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Bacterial genotyping of CNS tuberculosis in South Africa: heterogenic *M. tuberculosis* infection and predominance of lineage 4

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ABSTRACT

Background: Tuberculous meningitis (TBM), the most severe extra-pulmonary manifestation of tuberculosis, is caused by the pathogen *Mycobacterium tuberculosis*. The *M. tuberculosis* complex includes seven lineages, all described to harbour a unique geographical dissemination pattern and clinical presentation.

Method: In this study, we set out to determine whether a certain *M. tuberculosis* lineage demonstrated tropism to cause TBM in patients from Cape Town, South Africa. DNA was extracted from formalin-fixed paraffin embedded central nervous system (CNS) tissue from a unique neuro-pathological cohort of 83 TBM patients, collected between 1975 and 2012. *M. tuberculosis* lineages 1, 2, 3 and 4 were determined using an allele specific PCR and Sanger sequencing.

Results: Of the 83 patient specimen tested, bacterial characterization could be performed on 46 patients (55%). *M. tuberculosis* lineage 4 was present in 26 patients (56%) and non-lineage 4 was identified in 10 cases (22%). Moreover, genomic heterogeneity was detected in the CNS specimens of 7 adults and 3 children.

Conclusion: We could show that infection of the CNS is not restricted to a single *M. tuberculosis* lineage and that even young children with rapid progression of disease can harbour more than one *M. tuberculosis* lineage in the CNS.

KEY WORDS

CNS tuberculosis; tuberculous meningitis; genotyping, childhood tuberculosis; *Mycobacterium tuberculosis* lineage; mixed infection; genomic heterogeneity

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INTRODUCTION

The infectious disease tuberculosis (TB) is a major global health problem caused by infection with the pathogen *Mycobacterium tuberculosis*. Tuberculous meningitis (TBM), one of the most severe extra pulmonary manifestations of TB, can develop once *M. tuberculosis* disseminates from its primary pulmonary focus. TBM is generally accepted to be a consequence of pre-existing granulomatous lesions in the central nervous system (CNS), so-called Rich foci. Once these foci rupture into the subarachnoid space the subsequent inflammatory response induces meningitis (1, 2).

The *M. tuberculosis* complex (MTBC) includes seven large-sequence polymorphisms (LSP) based lineages which are human adapted. Lineages 2, also known as East-Asian lineage, and 4, the Euro-American lineage, show the broadest distribution worldwide. In contrast, lineage 5 and 6 (*Mycobacterium africanum*) are restricted to West-Africa and lineage 7 (Ethiopia lineage) is almost exclusively found in Ethiopia. Lineages 1 (Indo-Oceanic) and 3 (East-African-Indian) display a more intermediate distribution (3, 4). The geographical distribution of lineages might suggest that lineages are adapted to particular human population groups (4). In addition, individual lineages are linked to distinct patterns of pathology and disease severity. Several studies suggest that infections with lineage 2 strains are more frequently associated with extra-pulmonary TB and TBM (5–9). Other associations found with lineage 2 strains are a more acute presentation of disease and younger age, indicating that this lineage is the most pathogenic (10–12). In contrast, lineage 4 was associated with a higher prevalence of pulmonary TB compared to TBM, suggesting a protective association with this lineage (5, 11). In South Africa, a study among 285 children showed no difference in susceptibility to TBM between the prevalence of two dominant sub-strains belonging to lineage 2 and 4 (13), indicating that both strains might contribute to CNS involvement equally in this population.

Infection with *M. tuberculosis* is not restricted to infection with a single lineage. This so called mixed infection, or genetic heterogeneity, is present in 10-20% of sputum cultures from individual TB patients and can have an enormous influence on treatment outcome and can select for drug resistance (14–16). The presence of genomic heterogeneity has not been described in cerebrospinal fluid (CSF) of patients with TBM so far. Since detection is restricted to our limited ability to culture bacteria from the CSF and is dependent on the sensitivity of the genotyping method used (17–19), the actual number of patients with genomic heterogeneity in the CNS is most likely underestimated.

