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Mycobacterium tuberculosis and *Mycobacterium africanum* in stools from children attending an immunization clinic in Ibadan, Nigeria

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Summary
Background: Tuberculosis is a major cause of childhood morbidity and mortality in Nigeria. Diagnosis of childhood tuberculosis is a global challenge making early treatment a mirage. In this study we investigated the stools of children for the presence of mycobacteria.
Methods: Stool samples from children aged 3 days to 3 years who presented for postnatal immunization at a large university-based clinic in Nigeria, were subjected to Ziehl–Neelsen staining. Samples with acid-fast bacilli were further processed using mycobacterial culture, spoligotyping, and deletion typing.
Results: One hundred and ninety-two stool samples from different children were collected and processed. Thirty (15.6%) had acid-fast bacilli. Of these, eight had *Mycobacterium tuberculosis* and one had *Mycobacterium africanum*.
Conclusions: Approximately 5% (9/192) of apparently well children had evidence of potentially serious tuberculosis infection. The usefulness of stool specimens for diagnosing pediatric tuberculosis warrants further investigation.

Introduction

More than 2 billion people, approximately one-third of the global population, are infected with *Mycobacterium tuberculosis*, the major causative organism of tuberculosis (TB).[1]

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Mycobacterium africanum and *Mycobacterium bovis*, also members of the *Mycobacterium tuberculosis* complex, are much less frequent causes of TB in humans. The incidence and prevalence of pediatric TB varies significantly across the globe, driven largely by the burden of the disease in different countries. About 1 million children under 15 years of age develop TB worldwide annually, representing 11% of all TB cases.[2] The majority of these cases occur in low-income countries where the prevalence of HIV/AIDS is high.[3] Nigeria currently ranks fourth on the list of TB burdened nations globally,[1] and pediatric TB accounts for a substantial proportion of these cases.[4] Almost 2 million people per year die as a result of TB, mostly in developing countries like Nigeria, but the mortality in children is often underreported. Despite this, TB is one of the ten leading causes of childhood mortality.[5]

Young children and especially newborns are at a high risk when exposed to a contagious source.[6] A comprehensive review of the natural history of childhood TB showed that primary infection before 2 years of age frequently progressed to active disease within 12 months.[7] As such, pediatric TB is a sentinel event reflecting recent TB transmission from an infectious contact in the community. The number of children with TB in a community is an indirect parameter for assessing the effectiveness of the local TB control program.[3]

The diagnosis of pulmonary TB (PTB) in children is challenging.[8] Children rarely expectorate adequate amounts of sputum, and the limitations of using other specimens or techniques, such as first morning gastric aspirates (considered the best clinical specimens for young children with suspected PTB), nasopharyngeal swabs, sputum induction, and laryngeal swabs, are well known. Accordingly, there is a strong imperative to evaluate the diagnostic utility of clinical specimens that are more readily collectable. Some investigators have suggested that stool microscopy and culture for *M. tuberculosis* may be diagnostic in some children with tuberculosis,[9–12] but other investigators have described stool evaluation as 'worthless' since non-pathogenic acid-fast bacilli (AFB) may be found in the normal intestinal contents of adults.[13,14]

Following the identification of AFB in the stools of apparently well children who were being screened for cryptosporidiosis in our immunization clinic, we designed this study to characterize AFB in the stools of children attending the clinic.

Methods

Subjects

Subjects were consecutive children who presented for immunization at the University of Ibadan Health Services Clinic, Ibadan, Nigeria. Stool samples from the children were evaluated using AFB staining. All the AFB-positive stool specimens were evaluated further for the presence of mycobacteria.

Ethical approval

The Institutional Review Committee of the University of Ibadan and the University College Hospital, Ibadan, Nigeria approved the study. Oral informed consent was obtained from the parents of the children.

