputum sample (3%); the sputum was culture negative
- 1 tracheal aspirate (3%); the tracheal aspirate was culture negative

- 1 pus swab (3%); the pus swab was culture negative
- 1 case where both tissue and a tracheal aspirate sample were both submitted for TB culture (3%). Both tissue and the tracheal aspirate were TB culture positive.

Of the 7 cases where both sputum and tissue were submitted for TB culture: 3 cases were culture positive on tissue alone (42.9%), 2 cases were culture negative both on sputum and tissue (28.6) and 1 case was culture positive on both sputum and tissue (14.3%) (table 14). One of the cases cultured non-tuberculous mycobacterium on the sputum whilst the tissue sample was negative.

Table 14: Cases of necrotising granulomatous inflammation where both sputum and tissue samples were sent for TB culture

Cases with samples from both tissue and sputum submitted for TB culture (total=7)	Number of patients	% of patients
tissue+ sputum+	1	14.3
tissue- sputum-	2	28.6
tissue+ sputum-	3	42.9

+: Positive for Mycobacterium tuberculosis; -: Negative for Mycobacterium tuberculosis

Necrotising granulomatous inflammation and ZN vs TB culture

All the 39 cases that were reported as necrotising granulomatous inflammation had a ZN stain done (100%) (figure 2).

ZN positive

There were 28 positive stains (71.8%). Of the 28 cases of necrotising granulomatous inflammation with a positive ZN; 23 TB cultures were done of which 17 were TB culture positive (74%) (table 15).

Table 15: Cases of necrotising granulomatous inflammation with a positive ZN stain and positive TB culture

No. of cases with a ZN	No. of cases with a + ZN (%)	No. of cases with + ZN & TBC	No. of cases with ZN + &+ TBC (%)
39	28 (71.8)	23	17 (74)

No: Number; +: positive; ZN: Ziehl Neelsen stain; TBC: TB cultures

Of these 17 cases with a positive ZN and a positive TB culture:

- 13 cases had only tissue sent for TB culture
- 3 cases had both sputum and tissue submitted for TB culture; 1 case was culture positive on both samples and the 2 cases were culture positive on tissue alone
- 1 case had both tissue and tracheal aspirate submitted for cultures; both samples were positive.

There were 6 cases with a positive ZN and a negative TB culture. There was microbiologic and PCR evidence of TB in 3 cases (50%):

- 1 case was GXP positive
- 2 cases were positive by inhouse PCR (table 16 and table 17).

Table 16: Cases of necrotising granulomatous inflammation with a positive ZN stain and negative TB culture

No. of cases with a ZN	No. of cases with a + ZN (%)	No. of cases with + ZN & TBC	No. of cases with ZN + &- TBC (%)
39	28 (71.8)	23	6 (26)

No: Number; +: positive; -: negative; ZN: Ziehl Neelsen stain; TBC: TB cultures

Table 17: Cases of necrotising granulomatous inflammation with a positive ZN and negative TB cultures with proof of MTb.

No. of cases with + ZN and -TBC	No. of proven TB (%)	GXP + (%)	In-house PCR (%)
6	3	1 (33.3)	2 (66.7)

No: Number; +: positive; -: negative; ZN: Ziehl Neelsen stain; TBC: TB cultures; GXP: GeneXpert

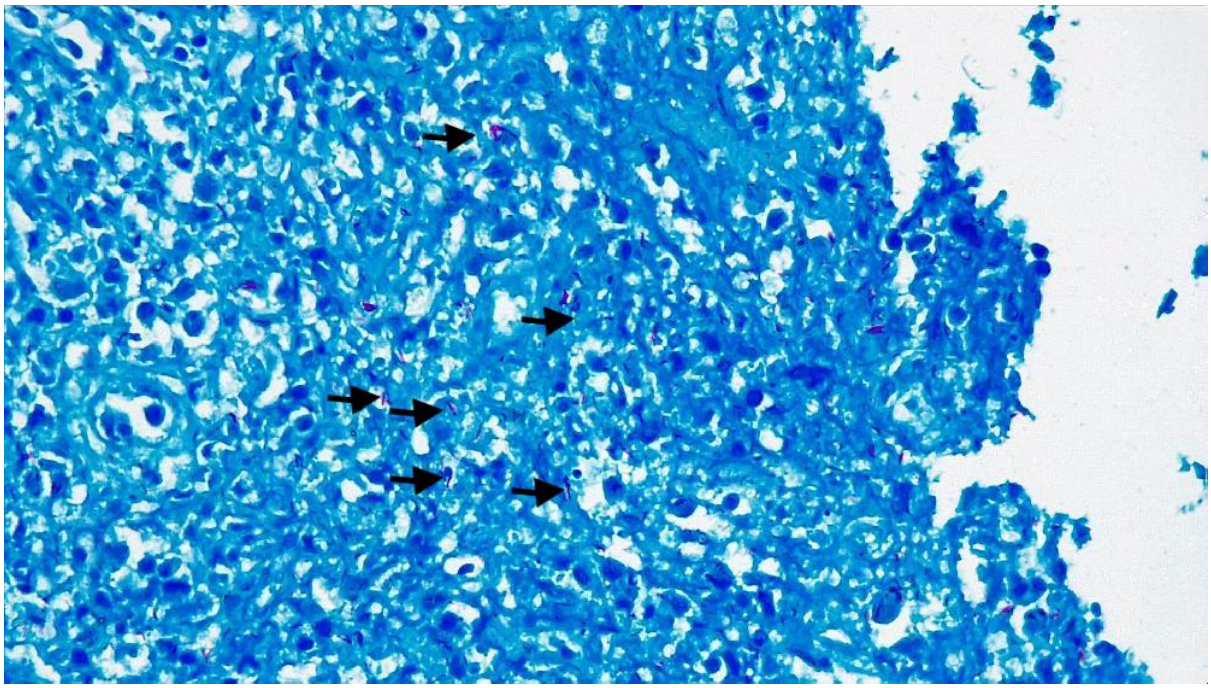

Figure 2: ZN stain showing multiple acid fast bacilli several highlighted by arrows (400X magnification)

ZN negative

There were 11 cases with a negative ZN (28.2%) and of these 9 cases had a TB culture result available. Of these 9 cases, 4 were TB culture positive (44.4%) (table 18).

There was 1 case that had a growth of non-tuberculous mycobacteria on the TB culture.

Table 18: Cases of necrotising inflammation with a negative ZN stain and positive TB culture

No. of cases with a ZN	No. of cases with a - ZN (%)	No. of cases with - ZN & TBC	No. of cases with ZN - &+ TBC (%)
39	11 (28.2)	9	4 (44.4)

No: Number; -: negative; +: positive; ZN: Ziehl Neelsen stain; TBC: TB cultures

Of the 4 cases with a negative ZN and positive TB culture:

- 3 cases were from tissue samples alone
- 1 case had both a sputum and tissue sample submitted for TB culture; the culture was positive on the tissue alone

Of the 9 cases with a negative ZN and a TB culture result available, there were 5 cases with negative TB cultures (table 19).

Table 19: Cases of necrotising granulomatous inflammation with a negative ZN stain and negative TB culture

No. of cases with a ZN	No. of cases with a - ZN (%)	No. of cases with- ZN & TBC	No. of cases with ZN - &- TBC (%)
39	11 (28.2)	9	5 (55.6)

No: Number; +: positive; -: negative; ZN: Ziehl Neelsen stain; TBC: TB cultures

There was evidence of MTb in 3 (60%) of the 5 cases with a negative ZN and negative TB culture.

- 1 tissue sample was sent for TB culture and GXP and the GXP was positive
- 2 cases had both tissue and sputum sent for GXP and TB cultures; both GXPs were positive on the tissue sample alone
- 1 tissue sample with a negative TB culture had an in-house PCR test performed, the PCR was negative (table 20).

Table 20: Cases of necrotising granulomatous inflammation with a negative ZN and negative TB cultures with proof of MTb elsewhere (GXP or inhouse PCR).

No. of cases with - ZN and -TBC	No. of proven TB (%)	GXP + (%)	In-house PCR + (%)
5	3 (60)	2 (66.7)	0

No: Number; +: positive; -: negative; ZN: Ziehl Neelsen stain; TBC: TB cultures; GXP: GeneXpert

Necrotising granulomatous inflammation vs GXP

Of the 39 cases reported as necrotising granulomatous inflammation, 29 samples were sent for GXP (74.3%). The MTb complex was detected in 26 out of the 29 cases (90%) via GXP.

There were 27 tissue samples (93%), 4 sputum samples (13.8%) and 1 tracheal aspirate (3%) sent for GXP. Some children had more than one sample sent for GXP. The MTb complex was detected in 24 out of the 27 (88.9%) tissue samples, 2 of the 4 sputum samples (50%) and in the 1 (100%) tracheal aspirate (table 21).

Table 21: Types of samples submitted for GXP in cases of necrotising granulomatous inflammation and results of GXP

Sample type submitted (Total no: of samples submitted=29)	No. of samples submitted for GXP (%)	No. of GXP + (%)
Tissue	27 (93)	24 (88.9)
Sputum	4 (13.8)	2 (50)
Tracheal aspirate	1 (3)	1 (100)

No.: Number; +: positive; GXP: GeneXpert

Necrotising granulomatous inflammation with ZN vs GXP

All 39 of the cases that were reported as necrotising granulomatous inflammation had a ZN stain done (100%).

ZN positive

There were 28 ZN stains that were positive for acid fast bacilli (71.8%) and of these there were 20 GXPs done and 19 were positive (95%).

Of these 19 cases with a positive ZN and a positive GXP:

- 2 cases had both sputum and tissue submitted for GXP; 1 case was GXP positive on both tissue and sputum and 1 cases was GXP positive on tissue alone
- 17 tissue samples were submitted for GXP; 16 tissue samples were GXP positive
- 1 tracheal aspirate was submitted for GXP and was GXP positive

Of the 20 cases with a positive ZN and a GXP result available, there was 1 case with a positive ZN and a negative GXP. There was no evidence of MTb on culture. The case was selected for the in-house PCR, however the FFPE tissue block was not retrieved.

ZN negative

There were 11 cases with a negative ZN (28.2%) and of which 9 had a GXP result available. Of these 9 cases there were 7 that were GXP positive (77.8%) (table 22).