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This present study aimed to determine the *M. tuberculosis* lineage most commonly found in the granulomas and inflammatory foci in brain tissue from patients in the Western Cape of South Africa. To achieve this, we use an historical cohort of brain specimen from children and adults with advanced stages of TBM. Within this paucibacillary tissue we demonstrate that lineage 4 is the most commonly detected lineage in our cohort. Furthermore, we could identify genomic heterogeneity within brain specimens and show for the first time that infection of the CNS is not restricted to a single *M. tuberculosis* lineage.

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MATERIALS AND METHODS

Study cohort

Material for this study was selected from a unique historical cohort study (Zaharie et al, unpublished data), consisting of brain specimen from children (<14 years, n=40) and adults (>14 years, n=43) with definite or probable TBM registered at the Department of Anatomical Pathology between 1975 and 2012. Patients with medical records, radiographic imaging and neuropathology brain specimen available were included (n=83), specimen were obtained post-mortem (n=76) or with diagnostic biopsy during life (n=7). For this study, one or two blocks from each patient were selected based on positive ZN staining. Boiled extracts from cultured *M. tuberculosis* isolates were included as technical controls for all tested bacterial lineages.

Sample preparation

The formalin-fixed paraffin embedded (FFPE) brain tissues were cut using a microtome (block size 8-54 $cm^{2)}$. To eliminate the possibility of cross-contamination, new microtome blades were used and the microtome was cleaned with xylene, 100% ethanol and 10% bleach between sectioning of each FFPE block. Blank, uninfected brain samples were tested as controls to exclude cross contamination on the microtome. Three to five slices of 10 µm (depending on block size) were stored for processing in 1.5 ml Eppendorf® Safe-Lock micro centrifuge tubes.

DNA extraction

DNA extraction was performed using the NucleoSpin® DNA FFPE XS kit (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions with the exception that the protocol was adjusted according to the amount of cut tissue.

Polymerase Chain Reaction Applications and Sanger sequencing

Extracted DNA was used for PCR amplification of the lineage specific alleles of lineage 1-4. Based on geographical distribution of *M. tuberculosis* lineages, we did not expect to find lineage 5-7 in the current cohort and focused on lineage 1-4. The QIAgen HotstarTaq® Plus Master Mix Kit (Qiagen, Hilden, Germany) was used with following cycling conditions: initial denaturation: 10 minutes, 95°C; 40 cycles of 90 seconds denaturation at 94°C; 30 seconds at the optimal T_m of the respective primers (Table S1) and 1 minute of extension at 72°C, with a final elongation at 72°C for 15 minutes.

Primers for the RD105 deletion were designed and adapted based on previous described work (20). Because of expected fragmentation of DNA, previously published primers could not be used. The

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primers were designed with Primer3 v. 0.4.0 and blasted on http://genolist.pasteur.fr/TubercuList/ using the flanking regions of RD239 (Lineage 1), RD105 (Lineage 2), RD181 (Sublineage 2), RD150 (Sublineage 2), RD142 (Sublineage 2) and RD750 (Lineage 3) (21). Details of all primer sequences and their regions are shown in supplemental table S1. PCR products were visualised with GR Green DNA dye (Labgene Scientific SA, Châtel-Saint-Denis, Switzerland) after electrophoresis in 2% agarose. Samples with one or two visible bands of expected size were identified as samples with intact DNA in the amplified region. In contrast, samples with a smear or no band (empty lane) were described as 'degraded DNA in the amplified region' or as 'absent *M. tuberculosis* DNA', respectively. Samples with degraded DNA in at least three different regions of the brain were not further tested. To identify lineage 4 strains, the presence or absence of the pks15/1 deletion was determined as previously described (22). PCR products with a clear band on agarose gel, were sent for Sanger Sequencing at Inqaba Biotech (South Africa).

Requirement for a reliable result was consistency between PCR result and sequencing. When a clear band on agarose gel is present and the sequencing is successful, results are called conclusive. If this is not the case, results are called inconclusive meaning that a lineage could not be determined for this sample.

H&E, RS and ZN-staining

Slides were stained according to conventional protocol with Hemotoxylin Eosin (H&E), Reticulin Silver (RS) and Ziehl-Neelsen (ZN) staining and analyzed by a skilled neuropathologist. Three categories were defined based on bacterial load per mm^2 identified on ZN staining with light microscopy Olympus BX43 (40x objective, $1mm^2$=4.2 high power field). Low bacterial load was defined as one or less bacteria per mm^2, medium bacterial load was defined as 2-20 bacteria per mm^2, and high bacterial load was defined as more than 20 bacteria per mm^2.