Table 22: Cases of necrotising granulomatous inflammation with a negative ZN stain and positive GXP

No. of cases with a ZN	No. of cases with a - ZN (%)	No. of cases with - ZN & GXP	No. of cases with ZN- & +GXP (%)
39	11 (28.2)	9	7 (77.8)

No: Number; -: negative; +: positive; ZN: Ziehl Neelsen stain; GXP: GeneXpert

Of the 7 cases with a negative ZN and positive GXP, samples were submitted from:

- 5 tissue samples
- 1 sputum sample
- 1 case had both a sputum and tissue sample submitted for GXP; the GXP was positive on the tissue alone

There were 2 cases with a negative ZN and a negative GXP:

- 1 case was culture positive for non-tuberculous mycobacteria
- 1 case had an inhouse PCR done and which was negative for the MTb complex.

Necrotising granulomatous inflammation vs GXP and TB cultures

There were 27 cases of necrotising granulomatous inflammation where samples were submitted for both TB cultures and GXP (69.2%). In 5 of these cases, the GXP was positive whilst the TB cultures were negative (17.9%). There were 2 cases where both TB cultures and GXP were negative (7.4%); a subsequent in-house PCR was performed on only 1 of these cases which was negative. The tissue blocks of the second case was not retrieved and therefore inhouse PCR could not be performed. In 20 of the 27 cases, both GXP and TB cultures were positive (74%).

Necrotising granulomatous inflammation without TB culture or GXP tests

Of the 39 cases reported as necrotising granulomatous inflammation, there were 4 cases where no samples for GXP or TB cultures were submitted.

All 4 of these cases had an inhouse PCR test performed and the MTb complex was detected in 2 cases (50%) (table 23).

Table 23: Cases of necrotising granulomatous inflammation without samples sent for GXP and TBC

No. of cases without TBC and GXP	No. of proven TB (%)	In-house PCR +
4	2 (50)	2

No: Number; +: positive; -: negative; ZN: Ziehl Neelsen stain; TBC: TB cultures; GXP: GeneXpert

3.2.3. Correlation of cases with non-necrotising granulomatous inflammation

There were 2 cases of non necrotising granulomatous inflammation, and both had ZN stains done which were positive for AFB (100%). Unfortunately, there were no corresponding TB culture or GXP results available. An in-house PCR was performed on the respective FFPE blocks and both were negative for the MTb complex (table 24).

Table 24: Histology cases of non-necrotising granulomatous inflammation and results of ancillary studies

No.	Site of biopsy	Histology	ZN	Cytology	TBC	GXP	Inhouse PCR
1	larynx	Non necrotising granulomatous inflammation	+	ND	ND	ND	-
2	ln	Non necrotising granulomatous inflammation	+	ND	ND	ND	-

No.: Number; LN: cervical lymph node; ZN: Ziehl Neelsen; ND: Not done; +: positive; -: negative; GXP: GeneXpert; TBC: TB culture

Folder review of the two children showed that they were both initiated on TB therapy and referred for continuation of treatment at peripheral clinics. The child with suspected laryngeal involvement also had enlarged hilar lymph nodes on chest X-ray.

Both children were started on therapy based on the histology results and clinical findings. Details regarding the clinical course were not available at the time of folder review.

3.2.4. Correlation of cases with necrotising inflammation

There were 4 cases reported as necrotising inflammation (table 25):

- 4 had ZN stains done (100%) and 2 were positive (50%)
- 4 had TB culture results available (100%)
- 1 case had a GXP result (25%).

There was microbiologic and PCR of MTb in 2 cases (table 26):

- 2 positive TB cultures
- 2 positive GXP results

Of the 2 cases without evidence of MTb:

- 1 case had a culture which was positive for *Mycobacterium bovis* on a lymph node tissue sample
- 1 case had a culture positive for *Staphylococcus aureus* on a tissue sample.

Table 25: Histology cases of necrotising inflammation and results of ancillary studies

No.	Site of biopsy	Histology	ZN	Cytology	TBC	GXP	Inhouse PCR
1	ln	Necrotising inflammation	-	ND	tissue + (Mb); gw: -	ND	ND
2	ln	Necrotising inflammation	-	ND	tissue, sputum- but Staph aur+	ND	ND
3	ln	Necrotising inflammation	+	ND	Tissue +	+	ND
4	Skin of neck	Necrotising inflammation - scrofuloderma	+	ND	Tissue, sputum +	Tissue, sputum +	ND

N, no: number; ZN: Ziehl Neelsen; +:positive; -: negative; GXP: GeneXpert; TBC: TB culture; ND: not done; gw: gastric washings; Staph aur: Staphylococcus aureus; Mb: Mycobacterium bovis

Table 26: Summary of the correlation of cases with necrotising inflammation with ZN stain against TB cultures, GXP and the in-house PCR for the diagnosis of TB

Histomorphologic feature (n)	no. of TB proven cases (%)	No. of TBC +	No. of GXP +
Necrotising inflammation (4)	2 (50%)	2	2

N, no: number; ZN: Ziehl Neelsen; +:positive; -: negative; GXP: GeneXpert; TBC: TB culture

3.2.4. Acute inflammation vs microbiologic culture and GXP

There were 9 cases that were reported with features of acute inflammation (table 27).

Of these 9 cases:

- 9 cases had samples sent for TB cultures (100%)
- 5 cases had samples sent for GXP (55.6%)
- 5 cases had the inhouse PCR test for MTb complex.

The ZN stain was done on 8 of these cases (89%). There were no acid fast bacilli identified on the ZN stains.

All cases had TB culture results available however two were contaminated (78%).

There was evidence of MTb in 4 of the 9 cases (43.3%) (table 28):

- *Mycobacterium tuberculosis* was cultured in 2 of the cases (66.7%)
- The MTB complex was detected on GXP in 3 of the cases (100%)

Of the 5 cases without evidence of MTb on GXP or TB culture :

- 5 cases had an inhouse PCR test done and all were negative for the MTb complex.
- 1 case had a culture which was positive for *Staphylococcus aureus.*

Table 27: Histology cases with features of acute inflammation and results of ancilliary studies

No.	Site of biopsy	Histology	ZN	Cytology	TBC	GXP	Inhouse PCR
1	ln	abscess	-	ND	Sputum, gw +	Sputum, gw +	ND
2	Left cheek (skin)	Organising abscess	-	ND	Tissue - ; St aur +	Tissue -	ND
3	Cervical vertebrae	inflamed granulation tissue	-	ND	Tissue +, sputum -	Tissue +	ND
4	submandibular ln	inflamed granulation tissue	-	ND	Tissue +	Tissue +	ND
5	neck soft tissue	abscess	-	ND	Contaminated	ND	-
6	tonsil	acute suppurative inflammation	ND	ND	Tissue -	ND	-
7	ln	Organising abscess	-	ND	Tissue -	ND	-
8	submandibular ln	Organising abscess	-	ND	Tissue, sputum -	Sputum -	-
9	ln	Organising abscess	-	ND	Contaminated	ND	-

N, no: number; ZN: Ziehl Neelsen; +:positive; -: negative; GXP: GeneXpert; TBC: TB culture; ND: not done; gw: gastric washings; St aur: Staphylococcus aureus

Table 28: Summary of the correlation of histology cases with acute inflammation with TB cultures, GXP and the in-house PCR for the diagnosis of TB

Histologic feature(n)	no. of TB proven cases (%)	No. of TBC +	No. of GXP +	Inhouse PCR
Acute inflammation (9)	3 (43.3)	2 (66.7)	3 (100)	0

N, no: number; ZN: Ziehl Neelsen; +:positive; -: negative; GXP: GeneXpert; TBC: TB culture

3.2.5. Correlation of histology for TB diagnosis

Overall 36 out of the 54 cases that were submitted for histology had proven TB (66.7%). There were 6 more cases of TB identified by GXP (31 cases) than by TB culture (69.4%). An additional 4 cases were identified by inhouse PCR (table 29). There were 25 histology cases with positive ZN stains that had TB proven (46%); again more cases were proven by GXP than by TB culture. Of the histology cases with a negative ZN there was evidence of TB in 11 cases (20.8%); with more cases proven by GXP than by TB culture.

Table 29: Summary of TB proven histology cases against reference standards

	No. of TB proven cases	No. of TB culture +	No. of +GXP	In house PCR+
Histology (n=54)	36 (66.7%)	25 (69.4%)	31 (86.1%)	4 (11%)
Histology +ZN	25 (47%)	19 (76%)	21 (84%)	4 (16%)
Histology -ZN	11 (20.8%)	6 (63.6%)	10 (91%)	0

N, no: number; ZN: Ziehl Neelsen; +:positive; -: negative; GXP: GeneXpert; TBC: TB culture

The sensitivity of histology was 85.2%, 90.3% and 89.2% against TB culture; GXP and combined (GXP, TB culture and PCR) reference standards respectively (table 30).

Table 30: Sensitivity and PPV of histology against the reference standards

		Sensitivity	PPV
Reference Test	Total	n/N (%)	n/N (%)
Histology vs TB culture	44	23/27 (85.2)	23/37 (62.2)
Histology vs GXP	38	28/31 (90.3)	28/30 (93.3)
Histology vs TBC, GXP & PCR	54	33/37 (89.2)	33/45 (73.3)

PPV: positive predictive value, n: index group; N: control group; GXP: GeneXpert; TBC: TB culture

3.3. Cytology Cases

3.3.1. Characteristics of the cytology cases

Cytomorphologic features

There were 28 children who had samples for cytology, 22 had cytology done only (78.6%) whilst 6 had both cytology and histology done (21.4%).

Of the 28 cytology cases available, 16 were reported as necrotising inflammation (57.1%); 5 as necrotising granulomatous inflammation (17.8%); 3 as acute inflammation (10.7%); 1 as non-necrotising granulomatous inflammation (3.6%), 1 as atypical spindle cells (3.6%); 1 as lymphocytic inflammation (3.6%); and 1 as a reactive lymph node (3.6%) (table 31).

Table 31: Cytomorphologic features from the cytology reports of suspected head and neck TB cases

Cytologic features	No. of patients (n=28)	% of patients
Necrotising inflammation	16	57.1
Necrotising granulomatous inflammation	5	17.8
Acute inflammation	3	10.7
Non-necrotising granulomatous inflammation	1	3.6
Other morphologies: atypical spindle cells 1; lymphocytic inflammation 1; reactive lymph node	3	10.7

N: number; No: Number

Cytomorphologic features consistent with TB

Of the 28 cytology cases, there were 21 that were reported with cytology features consistent with TB (78.6%) (table 32).

These features were necrotising inflammation (16 reports, 72.7%), necrotising granulomatous inflammation (5 reports, 22.7%) and non-necrotising granulomatous inflammation (1 report, 4.5%).

Table 32: Cytology cases with cytomorphologic features consistent with TB

	Number of cases	% of + cases
Number of cytology cases with features consistent with TB (Total number of cytology cases: 28)	22	78.6
Necrotising inflammation	16	72.7
Necrotising granulomatous inflammation	5	22.7
Non-necrotising granulomatous inflammation	1	4.5

+: positive

There was confirmed evidence of MTb in 20 of the 22 cytology cases with cytomorphological features consistent with TB (90.9%):

- 16 cases were proven via TB culture (84.2%)
- 18 cases were proven via GXP (94.7%)
- 1 case was proven by the in-house PCR test (5.2%).

ZN stain

Of the 28 cytology cases, there were 26 ZN stains done (92.9%) and of which 16 were ZN positive (57.7%) for acid fast bacilli (table 33).

Table 33: Number and percentage of ZN positive stains vs cytomorphologic features

Cytomorphologic feature	No. of ZN done (total number =26)	No. with ZN+	% of cases with ZN+
Necrotising inflammation	16	10	62.5
Necrotising granulomatous inflammation	5	4	80
Acute inflammation	3	0	0
Non-necrotising granulomatous inflammation	1	1	100
Atypical spindle cells	0	0	0
Lymphocytic inflammation	1	1	100
Reactive lymph node	1	0	0
Total number of ZN done	26	16	61.5

ZN: Ziehl Neelsen; No: Number

TB culture

There were 27 cytology cases with TB culture results available (96%). However, 1 culture was contaminated. Of the remaining 26 cytology cases with TB cultures, MTb was cultured in 20 of the 25 cultures (76.9%).

Of the 25 TB cultures there were 25 samples submitted from tissue (100%), 11 samples from sputum (44%), 3 samples from gastric washings (12%) 1 sample from a nasopharyngeal aspirate (4%) and 1 sample from an eye swab (4%).

Mycobacteria tuberculosis was cultured in 18 of the 25 tissue samples (72%), 5 of the 11 sputum samples (45%), 2 of the 3 gastric washings (66.7%) and in the single eye swab (100%). The nasopharyngeal aspirate was negative for MTb on culture (table 34).

Table 34: Origin of samples submitted for TB culture

Origin of samples submitted for TB culture (N:25)	No. (% of total TB cultures)	No. of + TB cultures (%)
Tissue	25 (100)	18 (72%)
Sputum	11 (42.3)	5 (45%)
Gastric washing	3 (11.5%)	2 (66.7%)
Nasopharyngeal aspirate	1 (3.8%)	0
Eye swab	1 (3.8%)	1 (100%)

N, No: Number; +: Positive

GXP

Twenty three of the 28 cytology cases had results from GXP available (85.2%) and the MTb complex was detected in 22 of the 23 GXP (95.7%).

There were in total 20 tissue samples (95%), 10 sputum samples (47.6%), 1 gastric washing (4.8%) and 1 nasopharyngeal aspirate (4.8%) submitted for GXP.

The Mycobacteria tuberculosis complex was detected in 17 of the 20 tissue samples (85%), in 5 of the 10 sputum samples (50%) and in the single gastric washing submitted (100%). The nasopharyngeal aspirate was negative for the MTb complex on the GXP (table 35).

Table 35: Summary of origin of samples submitted for GXP and breakdown of positive GXP results according to sites.

Origin of GXP	No. (% of total GXP)	No. of + GXP (%)
Tissue	20 (95%)	17 (85%)
Sputum	10 (47.6%)	5 (50%)
Gastric washing	1 (4.8%)	1 (100%)
Nasopharyngeal aspirate	1 (4.8%)	0

No: Number; +: Positive; GXP: GeneXpert

TB culture and GXP

There were 19 children with cytology who had samples submitted for both GXP and TB culture and of which 17 were positive on both TB culture and GXP (89.5%).

There were in total 19 tissue samples (100%), 8 sputum samples (42%), 1 gastric washing (5%) and 1 nasopharyngeal aspirate (5%) submitted for both GXP and TB culture. Mycobacteria tuberculosis complex was detected in 15 of the 19 tissue samples (80%), in 4 of the 8 sputum samples (50%) and in the single gastric washing submitted (100%). The nasopharyngeal aspirate was negative for the MTb complex both on GXP and culture (table 36).

Table 36: Summary of origin of samples submitted for both TB culture and GXP and breakdown of positive results according to sites.

Origin of material in cases where both GXP & TBC was submitted	No. of cases with GXP & TBC (% of total)	No. of + GXP & +TBC (%)
Tissue	19 (100%)	15 (80%)
Sputum	8 (42%)	4 (50%)
Gastric washing	1 (5%)	1 (100%)
Nasopharyngeal aspirate	1 (5%)	0

No: Number; +: Positive; GXP: GeneXpert; TBC: TB culture

Children with both cytology and histology investigations

Six of the 28 children who had FNAs for cytology also had biopsies done for histology (22.2%). All 6 of these histology cases were reported as necrotising granulomatous inflammation. From this cohort, 5 cytology cases were reported as necrotising inflammation and 1 case was reported as atypical spindle cells.

3.3.2. Correlation of cases with necrotising inflammation

There were 16 cytology cases that were reported as necrotising inflammation and of which :

- all had samples sent for TB culture (100%); however, one culture was contaminated
- 13 cases had samples sent for GXP (81.2%)
- 1 case had an additional biopsy for histology done and the FFPE block was sent for the in-house PCR (6.3%) (table 37).

Table 37: Cytology cases of necrotising inflammation and results from ancillary studies

No.	Site of biopsy	Cytology	Histology	ZN	TBC	GXP
1	ln	necrotising inflammation	ND	+	Sputum, tissue +	Sputum, tissue +
2	ln	necrotising inflammation	ND	+	Sputum, tissue +	Sputum, tissue +
3	ln	necrotising inflammation	Necrotising granulomatous inflammation; ZN+, mod ZN+	-	Tissue +	Tissue +
4	ln	necrotising inflammation	Necrotising granulomatous inflammation; ZN+	-	Tissue +	Tissue +
5	ln	necrotising inflammation	ND	+	Sputum +	Sputum +
6	cervical vertebrae	necrotising inflammation	Necrotising granulomatous inflammation; ZN-	+	Tissue -	Tissue +
7	ln	necrotising inflammation	ND	+	Tissue -	ND
8	ln	necrotising inflammation	ND	+	Tissue -	ND
9	neck soft tissue	necrotising inflammation	ND	+	Tissue: culture contaminated	Tissue +
10	ln	necrotising inflammation	Necrotising granulomatous inflammation; ZN+	-	Tissue -	ND
11	ln	necrotising inflammation	ND	+	Tissue +	Tissue +
12	ln	necrotising inflammation	ND	+	Tissue +, sputum -	Tissue +, sputum -
13	ln	necrotising inflammation	Necrotising granulomatous inflammation; ZN+	-	Tissue, sputum +	Tissue, sputum +
14	neck soft tissue	necrotising inflammation	ND	+	Tissue +	Tissue +
15	ln	necrotising inflammation	ND	-	Tissue +	Tissue +
16	Submandibular ln	Necrotising inflammation	ND	-	Tissue +	Tissue +

No: Number; ZN: Ziehl-Neelsen; +: Positive; GXP: GeneXpert; TBC: TB culture; - : negative; ND: Not done; ln: Cervical lymph node unless otherwise specified

Summary of cytology cases with necrotising inflammation

TB was proven in 14 of the 16 cases (87.5%); 11 cases were proven by TB culture (68.8%) and 13 cases were proven by GXP (81.3%).

There were 12 cases in which MTb was detected on samples sent for both GXP and TB culture (75%). In 2 cases MTb was detected on GXP alone; 1 TB culture was negative and 1 TB culture was contaminated.

Necrotising inflammation and the ZN stain

There was a ZN stain done on every case of necrotising inflammation (100%).

There were 10 reports of necrotising inflammation with a positive ZN stain and there was microbiologic and PCR confirmation of MTb in 8 of the 10 cases (80%):

- 6 cases were TB culture positive (85.7%)
- 8 cases were GXP positive (100%)

There were 6 cases of necrotising inflammation with a negative ZN stain but there was microbiologic and PCR confirmation of MTb in 5 cases (83.3%).

- 5 cases were TB culture positive (100%)
- 5 cases were GXP positive (100%) (table 38)

Table 38: Summary of the correlation of cytology with necrotising inflammation and the ZN stain with TB cultures and GXP for diagnosis of TB

Cytomorphologic feature (n)	no. of TB proven cases (%)	No. of TB culture +	No. of GXP +
Necrotising inflammation (16)	14 (87.5%)	11 (68.8%)	13 (81.3%)
Necrotising inflammation and ZN+ (10)	8 (80%)	6 (85.7%)	8 (100%)
Necrotising inflammation and ZN- (6)	6 (100%)	6 (100%)	6 (100%)

N, no: number; ZN: Ziehl Neelsen; +:positive; -: negative; GXP: GeneXpert

Necrotising inflammation vs TB culture

All 16 cases that were reported as necrotising inflammation had samples sent for TB cultures, however one of the cultures was contaminated. However, this case had samples sent for GXP as well and which was positive for MTb.

Mycobacterial tuberculosis was cultured in 11 of the 15 cases reported as necrotising inflammation with available TB culture results (73.3%).

There were in total 14 (93%) tissue and 5 sputum samples (33.3%) sent for TB culture. *Mycobacterium tuberculosis* was cultured in 9 out of the 14 (64%) tissue samples and in 4 of the 5 (80%) sputum samples submitted for culture (table 39).

Table 39: Sample types submitted for TB culture in cases reported as necrotising inflammation

Sample type submitted (Total no: of samples submitted=15)	No. of samples submitted for TB culture (%)	No. of TB culture + (%)
Tissue	14 (93)	9 (64)
Sputum	5 (33.3)	4 (80)

No.: Number; +: positive

There were 15 cases that was reported as necrotising granulomatous inflammation that had samples submitted for TB culture; the following samples were submitted for culture:

- In 4 cases both tissue and sputum were submitted together (26.7%)
- In 10 cases only tissue samples were submitted (66.7%)
- In 1 case only a sputum sample was submitted (7%).