Statistical analysis

The database with patient information was managed with IBMM SPSS Statistics, version 23.0.0.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis and creation of graphs was performed with the same program. Differences between children and adults were tested presenting p-values for Chi-square test (Fisher's Exact test for cell size less than five), Mann-Whitney U (median), and independent-samples t-test (mean). The correlation between sample age and PCR success rate, and between ZN positivity and PCR success rate was tested with Spearman's correlation. Significance was measured at p=0.05. These analyses were done with IBM statistics version 25 (IMB Corporation, South Africa).

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Ethics

The study was approved by the Human Research Ethics Committee of Stellenbosch University, Cape Town, Western Cape, South Africa (ethics approval number S12/11/298). Informed consent for this study was waived. The post-mortem collection of neuropathology material was originally collected during patient admission at Tygerberg Hospital as part of standard care and available because the patient or their family provided permission for initial autopsy at the time.

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RESULTS

Baseline characteristics

Bacterial characterization could be performed on FFPE neuro-pathology material of 46 out of 83 TBM patients (55%) with conclusive results. On pathological examination, the diagnosis TBM was probable when clear parenchymal inflammation and granulomas were found, and definite when additional ZN positive bacteria were found or when the CSF culture was positive for bacteria. Of the 46 patients included for analyses, 28 were male (61%) and 33 were of mixed ancestry (77%). The median age was 5.6 years (0.5-65.5). The majority of patients presented with stage 3 TBM (61%) on admission and had definite TBM (62%) and the duration between start of therapy until autopsy was 7 days (range 0-153). Data on immunization was not available for most children (1/9, 11.1%, received BCG), and 11/21 (52%) had a registered TB contact in the household. 17 children were on the local standard 4-drug regimen of isoniazid, rifampicin, pyrazinamid and ethionamide (74%) (Table 1). Ethionamide is used instead of (WHO recommended) ethambutol within a short intensified drug regimen, because of better CSF penetration and good experience in respect to mortality and outcome (23). Definite TBM with a positive ZN staining was more frequently seen in children than adults. Due to the historical time frame of this cohort, HIV co-infection and MDR or XDR *M. tuberculosis* was not recorded.

Paucibacilarity of tissue limits PCR based genotyping

In order to genotype *M. tuberculosis*, DNA was extracted from the available samples and bacilli were genotyped using a targeted PCR-based method. Successful PCR amplification followed by sequencing was only observed in 46 patient specimens. This could be related to the fact that the tissue had been stored in either buffered or unbuffered formalin for an unknown time period before embedding in paraffin. Furthermore the median sample age was 27.7 years (IQR: 23.2-32.6, range 6.2-39.7), however, the age of the block was not associated with success of PCR-based genotyping ($p>0.05$). The bacilli burden was not associated with positive PCR amplification; 19/46 (41%) patient specimens were ZN negative and 27/46 (59%) were ZN positive. Additionally, a low ZN positivity was found in the majority of these patients 23/27 (85%), in comparison to 3 and 1 patient with medium respectively high ZN positivity.

Lineage 4 is most prevalent in CNS granulomas between 1975 and 2012

Lineage 4 was identified in 26/46 samples (56.5%), non-lineage 4 was found in 10 cases (22%) and a genomic heterogenic infection of lineage 4 and non-lineage 4 was found in another 10 cases (22%) (Fig. S1). Age of the sample was not associated with detected lineage ($p>0.05$) (Figure 1). Genotyping

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for lineage 1 (RD 239 deletion), lineage 2 (RD 105 deletion and sub-lineages with deletion in RD181, 150 and 142) and lineage 3 (RD 750 deletion) resulted in large number of inconclusive results. Lineage distribution could therefore not be determined in the non-lineage 4 group or group with genetic heterogenic infection (Fig. S1).

Genomic heterogenic infection in children

Genomic heterogeneity was identified in 7 patients > 14 year and 3 children < 14 years (total 10 patients=22%). Since mixed infection has not previously been described in children, these three cases are described in more detail. The children were between 2 and 3 years of age and their samples were collected in 1984, 1986 and 1992, respectively (Table 2). Case 1 and 2 presented with TBM stage IIa, in which mild neurological symptoms were present. Case 3 presented with TBM stage III and showed improvement of staging during treatment. Both Case 1 and 3 experienced a rapid disease progression with miliary TB. Both patients deceased within 3 weeks of presentation, despite intensive treatment with at least four different tuberculous drugs. Case 2 deceased without involvement of other organs and after longer treatment duration of 52 days.

On neuropathological examination, all three children showed chronic inflammation in the leptomeninges, poorly formed granulomas and fibrosis. Acute inflammation was present in patient Case 2. All three cases were ZN negative (Figure 2).

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DISCUSSION

In this study, we successfully identified lineage 4 as predominant *M. tuberculosis* strain in CNS granulomas. At Tygerberg Hospital, the hospital where this study was performed, 15% of all culture confirmed TB cases under the age of 14 years are TBM (24). However, little information was available about *M. tuberculosis* lineage specificity until recently. Based on previous studies performed in the same geographical region, lineage 4 was expected to be most prevalent in pulmonary TB (25) and lineage 2 was expected to be most prevalent in children with TBM (6). However, additional studies show an equal distribution of lineage 2 and 4 for both pulmonary and extra-pulmonary TB (Brites and Gagneux, 2017; Nicol et al., 2005).

Lineage 4, also known as the Euro-American lineage, is geographically the most wide-spread lineage and shows substantial variation among different ethnic groups and in different clinical settings (4). This diversity might be explained by the fact that lineage 4 consists of at least ten different sub-lineages which have evolved independently and thereby may have adapted to specific host populations. The overrepresentation of lineage 4 in our TBM samples might be unexpected and is in contrast with previous results. However, most of these studies were performed in Vietnam, China and Thailand, thereby representing patients with a different ethnic background. The wide-spread geographical variation of strains suggests the possibility of a different *M. tuberculosis* genotype distribution among the South African population as described here (5–8, 10–12, 26). The predominance of lineage 4 is a logical consequence of it being the most dominant lineage in Cape Town. In addition, it can be explained by the hypothesis that lineage distribution is subject to time in addition to geography. A study performed in Cape Town with archived post-mortem samples reported an increase in infection with Beijing strain over the past decades. This strain was never found before 1966, but prevalence significantly increased after 1995. Simultaneously, the proportion of Euro-American strains declined (25). However, Beijing prevalence never exceeded lineage 4. Although a correlation between time and prevalence was not found in the current cohort, the effect of altering prevalence of specific strains cannot be completely excluded.

The second finding in this study is the presence of more than one *M. tuberculosis* lineage within the CNS of a single patient, even when patients were ZN negative during initial histopathological examination. Genetic heterogeneity in lung tissue, in which multiple lineages can be found in a single individual at the same time, is in fact not new. Numerous reports, including a South African cohort in the same region as the current study, report 10-20% of sputum cultures containing more than one *M. tuberculosis* lineage (15–18, 20). However, this is likely an underestimation of the actual amount of

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genetic heterogenic infections due to the difficulty to isolate these strains and differences in genotyping method (17, 18, 27). In addition, to the presence of multiple strains in sputum samples, heterogenic populations of strains were also found in spleen, blood and lymph nodes (17, 28–30), indicating that genetic heterogeneity is not restricted to the lung. The current study is the first to identify genetic heterogeneity in the CNS in both adults and children, in ten patients out of 46 patient, similar to 22% occurrence described in literature (15–18, 20). Laboratory contamination during sample processing cannot be ruled out completely but it can be assumed to only contribute to a small proportion of genetic heterogeneity. This is based on a previous study that showed a maximum of 4% of contamination in cultures (16), and the negatively tested blank controls in the current study.