In the 4 cases where both sputum and tissue were sent for TB culture: 3 cases were positive on both sputum and tissue (75%) and in 1 case, only the tissue was positive (25%) (table 40).

Table 40: Cases of necrotising inflammation where both sputum and tissue samples were sent for culture

Cases with samples from both tissue and sputum submitted for TB culture (total=4)	Number of patients	% of patients
tissue+/ sputum+	3	75
tissue- /sputum+	0	0
tissue+/ sputum-	1	25

+: Positive for Mycobacterium tuberculosis; -: Negative for Mycobacterium tuberculosis

Necrotising inflammation and ZN vs TB culture

All 16 of the cases that were reported as necrotising inflammation had a ZN stain done (100%) and of which 10 were positive for acid fast bacilli (66.7%).

Necrotising inflammation and positive ZN vs TB culture

All 10 of the cases of necrotising inflammation with a positive ZN stain had TB cultures done and of which 6 were positive (60%).

Of these 6 cases with a positive ZN and a positive TB culture:

- 3 cases had samples from both sputum and tissue sent for TB culture; 2 cases were positive on both sputum and tissue (66.7%) and 1 case was positive on tissue alone (33.3%).
- 5 cases had only tissue samples sent for TB culture and 2 cases were positive (40%)
- 1 case had only sputum sent for TB culture and was positive (100%).

There were 4 cases with a positive ZN and a negative TB culture. However, in 2 cases MTb was detected on GXP (50%):

- 1 case had both sputum and tissue sent for TB culture; the culture was contaminated; however, there was material which was submitted for GXP and which was positive for the MTb complex
- 2 cases were TB culture negative on tissue and no samples were sent for GXP
- 1 case had a tissue sample with a negative TB culture but a positive GXP.

Necrotising inflammation and negative ZN vs TB culture

There were 6 cases of necrotising inflammation with a negative ZN. These cases all had TB culture results available. Of these 6 cases, 5 were TB culture positive (83.3%). Of the 5 cases with a negative ZN and positive TB culture:

- 4 cases had tissue samples submitted for TB culture
- 1 case had both sputum and tissue samples submitted; the TB cultures were positive on both samples.

Necrotising inflammation vs GXP

There were 13 cases of 16 cases with necrotising inflammation that had samples submitted for GXP (81%). All 13 of the GXPs were positive for the MTb complex (100%).

There were in total 12 tissue samples (92.3%) and 5 sputum samples (38.5%) submitted for GXP (table 41).

Table 41: Sample types submitted GXP in cases of necrotising inflammation

Sample type submitted (Total no: of samples submitted=13)	No. of samples submitted for GXP (%)	No. of GXP + (%)
Tissue	12 (92.3)	10 (71.4)
Sputum	5 (38.5)	4 (80)

No.: Number; +: positive

Of the 13 cases reported as necrotising inflammation with a GXP result:

- 8 cases had only tissue samples (61.5%) submitted to GXP
- 1 case had only a sputum sample (7.7%) submitted to GXP
- 4 cases had samples from both sputum and tissue submitted for GXP (30.8%); 3 cases were GXP positive on sputum and tissue. One case was positive on GXP on tissue alone.

All of the cases with a GXP result had a TB culture result as well. All but one case has a discrepant correlate; where MTb was detected on GXP whilst TB culture was negative (i.e., sputum was TB culture and GXP negative; tissue was TB culture and GXP positive).

Necrotising inflammation and ZN vs GXP

There were 13 cases reported as necrotising inflammation that also had a ZN stain done with GXP results available. All 13 GXPs were positive (100%).

Necrotising inflammation and positive ZN vs GXP

There were 8 cases that were reported necrotising inflammation with a positive ZN result (80%). All 8 cases with a positive ZN had a positive GXP (100%).

Of these 8 cases with a positive ZN and a positive GXP:

- 3 cases had both sputum and tissue submitted; 2 of the 3 cases were positive on both sputum and tissue samples (66.7%) and in 1 case the GXP was positive on tissue only (33.3%)
- 1 case was from a sputum sample
- 4 cases were from tissue samples alone, in one case the TB culture was negative while the GXP was positive (25%).

Necrotising inflammation and negative ZN vs GXP

There were 5 cases of necrotising inflammation with a negative ZN result and with a GXP result available. All 5 GXPs were positive.

Of the 5 cases with a negative ZN and positive GXP:

- 4 cases had tissue samples submitted
- 1 case had both a sputum and tissue sample submitted; the GXP was positive on both samples.

There was one case where a GXP was not done and the TB culture that was submitted was negative. This case also had a histology result available and which was reported as necrotising inflammation with a positive ZN.

An in-house PCR was done on the FFPE blocks from this case and was positive for the MTb complex.

Necrotising inflammation and histology

There were 5 children who had cytology reported as necrotising inflammation who also had histology results available (37.5%). All 5 of the histology cases were biopsies from the cervical lymph nodes and which were all reported as necrotising granulomatous inflammation. All 5 histology cases also had a ZN stain done, of which 4 were positive for acid fast bacilli (80%). None of the ZN results were congruent between the cytology and histology cases:

- All the histology cases with a positive ZN had a negative ZN on cytology
- In one case, the ZN on cytology was positive whilst the ZN on histology was negative.

3.3.3. Correlation of cases with necrotising granulomatous inflammation

There were 5 cytology cases that were reported as necrotising granulomatous inflammation (figure 4). One cell block was done which was non-contributory. All ZN stains done. All had samples submitted for TB culture and GXP (table 42).

Table 42: Cytology cases reported as necrotising granulomatous inflammation and results from ancillary studies

No.	Site of biopsy	Cytology	Histology	ZN	TBC	GXP
1	ln	necrotising granulomatous inflammation	ND	-	Tissue +; Sputum -	Tissue +
2	Submandibular ln	necrotising granulomatous inflammation	ND	+	Tissue +; Gastric washing -	Tissue +
3	ln	necrotising granulomatous inflammation	ND	+	Tissue, gastric washing, eye swab+; sputum -	Tissue +
4	ln	necrotising granulomatous inflammation	ND	+	Tissue +; Sputum -	Tissue +; Sputum -
5	ln	necrotising granulomatous inflammation	ND	+	Tissue -; Gastric washing +	Gastric washing +; tissue -

No: Number; ZN: Ziehl-Neelsen; +: Positive; GXP: GeneXpert; TBC: TB culture; - : negative; ND: Not done; ln: Cervical lymph node unless otherwise specified

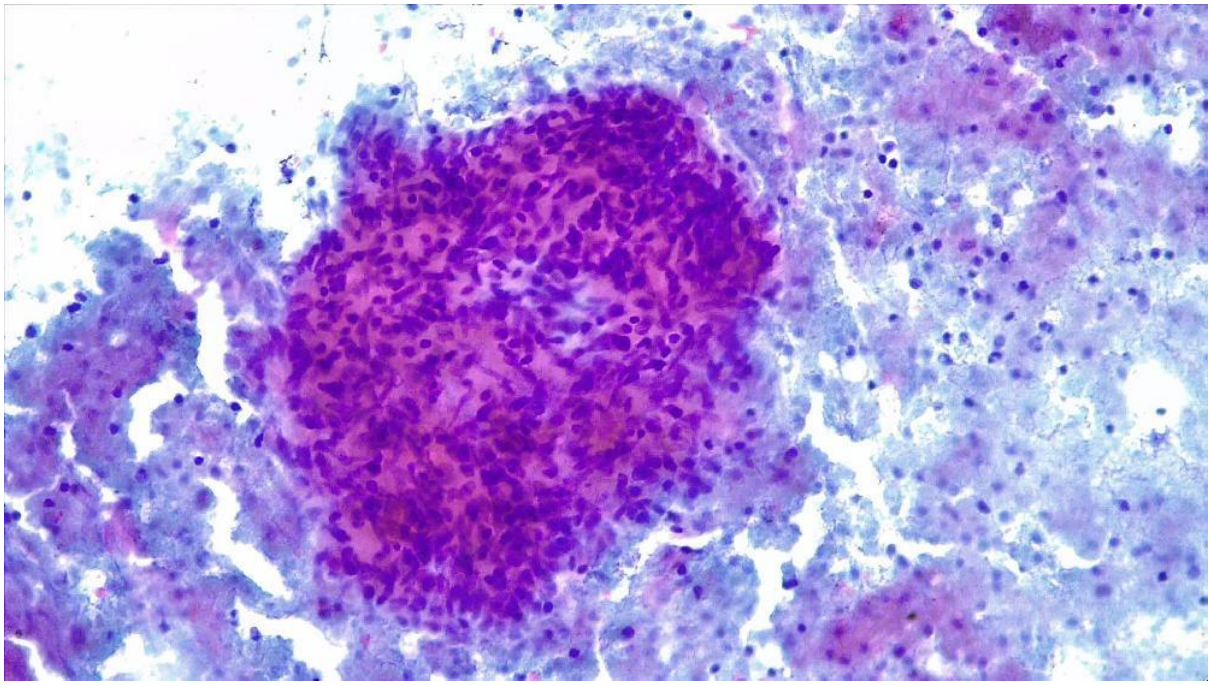

Figure 4: Cytology photomicrograph of granulomatous inflammation within a necrotic background (200x magnification)

Summary of correlation of cases with necrotising granulomatous inflammation

All 5 cases that were reported as necrotising inflammation had TB proven via both TB culture and GXP (100%) (table 43).

Table 43: Cytology with necrotising granulomatous inflammation and correlation with reference standards

Cytomorphologic feature	No. of TB proven cases	No. of TB Culture +	No. of GXP +
Necrotising granulomatous inflammation (n=5)	5 (100%)	5 (100%)	5 (100%)
Necrotising granulomatous inflammation, ZN+ (4)	4 (100%)	4 (100%)	4 (100%)
Necrotising granulomatous inflammation, ZN- (1)	1 (100%)	1 (100%)	1 (100%)

No: Number; ZN: Ziehl-Neelsen; +: Positive; GXP: GeneXpert

Necrotising granulomatous inflammation vs TB culture

All the cases had samples sent for TB cultures and which were all positive (100%). There were in total 5 tissue samples (100%), 3 sputum samples (60%), 2 gastric washings (40%) and an eye swab (20%) sent for TB culture.