Genetic heterogeneity is thought to be a consequence of reinfection or superinfection after an (incomplete) recovery of a previous *M. tuberculosis* infection and it has been hypothesized that this second strain may reactivate the primary infection (17, 20, 31). In young children, the amount of genomic heterogenic infection is thought to be reduced due to the rapid progression of disease and the small time frame in which reinfection can occur (18, 19). Therefore, the genomic heterogeneity identified in the CNS of three young children was a major surprise. Hypothetically, a high rate of reinfection can exist in areas with a high *M. tuberculosis* burden. Subsequently, once the first strain causes a latent CNS infection, a reinfection can easily trigger progression of disease in the vulnerable brain of a child with a relative immature immune system. Another possibility is exposure of the child to two different strains at once. Children are in close contact with adults, in which mixed infection in sputum has been shown before. Infection with more than one strain might than promote extra-pulmonary dissemination.

Heterogenic infection was concentrated in the first half of the study period and it would be likely that a higher TBM prevalence increases the probability for finding genomic heterogeneity. Nevertheless, exact incidence rates for childhood TB and TBM are scarce, due to diagnostic challenges and lack of disease reports in rural areas. One study performed between 1985-1994 in two rural areas of Cape Town, reported no increase or decrease in incidence of childhood TB over this time period (32). Furthermore, clinicians at Tygerberg Hospital report an increasing incidence of TBM over the last 5-10 years (personal communication). Therefore, the concentration of genomic heterogeneity in the first half of the study is rather an effect of a larger amount of samples collected during that time than a consequence of a higher TBM prevalence.

This study has certain limitations. Although genomic heterogeneity was identified, the combination of the historical sample collection with partly degraded DNA, the paucibacillary nature of the samples and the chosen genotyping methods did not allow for a further characterisation of sub-lineages or strain classification at the sub-lineage level. Ideally, the genotyping performed in this study was

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repeated to confirm the findings, but the quality of the samples did not allow for additional testing. Also, CSF of patients with active TBM have an extremely low bacterial load (19). Therefore, it is questionable if it were possible to identify a large number of bacteria at a single location in an individual patients' brain specimen, which might lead to an underestimation of the amount of mixed infections. Another limitation was the archived FFPE samples which were stored in buffered or unbuffered formalin for an unknown period of time under unknown circumstances with most likely damaging effects on the samples (33). Given the paucibacillarity of the specimen and previous genotyping studies describing inconclusive results on FFPE material with spoligotyping (21), PCR based genotyping seemed the best option to distinguish the different lineages. However, this method resulted in a large proportion of inconclusive results as well, perhaps explained by the expected high rate of fragmentation in the DNA in combination with the size of the PCR products. This is supported by the observation that the primers generating the smallest products were the most reliable. Nevertheless, results concerning lineage 4 were conclusive. The sequences of the PCR products made it possible to clearly distinguish between lineage 4, non-lineage 4 and a mixed infection with lineage 4 and another lineage. However, since these primers group many different sublineages 4, it is likely that this method results in an underestimation of mixed infection of more than one sublineage 4.

Taken together, this unique cohort reveals valuable insight in the overrepresentation of lineage 4 and the presence of genomic heterogenic infection in the CNS of young children in TBM samples in the Western Cape of South Africa over the past decades. This might explain the rapid progression of disease and insufficient response to standard therapy seen in this population. Not only the advanced stage of TBM can explain outcomes of this patient group, also the genomic heterogeneity may contribute to treatment unresponsiveness leading to unfavourable outcome.

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SUPPLEMENTAL INFORMATION

Supplemental information includes one table.

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Author Contributions Statement

PV, ES and MvdK conceived and designed the experiments, with input from LvL, RW and AvF. LvL, PV, AJ and ES. SDZ collected and analyzed neuropathology material. SvE and MvdK managed the project. LvL and PV analyzed the data, LvL and SvE performed statistical tests. LvL and PV wrote the paper with input from SvE, RW, MvdK and AvF.

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Tables & Figures legends

Table 1 – Baseline characteristics

Baseline characteristics for < 14 years old versus > 14 years old with probable or definite tuberculous meningitis of which samples are used in this study. *chi-square used unless stated otherwise (FE = Fisher's Exact test, MW = Mann-Whitney U test); **Significance level between mixed ancestry and African black; ***Tuberculous meningitis stage at presentation is based on the 'refined' British Medical Research Council scale (34); Stage 1: Glasgow Coma Scale (GCS) of 15, without focal neurological deficits; Stage 2a: GCS of 15, with focal neurological deficits, or GCS of 13–14, with or without focal neurological deficits; Stage 2b: GCS of 10–12, with or without focal neurological deficits; Stage 3: GCS <10, with or without focal neurological deficits, p-value represents difference between IIb or III and IIa; † Probable based on histopathology, Proven based on histopathology & ZN positivity; ‡Time between treatment commenced until biopsy/death in days.