Mycobacterium tuberculosis was cultured in all 5 tissue samples (100%), 1 of the 2 gastric washings (50%), in 1 eye swab (100%) and in none of the 3 sputum samples (0%) (table 44).

Table 44: Samples from cases of necrotising granulomatous inflammation submitted for TB culture

Sample type submitted	No. of samples submitted for TB culture	No. of culture positive (%)
Tissue	5	5 (100%)
Sputum	3	0
Gastric washings	2	1(50%)
Eye swab	1	1 (100%)

No.: Number

Necrotising granulomatous inflammation and ZN vs TB culture

All 5 cases had a ZN done and in 4 cases the ZN was positive for acid fast bacilli (80%). All of the 4 cases had a positive TB culture result (100%). In addition the ZN negative case was TB culture positive.

Of these 4 cases with a positive ZN and a positive TB culture:

- 2 cases had both a gastric washing and tissue sent for TB culture; in both cases only the tissue samples were positive
- 1 case had both tissue and sputum sent for culture and only the tissue was positive
- 1 case had samples from tissue, gastric washings, sputum and an eye swab sent for culture; the tissue, gastric washing and eye swab were positive.

In the case with a negative ZN, samples from both tissue and sputum were sent for TB culture and the tissue sample was positive.

Necrotising granulomatous inflammation vs GXP

All cases had samples sent for GXP and all were positive (100%).

The GXP samples from the 5 cases were obtained from 5 tissue samples, 2 sputum samples and 1 gastric washing:

- In 2 cases samples from both tissue and sputum were sent for GXP
- In 2 cases samples from tissue alone was sent for GXP
- In one case, both a tissue sample and a gastric washing was sent for GXP.

The MTb complex was detected on GXP in 5 of the 5 tissue samples (100%) and in the single gastric washing (100%). All 2 sputum samples were GXP negative (table 45).

Table 45: Samples from cases of necrotising granulomatous inflammation submitted for GXP

Sample type submitted	No. of samples submitted for GXP	No. of GXP positive (%)
Tissue	5	5 (100%)
Sputum	2	0
Gastric washings	1	1(100%)

No.: Number

Necrotising granulomatous inflammation and ZN vs GXP

There were 4 cases with a positive ZN result. All 4 of these cases also had a positive GXP result (100%).

Of the 4 cases with positive ZN and a positive GXP:

- 2 cases had tissue alone submitted for GXP; both had positive GXP results
- 1 case had samples from a gastric washing and tissue submitted for GXP; only the gastric washing was GXP positive
- 1 case had samples from sputum and tissue submitted and only the tissue was GXP positive.

There was 1 case with a negative ZN result that had a positive GXP. This case had a sample from both tissue and sputum which was submitted to GXP; however only the tissue sample was GXP positive (table 46).

Table 46: Cases of necrotising granulomatous inflammation and ZN submitted for GXP

Necrotising granulomatous inflammation with ZN	No. of GXP done	No. of GXP + (%)
ZN+	4	4 (100%)
ZN-	1	1 (100%)

No.: Number; ZN: Ziehl Neelsen; +: positive; -: negative

3.3.4. Correlation of cases with acute inflammation

There were 3 cases reported with features of acute inflammation; 2 cases were reported as acute inflammation and 1 case was reported as an abscess. All 3 cases had a ZN stain; however, all were negative. All 3 cases had a TB culture and GXP result (table 47).

Table 47: Cytology cases reported with features of acute inflammation and results from ancillary studies

No.	Site of biopsy	Cytology	Histology	ZN	TBC	GXP
1	ln	Abscess	ND	-	Tissue, NPA, sputum -	Tissue, NPA, sputum -
2	neck soft tissue	Acute inflammation	ND	-	Tissue +	Tissue +
3	ln	Acute inflammation	ND	-	Tissue +	Tissue +

No: Number; ZN: Ziehl-Neelsen; +: Positive; GXP: GeneXpert; TBC: TB culture; - : negative; ND: Not done; ln: Cervical lymph node unless otherwise specified; NPA: Nasopharyngeal aspirate

There was TB proven disease in 2 cases (66.7%):

- 2 cases were proven via TB cultures from tissue samples
- 2 cases were proven via GXP from tissue samples.

There was no proven TB in the case reported as an abscess on material submitted from both TB culture and GXP (table 48).

Table 48: Cytology cases of acute inflammation and ZN vs TB proven via reference standards

Cytomorphologic feature	No. of TB proven cases	No. of TB Culture +	No. of GXP +
Acute inflammation (n=3)	2 (67%)	2 (100%)	0
Acute inflammation, ZN- (3)	2 (67%)	2 (100%)	0

No: Number; ZN: Ziehl-Neelsen; +: Positive; GXP: GeneXpert; TBC: TB culture; - : negative

3.3.5. Correlation of cases with non-necrotising granulomatous inflammation

There was a single cytology case that was reported as non-necrotising granulomatous inflammation (figures 5 and 6). A ZN stain was done and was positive. There was evidence of MTb in this case. Samples from both tissue and sputum were sent for TB culture and GXP. The tissue sample submitted for both TB culture and GXP were positive for MTb. The sputum was negative. A cell block was also made; however, was reported as non-contributory

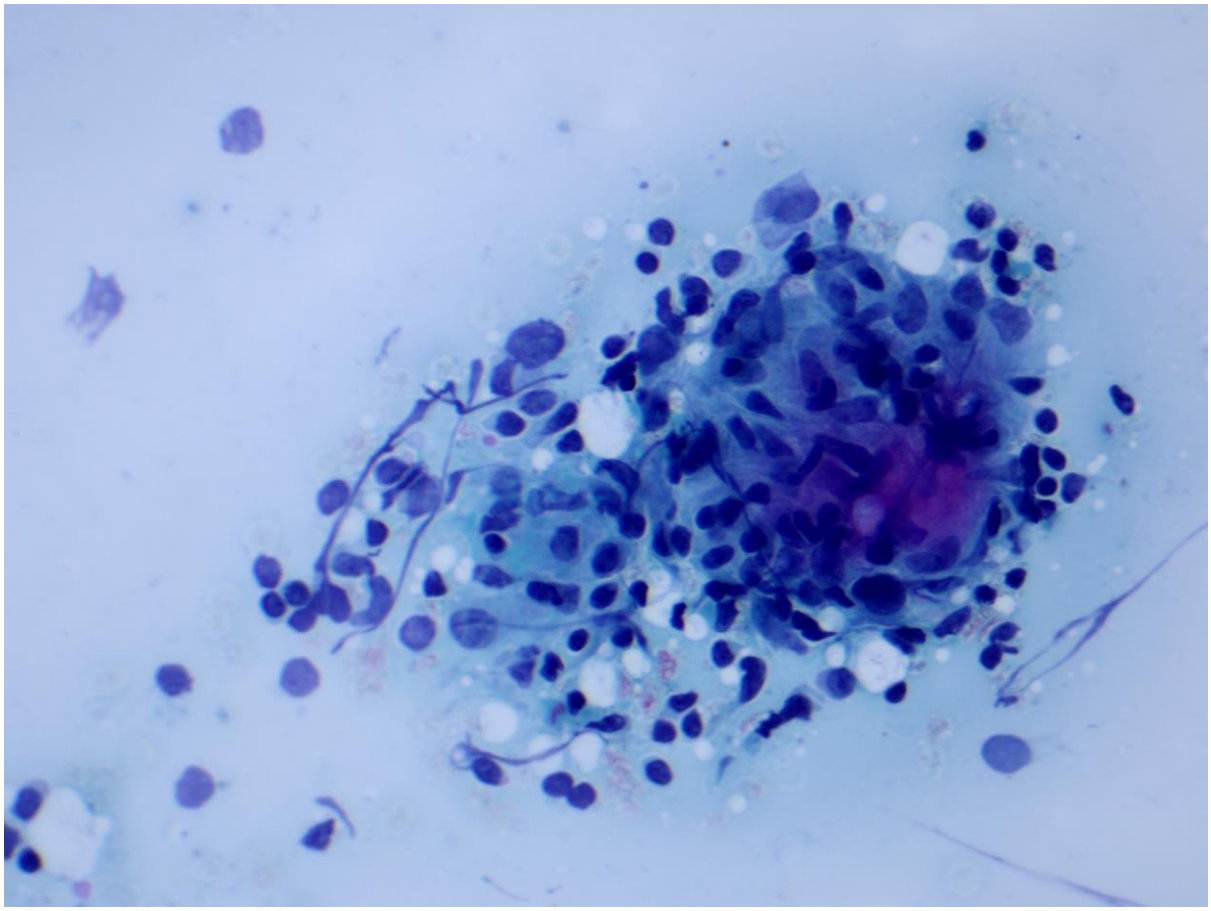

Figure 5: Photomicrograph of a non-necrotising granuloma on cytology (200x magnification)