Figure 1 – Distribution of lineages between 1975 and 2012

Identification of lineage 4, non-lineage 4 or heterogenic infection in patients < 5yr (small blue circles) age 5 – 14 yrs (medium green circles) and > 14 yrs (large red circles) per year between 1975 and 2012. Number indicates multiple patients at a specific time point for the youngest group. No association was found between presence of a specific lineage and year of admission.

Table 2 – Patient characteristics of three children with heterogenic infection

Three children were found to have a heterogenic population of lineages in CNS granulomas. Characteristics of the individual patients are presented in this table. * Tuberculous meningitis stage at presentation is based on the 'refined' British Medical Research Council scale (34).

Figure 2 - Neuropathological findings in three children with tuberculous meningitis and heterogenic infection.

Characteristics of the three cases presented are specified in table 2. A. Case 1, Medulla oblongata; multiple intraparenchymal granulomas. H&E x40 magnification. B. Case 1, Medulla oblongata; granulomas with lymphocytes (chronic inflammation). H&E x100 magnification. C. Case 1, Leptomeninges; lymphocytes (chronic inflammation), no granulomas present. H&E x100 magnification. D. Case 2, Leptomeninges; poorly formed granulomatous inflammation. H&E x100 magnification. E. Case 2, Leptomeninges; lymphocytes (open arrow, chronic inflammation) and macrophages (closed arrow). H&E x400 magnification. F. Case 2, Leptomeninges; fibrosis and

vascular proliferation (arrow). Reticulin silver stain x100 magnification. G. Case 3, Brain parenchyma and leptomeninges; poorly formed granuloma (closed arrow) and foreign body giant cell (open arrow). H&E x100 magnification. H. Case 3, Brain parenchyma and leptomeninges; Langhans giant cell (arrow). H&E 400x magnification. I. Case 3, Brain parenchyma and leptomeninges; fibrosis (arrow) and vascular proliferation. Reticulin silver stain x100 magnification. J. Case 3, Brain parenchyma; infarct. H&E x40 magnification. K. Case 3, Brain parenchyma; infarct with vascular proliferation. Reticulin silver stain x40 magnification. L. Case 3, Brain parenchyma; infarct with vascular proliferation (arrows). Reticulin silver stain x100 magnification.

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539 Table 1 – Baseline characteristics

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	Total	Age < 14 year	Age > 14 year	
	n (%)	n (%)	n (%)	p-value*
Total number of patients	46	23	23	
Age (years):				
Median [IQR]; range	5.6 [2.3-32.6]; 0.5-65.5	2.6 [2.0-4.2]; 0.5-11.4	40.7 [23.8-48.5]; 14.3-65.5	-
Gender:				
Male	28 (60.9)	15 (65.2)	13 (56.5)	-
Female	18 (39.1)	8 (34.8)	10 (43.5)	0.546
Race:**				
Caucasian	1 (2.3)	0 (0)	1 (5.0)	-
Mixed ancestry	33 (76.7)	21 (91.3)	12 (60.0)	-
African black	9 (20.9)	2 (8.7)	7 (35.0)	0.055 FE
BCG immunization:				
Yes	1 (11.1)	8 (88.9)	-	-
No	8 (88.9)	1 (11.1)	-	-
Possible TB household contact:				
No/unknown	10 (47.6)	10 (47.6)	-	-
Yes	11 (52.4)	11 (52.4)	-	-
TBM-stage*:**				
I	2 (4.9)	0 (0)	2 (10.5)	-
IIa	8 (19.5)	4 (18.2)	4 (21.1)	-
IIb	6 (14.6)	1 (4.5)	5 (26.3)	0.301 FE
III	25 (61.0)	17 (77.3)	8 (42.1)	0.420 FE
TBM†:				
Probable	17 (37.8)	4 (17.4)	13 (59.1)	-
Proven	28 (62.2)	19 (82.6)	9 (40.9)	0.004
Therapy received:				
Isoniazid	34 (87.2)	21 (91.3)	13 (81.3)	0.631 FE
Rifampicin	34 (87.2)	22 (95.7)	12 (75.0)	0.139 FE
Pyrazinamid	29 (74.4)	19 (82.6)	10 (62.5)	0.264 FE
Ethionamide	18 (46.2)	17 (73.9)	1 (6.3)	<0.001
Ethambutol	20 (51.3)	10 (43.5)	10 (62.5)	0.242
Penicillin	28 (71.8)	19 (82.6)	9 (56.3	0.146 FE
Streptomycin	8 (20.5)	3 (13.0)	5 (31.3)	0.235 FE

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Therapy duration (days)‡:				
Median [IQR]; range	7 [4-13]; 0-153	8 [4-25]; 0-153	7 [3-9]; 2-28	0.167 [MW]

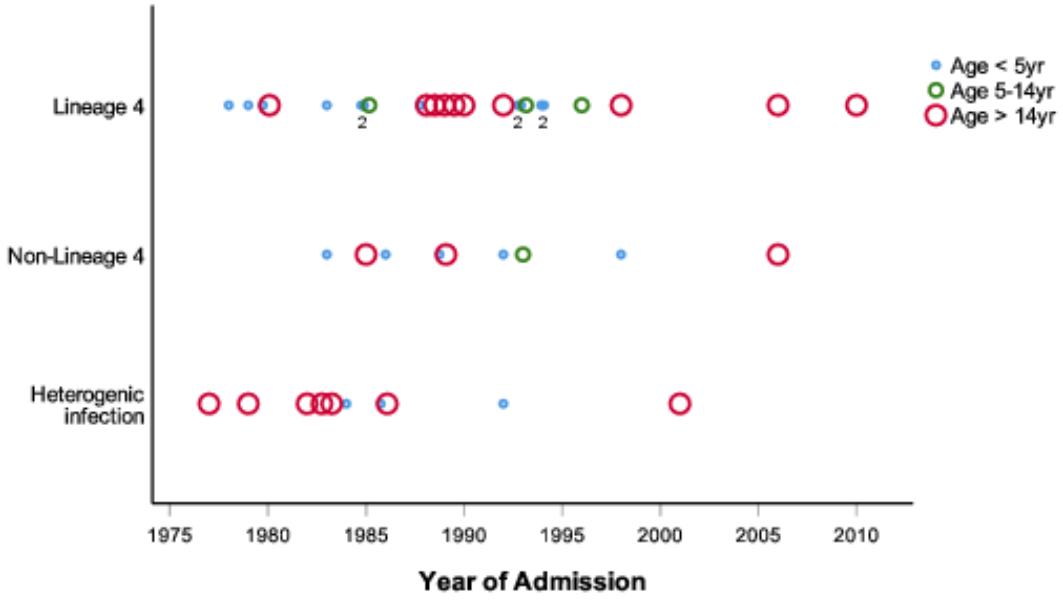
Table 2 – Patient characteristics of three children with heterogenic infection

	Case 1	**Case 2**	**Case 3**
Year of administration	1984	1986	1992
General characteristics			
Age in months	32	27	26
Gender	Male	Male	Female
Race	Black	Black	Mixed ancestry
BCG immunization	Unknown	Unknown	Yes
Possible TB household contact	Yes	Unknown	Unknown
TBM-stage*			
At presentation	IIa	IIa	III
During treatment	IIa	III	IIa
Before death	III	III	III
Outcome	Death	Death	Death
TBM diagnosis	Proven	Probable	Proven
Based on	Histology	Clinical symptoms	Histology
ZN positivity	Yes	No	Yes
Additional disease			
Pulmonary TB	Yes	Yes	Yes
Miliary TB	Yes	Unknown	Yes
Organs involved	Thyroid, spleen, hilary lymph nodes	Unknown	Kidney, spleen, liver
Treatment			
Tuberculostatics received	Rifampicin, isoniazide, pyrazinamide, ethionamide	Rifampicin, isoniazide, pyrazinamide, ethionamide, ethambutol	Rifampicin, isoniazide, pyrazinamide, ethionamide
Duration until death in days	17	52	13
Prednisone received	Yes	Yes	No

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