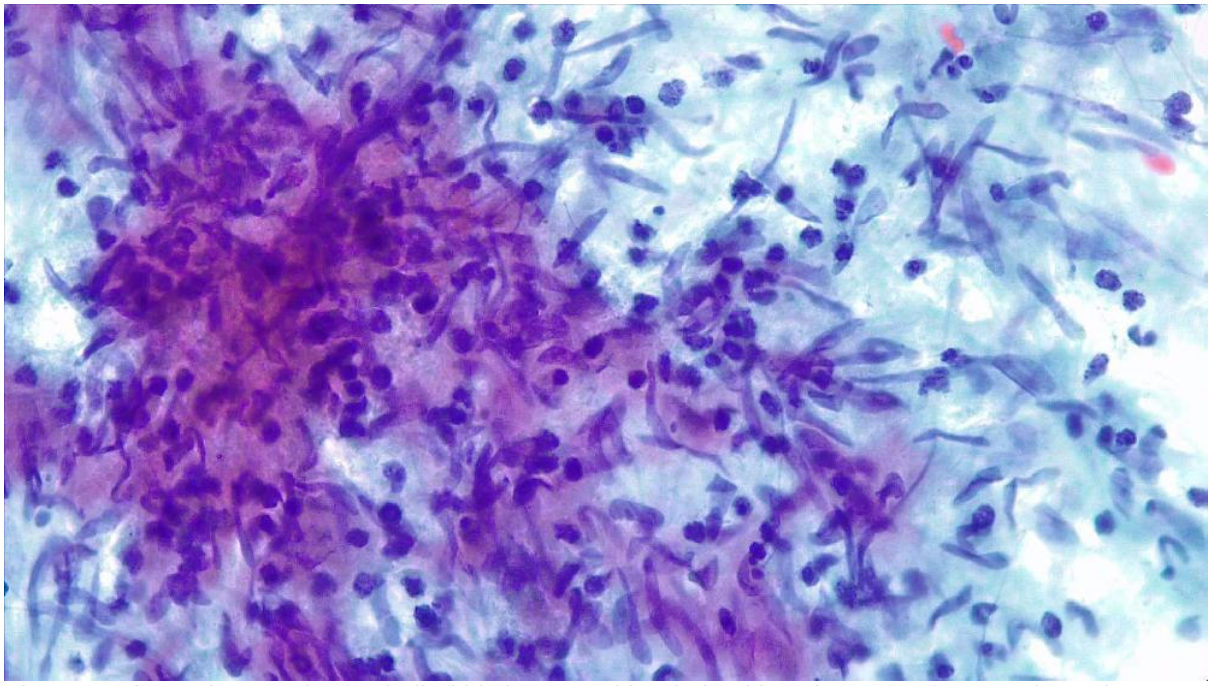

Figure 6: Photomicrograph of spindled histiocytes with admixed lymphocytes of a granuloma (400x magnification)

3.3.6. Correlation of cases with other morphologies

Atypical spindle cells

There was 1 case that was reported as atypical spindle cells from a cervical lymph node aspirate. A biopsy for histology was done and was reported as necrotising granulomatous inflammation with a positive ZN stain. There were no samples submitted for TB culture or GXP. An in-house PCR was done which confirmed the presence of MTb complex.

Reactive lymphocytes

There were 2 cytology cases that were reported as reactive lymphocytes. Both had ZN stains done with 1 case having a ZN positive result. In addition, a single cell block on one case was performed but was reported as non-contributory. There was evidence of MTb in both cases on TB culture and GXP. Samples from both tissue and sputum were sent for TB culture and GXP (table 49).

Table 49: Cytology cases reported with other morphologies and results from ancillary studies

No.	Site of biopsy	Cytology	Histology	ZN	TBC	GXP	In-house PCR
1	ln	Atypical spindle cells	Necrotising granulomatous inflammation, ZN+	ND	ND	ND	+
2	ln	Reactive lymphocytes	ND	+	Tissue, sputum +	Tissue, sputum +	ND
3	ln	Reactive lymphocytes	ND	-	Tissue +	Tissue +	ND

No: Number; ZN: Ziehl-Neelsen; +: Positive; GXP: GeneXpert; TBC: TB culture; - : negative; ND: Not done; ln: Cervical lymph node; PCR: polymerase chain reaction

3.3.7. Correlation of cytology with reference standards for the diagnosis of TB

There were in total 28 children with suspected TB who had FNAs for cytology done. There was proven TB via TB culture and/or molecular investigations in 24 of the 28 cases (85.7%).

MTb was demonstrated by:

- TB culture in 20 of the 24 TB proven cases (88.9%)
- GXP in 22 of the 24 TB proven cases (91.7%)
- Both GXP and TB cultures in 17 of the 24 TB proven cases (70.1%)
- In-house PCR in 2 of the 24 TB proven cases (8%).

There were 26 ZN stains done; 15 ZN stains were positive (55.6%) and 11 ZN stains were negative for acid fast bacilli (40.1%). There were 2 cases where a ZN was not done.

Of the 15 cytology cases with a positive ZN; there was microbiologic and PCR evidence of MTb in 13 of the cases (86.7%).

MTb was demonstrated by:

- TB culture in 12 of the 13 TB proven cases (86.7%)
- GXP in all 12 of the 13 TB proven cases (100%)
- Both GXP and TB culture in 10 of the 13 TB proven cases (77%)

Of the 11 cytology cases with a negative ZN; there was microbiologic and PCR evidence of MTb in 10 cases (91%).

MTb was demonstrated via:

- TB culture in 8 of the 10 TB proven cases (80%)
- GXP in all 7 of the TB proven cases (70%)
- Both GXP and TB cultures in 7 of the 13 TB proven cases (70%)
- In-house PCR in one case (10%) (table 50).

Table 50: Cytology cases with TB proven by reference standards

	No. of TB proven cases (%)	No. of + TB culture cases (%)	No. of + GXP cases (%)	No. of + GXP & + TB culture (%)	No. of in-house PCR+ (%)
Cytology (n=28)	24 (88.9%)	20 (88.9%)	22 (91.7%)	17 (70.1%)	2 (8%)
Cytology & ZN + (n=15)	13 (86.7%)	11 (84.6%)	13 (100%)	10 (77%)	0
Cytology & ZN – (n=11)	10 (91%)	8 (80%)	9 (81.8%)	7 (70%)	1 (10%)
Cytology without ZN (n=2)	2 (100%)	1 (50%)	0	0	1 (50%)

No: Number; ZN: Ziehl-Neelsen; +: Positive; GXP: GeneXpert; TBC: TB culture; - : negative; ND: Not done; ln: Cervical lymph node; PCR: polymerase chain reaction; TB: Tuberculosis

The sensitivity of cytology for TB diagnosis was 79.2% against the combined reference standards, 81.8% against GXP and 77.3% against TB cultures. The PPV of cytology for TB diagnosis was 86.4% against the combined reference standards, 94.7% against GXP and 81% against TB cultures (table 51).

Table 51: Sensitivity and PPV of cytology against the reference standards

		Sensitivity	PPV
Reference Test	Total	n/N (%)	n/N (%)
Cytology vs TB culture	25	17/22 (77.3)	14/21 (81)
Cytology vs GXP	21	18/22 (81.8)	18/19 (94.7)
Cytology vs TBC, GXP, PCR	28	19/24 (79.2)	19/22 (86.4)

PPV: positive predictive value, n: index group; N: control group; GXP: GeneXpert; TBC: TB culture

Chapter 4: Discussion

The diagnoses of pulmonary and extrapulmonary TB are difficult to confirm in children. This is due to the paucibacillary nature of paediatric TB disease and the difficulty in obtaining samples for microbiological confirmation. Masses in children with suspected cervicofacial TB are amenable to FNA or surgery for further cytology and histology assessment. It is therefore important to understand how well the morphologic features (from cytology and histology) correlate with defined reference standards (TB culture and molecular evidence of MTb) for the diagnosis of TB and how well one can rely on these features.

4.1. Case characteristics

Demographic information

There were 3 children less than 1 years of age included in the study and TB was proven in 1 of these children. A 1-month-old child had both TB culture and GXP confirmed TB of the cervical lymph node, a 3-month-old child had a cervical lymph node biopsy for histology which showed necrotising inflammation with a negative ZN but with a TB culture positive for M. *bovis*. This most likely represents a manifestation of BCG lymphadenitis secondary to the BCG vaccine which is administered after birth. Typically, involvement of the axillary lymph nodes is seen, but cases of cervical disease have been documented (52). Although surgical excision is not recommended, surgery is curative obviating the need for antituberculous drugs (53).

Lastly, an 8-month-old had a cervical lymph node biopsy for histology which showed organising granulation tissue compatible with the wall of an abscess; subsequent TB cultures and the in-house PCR was negative for the MTb complex.

There was slightly more female (43 cases, 56.6%) than male (33 children, 43.4%) distribution in the study; in keeping with multiple studies that shows a slight female predominance in cases of head and neck TB (30).

There were more children that had biopsies done for histology (48 children) than cytology (22 children) in this study. Red Cross Children's Hospital is a tertiary center that receives referral patients from clinics and secondary hospitals. It is likely that children from peripheral medical centers were referred to Red Cross Children's Hospital for excisional biopsies of clinically suspected TB lesions of the head and neck region. Other diagnoses may have also been clinically suspected (such as lymphomas or salivary gland neoplasms) thus prompting referral for an excisional biopsy. One child was referred to ENT surgery because of upper airways obstruction.

There were results from 68 TB cultures and 56 GXPs available. There were more TB cultures than GXP done, as the GXP was only fully implemented in the country in 2013 (54).

Sites of involvement by EPTB

The cervical and the submandibular lymph nodes were the most frequently involved sites by EPTB. This is in keeping with literature where the cervical lymph nodes are the most common sites of involvement by EPTB in the paediatric population (30,55)

The larynx is the second most commonly affected site by TB (30). In this study, there were 2 cases of suspected laryngeal involvement with histology suggestive of TB (necrotising granulomatous inflammation); however, TB cultures and PCR were negative and therefore TB disease could not be confirmed in these cases. This may be due to the submission of material which may have been too small or under-sampled.

Involvement of other sites in the head and neck area by EPTB are rare (30); however, in this study there was TB confirmed disease involving the cervical vertebrae (3 cases) retro-pharynx (1 case), soft tissues (5 cases), skin (3 cases), and mastoid bone (2 cases).

Of the 5 confirmed soft tissue TB cases, 4 were from the neck and 1 was from the scalp. 2 cases showed necrotising granulomatous inflammation on histology with negative ZN stains, 2 cases showed necrotising inflammation on cytology and 1 case showed acute inflammation on cytology. These soft tissue TB cases probably represent extension of underlying complicated TB lymphadenitis. There was 1 case of suspected TB soft tissue involvement that showed features of an abscess on histology but had a contaminated TB culture, negative GXP and negative in-house PCR. This case probably represented a true abscess and not TB as suspected.

One of the skin biopsies was reported as necrotising granulomatous inflammation consistent with a papulonecrotic tuberculid on histology, the ZN was negative and no material was submitted for TB culture or GXP. A subsequent in-house PCR was performed on the FFPE blocks which was negative for the MTb complex. Papulonecrotic tuberculids are hypersensitivity reactions to mycobacterial antigens often as a result of pulmonary tuberculosis (18). Therefore mycobacteria are not detected on the ZN stains, TB cultures or GXP of samples from papulonecrotic tuberculids.

Another skin biopsy was also reported as necrotising granulomatous inflammation consistent with papulonecrotic tuberculid with a negative ZN; however, the GXP performed on a sputum samples was positive confirming pulmonary disease and thus highlighting the immunologic nature of tuberculid disease.

In contrast, a skin biopsy from the neck was reported as necrotising inflammation features consistent with scrofuloderma with a positive ZN, and had TB cultures and GXP performed on both tissue and sputum samples which were positive for MTb confirming tuberculosis. In this case, scrofuloderma probably resulted from cutaneous involvement from underlying cervical TB lymphadenitis (18).

Involvement of the mastoid bone, cervical spine and retropharynx are rare forms of EPTB and untreated disease can result in sequelae such as neurological fallout, intracranial dissemination of disease and permanent disability (29, 33). A prompt diagnosis and rapid initiation of appropriate therapy is mandatory.

TB drug resistance

The prevalence of drug resistant remains high in children in the Western Cape. Of the 52 cases of TB proven by GXP and/or TB cultures, there were 3 cases of drug resistant TB (5.8%). There were 46 cases of Rifampicin sensitive TB, 1 case of Rifampicin resistant TB and 2 cases of TB resistance to Isoniazid and Rifampicin. The GXP provided information regarding sensitivity to Rifampicin in 47 cases (90.3%). The GXP has the advantage of being able to detect the MTb complex and resistance to Rifampicin on the same specimen on one test. The GXP has a shorter turnaround time (48 hours) when compared to TB culture (up to 8 weeks). Thus allowing for rapid initiation of appropriate therapy. However, a disadvantage of the GXP test is that it only detects resistance to Rifampicin.

A disadvantage of the in-house PCR test which was performed on FFPE samples is that TB drug resistance cannot be identified.

GXP vs TB Culture

The study showed that the yield of MTb is greater with GXP than TB culture (87.9% vs 63.5%). The GXP was able to identify MTb in 5 cases that were TB culture negative. These findings adds weight to the WHO recommendation for the use of the GXP for diagnosis of EPTB. Possible reasons for a negative TB culture/ a positive GXP result includes the uneven distribution of bacilli in tissue, the paucibacillary nature of EPTB and possible amplification of non-viable MTb DNA.

The GXP has a quicker turnaround time (a few days) than TB culture (up to 6-8 weeks) and also provides a result of Rifampicin sensitivity. However, the TB culture provides information regarding mycobacterial speciation assisting in treatment guidance.

The TB culture had the highest yield on samples received from tissue (43 of 66 samples were culture positive; 65%) compared with samples from sputum and

tracheal aspirates (10 of 28 samples were culture positive; 23.1%). Likewise there were more patients that had GXP positive results from tissue samples (45 of 52 samples; 85.6%) than on samples from sputum and tracheal aspirates (11 of 18 samples; 61.1%). Of the children who had samples from both tissue and respiratory samples (sputum and tracheal aspirates) submitted for GXP and TB culture, in 6 cases only the tissue samples were positive. These findings suggest that a proportion of the children with head and neck TB probably did not have concomitant pulmonary involvement. Therefore samples from tissue of the affected sites should always be submitted for TB culture and GXP.

4.2. Cytology

TB disease was proven by either TB culture or GXP in 20 of the 22 cytology cases with cytomorphological features compatible with TB.

There was proven TB in 14 of the 16 cases reported as necrotising inflammation (87.5%), in all 5 cases reported as necrotising granulomatous inflammation (100%) and in the 1 case reported as non-necrotising granulomatous inflammation (100%). This is in keeping with the cytology findings of TB lymphadenitis described in the literature (6). The morphologic finding that correlated the best with proven TB in this study was necrotising inflammation rather than granulomatous inflammation. This finding may be reflective of the affected child's immature immunity where a necrotising inflammatory response is elicited rather than a granulomatous response (6).

All 22 cases showing morphologic features compatible with TB had a ZN stain done; of which there were 15 ZN positive stains (68.2%) and there were 7 ZN negative stains (31.8%). Of these cases with a positive ZN, TB was proven in 13 cases (86.7%). The 2 cases without proof of TB were reported as necrotising inflammation with negative TB cultures only and no material was sent for GXP. The TB cultures in

these cases may have yielded a false negative result as it is possible that not enough viable mycobacteria were present to allow for growth in the TB culture. Also mycobacteria are non-uniformly distributed and may not have been sampled. If a cellblock was done on these cases, the FFPE of the cell block could have been sent for in-house PCR testing. This highlights the potential value and utility of having a cellblock done in cases with cytomorphology findings consistent with TB but without TB culture or GXP proof of TB; the cellblock provides material for additional ancillary studies (i.e. molecular tests) to be done.

In cases with a negative ZN, TB was proven in all 7 cases (100%). This result indicates that a negative ZN stain does not rule out TB especially in the presence of typical cytomorphological findings. This also highlights the fairly good specificity but low sensitivity of the ZN stain (25, 36, 51). Therefore material should always be submitted for both TB cultures and GXP in all cases of clinically suspected TB.

FNA is easy and safe to perform; however, the cytomorphological features may not always be specific for TB (6, 25, 36). The pathological lesions in affected areas may not be evenly distributed and thus the FNA may not directly sample these pathological sites and may not be present in aspirates. In our study, there were cases that showed features not typical for TB (acute inflammation, reactive lymphocytes and atypical spindle cells) but had supported evidence of TB (5 of 6 cases). Mycobacterial lymphadenitis has been described with features of acute suppurative inflammation without granulomas, and in this study there were 2 out of the 3 cases that were reported as acute inflammation that had confirmed TB (66.7%) (6). The third case had no TB culture or GXP proof of TB and likely represented a true abscess.

In the case that was reported with atypical spindle cells there was also histology done. The histology was reported as necrotising granulomatous inflammation with a positive ZN. A subsequent in-house PCR was positive for MTb. The spindle cells likely represented epithelioid macrophages or fibroblasts, which was likely part of a granuloma which was not sampled on the FNA. Although there was material submitted for a cellblock, the cellblock was inadequate for definitive diagnosis probably due to under sampling of the affected area.

The sensitivity of cytomorphology for the diagnosis of TB has been reported with a sensitivity of 78% against TB culture (25, 36). In our study, the sensitivity was 77.3%. This result may be confounded by selection bias and the high incidence of TB in the Western Cape and in South Africa.

The sensitivity of cytomorphology against GXP (81.8%) a reference standard was slightly higher than that of cytomorphology against TB culture (77.3%) for a TB diagnosis. This is in keeping with a meta-analysis which showed a sensitivity of 96% with the use of GXP on lymph node aspirates for TB diagnosis (56). Ligthelm showed that the GXP on FNA samples had a sensitivity of 96.7% and a specificity of 88.9% when compared against positive cytomorphology and/or positive TB culture as combined reference standards (46). The positive predictive value of cytology and GXP (94.7%) was higher than that of cytology and TB culture (81%). In addition, there were 2 more cases of TB detected by cytology analysis along with GXP compared to TB culture. Antel reported that yield of MTb was only 17% by TB culture on FNA material thus limiting its usefulness on FNA samples (51).

The use of FNA cytology and a GXP is an excellent first line test for TB. The GXP has the added advantage over TB culture of providing a rapid diagnosis as well as

providing information regarding Rifampicin resistance directly from a single FNA specimen.

Recommendations from our cytology findings:

If the cytomorphology is consistent with mycobacterial infection (necrotising granulomatous, granulomatous or necrotising inflammation), regardless of the ZN stain result; TB is a likely diagnosis. All FNA samples should have cell block done, as this allows for possible further PCR tests to be done. Material should be sent for both GXP and TB cultures; however, in the interim, TB therapy can empirically be started. Excisional biopsies for histology should be done if the initial FNA and GXP are negative in a patient with suspected head and neck EPTB.

4.3. Histology

The classically described histomorphology of TB is necrotising granulomatous inflammation (23, 57). In this study, 79.5% of the cases that were reported as necrotising granulomatous inflammation had proven TB. In 7 cases reported as necrotising granulomatous inflammation in this study, there was no definitive proof of TB; however on folder review, there was recorded clinical improvement in 2 children after initiation of therapy and thus it can be presumed that these children had TB. In an endemic area such as the Western Cape, empiric TB treatment can be started based on characteristic morphology and a positive ZN stain; however material for both TB cultures and GXP should always be submitted if possible. If the facilities are available and samples for TB culture and GXP weren't submitted, the inhouse PCR is an option in order to demonstrate the presence of MTb. The reported sensitivities for the use of TB PCR on FFPE in literature have ranged from 51.1% to 87.5% and the specificity has ranged from 90% to 100%. Of the 10 cases in which the PCR was done, 1 was inadequate and in 4 cases MTb was detected. However, the PCR does not discriminate between live and dead bacilli, therefore clinical and TB culture (if possible) correlation should be made before TB treatment is started. There were 5 negative PCR tests. In 2 cases there was reported clinical improvement on follow up visits. One child had laryngeal involvement with subsequent suspected PNT lesions, it can thus be assumed that this child had true TB.

Possible reasons for a negative PCR even in the face of suggestive morphology includes:

- some strains of MTb do not contain the IS6110 sequence which would result in a negative PCR reaction (58)

- possible temperature related degradation of DNA in the FFPE tissue blocks (41)
- formalin fixation induced degradation of DNA (47)
- small biopsy size leading to insufficient DNA quantity for proper evaluation and lesional tissue which may not have been included in the section for PCR (41, 47).

One study showed that the ZN stain had a specificity of 97% therefore in the remaining 4 children with negative PCR results but positive ZN stains, although there is no definitive proof of TB it can be assumed that these cases were probable TB infections (59).

One of the cases that was reported as necrotising granulomatous inflammation had a negative ZN but a positive modified ZN proved on culture to be non-tuberculous mycobacteria. The histology of non-tuberculous mycobacterial infection shows necrotising granulomatous inflammation, similar to MTb infection; but collections of foamy histiocytes may be seen (6).

Mycobacterium avium intracellulare, Mycobacterium scrofulaceum and Mycobacterium kansasii can cause localised neck lymphadenopathy in children, which resolves with drainage alone (22). Morphologically the bacilli are identical to MTb. A high level of suspicion is required in order to diagnose NTBM infection and a modified ZN should be requested in addition to the ZN stain.

Necrotising granulomatous inflammation can also be seen in fungal infections such as histoplasmosis and cryptococcosis (57). Thus requesting fungal stains (PAS and Grocott) should be done on all cases of necrotising granulomatous inflammation if the ZN and modified ZN stains are negative. Jain and Mukhopadhyay have recommended

avoiding the use of the term “caseating” granulomas to avoid implying that this morphological pattern is specific for MTb (57).

All children with histology reported as necrotising granulomatous inflammation had a ZN stain done (39), of which 28 were AFB positive and 11 were AFB negative.

So although the ZN is a relatively specific stain, the reported sensitivity of the ZN is low. Studies have reported the sensitivity ranging from 20 to 43% (60). The detection of acid fast bacilli in granulomas is dependent on the child’s immune system, and where there is a prominent granulomatous response acid fast bacilli are scarce which could account for the false negative ZN results in cases of necrotising granulomatous inflammation.

There was proof of TB in 8 of the 11 negative ZN cases (72.7%). Thus TB cannot entirely be excluded even if the ZN is negative. It is also important to inform clinicians that TB cultures may be positive even if ZN stains are negative.

Half of the cases of necrotising inflammation without granulomas had proven TB (2 of 4 cases, 50%). Of the 2 cases without proven TB, 1 case cultured Staphylococcus aureus and therefore was representative of an abscess while the other case cultured Mycobacterium bovis (as discussed above). The 2 cases that had proven TB were ZN positive whilst the other 2 cases were ZN negative. Thus it is important to keep in mind other causes of necrotising inflammation, especially when the ZN is negative. Additional stains such as the modified ZN, Warthin-Starry, PAS and Grocott should be done to exclude atypical mycobacteria, Bartonella henselae and fungal organisms. Non-infectious causes of necrotising lymphadenitis such as granulomatosis with polyangiitis, Kikuchi’s lymphadenitis and rheumatoid nodule, although less likely in children may be considered (61).

Surprisingly none of the cases reported as non-necrotising granulomatous inflammation (2 cases) had proven TB even though both cases were ZN positive. These cases may represent false negative cases. EPTB is paucibacillary and the bacilli are not evenly distributed in tissue. It is possible that samples from these cases did not contain any bacilli. So although there is absence of microbiological and molecular proof of TB, it is probable that these cases represented TB disease and that the patients best be managed with anti-TB drugs. The patient with laryngeal involvement had enlarged hilar lymph nodes, suspicious for concomitant pulmonary TB disease. Unfortunately details regarding clinical response to TB therapy was not available. Other stains should be done in addition to the ZN to exclude fungal infections (PAS and Grocott stains). Other infectious causes that may be considered include CMV lymphadenitis, Schistosoma sp. and Toxoplasma gondii infection (61). Thereafter non-infectious causes of granulomatous infection can be considered.

TB may also show an acute inflammatory type appearance which may or may not be part of necrotising granulomata. In our study 4 out of 9 cases that were reported with features of acute inflammation proved to be TB. All 4 of these cases were ZN negative, highlighting the poor sensitivity of the ZN stain. The remaining 5 cases all proved to be negative for TB on microbiology and molecular studies and most likely abscesses in evolution rather than TB. However, it is important to exclude fungal organisms when there is suppuration present (23). Stains for bacteria and fungi should therefore be done. Notwithstanding, an acute inflammatory type pattern may still prove to be TB even if ZN stains are negative. Therefore material should be sent for TB cultures and GXP even in abscesses in the clinical context suspicious for TB.

TB cultures are sensitive and are the WHO-defined gold standard for TB diagnosis (1,2). However, in this study there were more cases of TB detected by GXP rather

than by TB culture. In addition the sensitivity of histology is higher when GXP was used as a reference standard compared with TB cultures (90.3%% vs 85.2%). Of the 36 histology cases with proven TB, there were 6 more cases identified by GXP than by TB culture. Because Red Cross Children's Hospital is a specialty referral centre, children who were referred from peripheral hospitals and clinics may have had prior TB treatment which would decrease the density and weaken the TB bacilli resulting in negative TB cultures. The GXP and molecular studies are able to detect both live and dead or weakened bacilli which results in a higher detection rate by these methods (62). Due to the paucibacillary nature of extrapulmonary TB, the sensitivity of TB cultures are limited.

Overall the use of histology for TB diagnosis has shown to be a sensitive test; however it requires an invasive procedure, that needs to be performed by medical practitioners with surgical skill and with potential complications including anaesthetic complications, wound infection, sinus formation and scarring.

Histology vs Cytology for TB diagnosis

In this study, against combined reference standards, histology was slightly more sensitive than cytology for TB diagnosis (89.2% vs 79.2%), but cytology had a higher positive predictive value (86.4% vs 73.3%). However, because FNA for cytology is a safer and simpler procedure to perform than biopsy for histology, the use of FNA as an initial investigation for children with suspected EPTB, is appropriate. FNA can be done in an outpatient setting. An extra advantage of the FNA is that repeated aspirations can be done for therapeutic drainage of lesions in addition to providing material for TB cultures and GXP. The calculated specificity and negative predictive values were not included due to the negligible numbers of non-TB cases included in the study.

Study Strengths

Red Cross Children's Hospital is a dedicated paediatric centre in South Africa and the results from this study are probably representative of TB cases from this region. This is also one of the first studies to investigate the correlation of histology and cytology for the diagnosis of EPTB in the head and neck region in children.

Study Limitations

The study had several limitations including a small sample size, the lack of clinical detail regarding follow up after initiation of therapy and the retrospective nature of the study design. The quality of the data included in the study was dependent on the reports from the information systems database. Future studies should include a larger sample size to achieve statistical significance. Not all children had a GXP performed in this study, so it is difficult to make direct comparisons between groups between groups who had TB cultures and the GXP test. There were several potential sources of bias including selection bias, as patients who had clinical suspicion of EPTB were specifically selected for the study and this could lead to an overestimation of calculated sensitivities and inaccurate estimation of the specificity. The study only included a few patients with head and neck lesions in which TB was excluded. If funds allowed, all the cases in this study could have had in-house PCR performed to allow for comparisons of performance against GXP and TB culture.

Suggestions for future studies

The GXP ultra has greater sensitivity than the GXP for the detection of MTb which is beneficial for use in paucibacillary samples such as lymph node tissue. There has been limited studies on the performance of GXP ultra on FFPE tissue for a TB diagnosis. A study by Amira has shown promising results for GXP ultra use on

FFPE for TB cervical lymphadenitis diagnosis (51). A potential study could be to compare the performances of GXP against the GXP ultra for TB diagnosis on FFPE tissues.

CONCLUSION

Even though tuberculosis is a curable disease, it continues to impact the lives and development of millions of children. It is therefore important that a rapid and accurate diagnosis of TB is made so that appropriate treatment is provided.

TB cervical lymphadenitis is the most common presentation of EPTB in the head and neck.

FNAB for cytology is a minimally invasive, safe, and inexpensive procedure which can be done in the outpatient or primary health care setting by not only doctors, but also trained nurse practitioners for a rapid and accurate diagnosis of TB. The cytomorphologic features of necrotising inflammation with or without granulomas and a positive ZN stain correlates well with both TB cultures and GXP for the diagnosis of TB.

A FNAB also provides material for both GXP and TB cultures. Although TB cultures are still the WHO-defined gold standard for diagnosis, it takes up to 8 weeks for a result to be issued. However, the GXP offers both a rapid TB diagnosis as well as a Rifampicin sensitivity results. Clinicians should also be made aware of the potential utility and are encouraged to perform cellblocks, which can also be used for inhouse PCR tests for MTb detection. Therefore, a FNAB for cytology of the affected area in the head and neck is a good first line procedure when EPTB is suspected in children.

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Appendix

Ethics approval letters

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

HUMAN RESEARCH ETHICS COMMITTEE
09 SEP 2020
UNIVERSITY OF CAPE TOWN

Form FHS007: Amendment – study staff

HREC office use only (FWA00001637; IRB00001938)			
☑ Approved			
This serves as notification that all changes to the study staff and documentation described below are approved.			
Chairperson of the HREC signature/ Designee		Date	10/9/20

Note:
Please note that incomplete amendment submissions will not be reviewed.

Please email this form and supporting documents (if applicable) in a combined pdf-file to hrec-enquiries@uct.ac.za

Please clarify your plan for research-related activities during COVID-19 lockdown.

Principal Investigator to complete the following:

1. Protocol Information

Date (when submitting this form)	2/9/2020
HREC REF Number	035/2020
Protocol title	Cytohistological correlation of cervicofacial head and neck extra-pulmonary tuberculosis in children: A 5 year retrospective case series
Protocol number (if applicable)	
Principal Investigator	Dr Shazia Peer
Department / Office Internal Mail Address	Shazia.peer@uct.ac.za

1.1 Does this protocol receive US Federal funding?	☐ Yes	☐ No

2.1 Staff changes (tick ✓)

Are new personnel being added to this research?	☐ Yes	☐ No
Are current personnel being removed from this research?	☐ Yes	☐ No
Is the principal investigator for this research being changed? If yes, please attach revised conflict of interest and PI declaration statements. (Refer: sections 7 and 8.3 in the New Protocol Application Form - FHS013)	☐ Yes	☐ No

UNIVERSITY OF CAPE TOWN FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS016: Annual Progress Report / Renewal

HREC office use only (FWA00001637; IRB00001938) This serves as notification of annual approval, including any documentation described below.			
☑ Approved	Annual progress report	Approved until/next renewal date	28.02.22
☐ Not approved	See attached comments		
Signature Chairperson of the HREC		Date Signed	18/5/2021

Comments to PI from the HREC

Principal Investigator to complete the following:

1. Protocol Information

Date (when submitting this form)	10/05/2021		
HREC REF Number	035/2020	Current Ethics Approval was granted until	28 Feb 2021
Protocol title	Cytohistological Correlation of cervicofacial head and neck extrapulmonary tuberculosis in children: A 5 year retrospective case series.		
Protocol number (if applicable)			
Are there any sub-studies linked to this study?	☐ Yes ☑ No		
If yes, could you please provide the HREC Ref's for all sub-studies? **Note:** A separate FHS016 must be submitted for each sub-study			
Principal Investigator	DR SHAZIA PEER		
Department / Office Internal Mail Address	SHAZIA.PEER@UCT.AC.ZA.		

(Note: Please complete the Closure form (FHS01D) if the study is completed within the approval period)