Clinical specimens

Stool samples were collected from each child into a sterile plastic container and kept in the refrigerator at 4 °C prior to processing using Ziehl–Neelsen (ZN) staining. The ZN stain was carried out as described by Shrestha et al.[15]

Processing of samples

From the stool samples positive by ZN staining, 2–3 g was suspended in 5 ml of sterile distilled water, mixed, and left for 15 min to separate, after which 3 ml of the supernatant was processed. Using a sterile centrifuge tube, equal amounts of specimen and activated *N*-acetyl-L-cysteine (NALC)–NaOH (3 ml each) was added. The contents of the tube were mixed until the specimen was liquefied, and then allowed to stand for 15 min. Phosphate buffer was added to the 10 ml mark on the centrifuge tube and mixed, followed by centrifugation for 15 min at 3000 × *g*. The supernatant was decanted; 2 ml of phosphate buffer pH 6.8 was added to resuspend the pellet. The suspension was inoculated onto Lowenstein–Jensen slopes with pyruvate and/or glycerol and incubated at 37 °C for between 8 and 12 weeks. Isolates were harvested for molecular typing analysis by scraping the growth from slopes into 200 μl of sterile distilled water and heating at 80 °C for 1 h.

Spoligotyping

This was carried out as previously described with minor modifications.[16] The direct repeat (DR) region was amplified by PCR with oligonucleotide primers derived from the DR sequence. Twenty-five microliters of the following reaction mixture was used for the PCR: 12.5 μl of HotStarTaq Master Mix (Qiagen; this solution provides a final concentration of 1.5 mM $MgCl_2$ and 200 μM each deoxynucleoside triphosphate), 2 μl of each primer (20 pmol each), 5 μl of the suspension of heat-killed cells (approx. 10–50 ng), and 3.5 μl of distilled water. The mixture was heated for 15 min at 96 °C and subjected to 30 cycles of 1 min at 96 °C, 1 min at 55 °C, and 30 s at 72 °C. The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed twice for 10 min in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (pH 7.7))–0.5% sodium dodecyl sulfate (SDS) at 60 °C and then incubated in 1:4000-diluted streptavidin-peroxidase conjugate (Boehringer) for 45 to 60 min at 42 °C. The membrane was washed twice for 10 min in 2× SSPE–0.5% SDS at 42 °C and rinsed with 2× SSPE for 5 min at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence method (Amersham) and by exposure to X-ray film (Hyperfilm ECL; Amersham) as specified by the manufacturer.

Deletion typing

The use of deletion analysis for the typing of *M. tuberculosis* complex strains has been previously described.[17,18] For this work, the deletion typing method described by Warren et al.[19] was used. In our analysis, we used primers directed against the RD4 and RD9 loci to generate a deletion profile

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Table 1 Profile of pediatric patients with a positive culture result for *Mycobacterium* species

ID	Sex	Age	SpolDB4 type	Family in SpolDB4 type	Species
JC1	F	2 months	52	T2	*M. tuberculosis*
JC2	F	1 week	53	T1	*M. tuberculosis*
JC3	M	6 weeks			*M. tuberculosis* (deletion analysis)
JC4	F	5 weeks	61	LAM10_CAM	*M. tuberculosis*
JC5	M	3 months	774	T1	*M. tuberculosis*
JC6	F	1 year, 3 months	61	LAM10_CAM	*M. tuberculosis*
JC7	M	4 months			*M. africanum* (deletion analysis)
JC8	F	3 months	Not in SpolDB4	New strain (probably evolved from LAM10_CAM)	*M. tuberculosis*
JC9	F	6 months	358	T1	*M. tuberculosis*

that would allow speciation of the isolate. The multiplex master mix system from Qiagen was used for the PCRs, with primers previously described by Warren and colleagues.[19] The PCR mixture was a multiplex reaction, with each PCR reaction containing 1 μl of DNA template, 5 μl Q-buffer, 12.5 μl multiplex master mix (Qiagen), and 0.5 μl of each primer (50 pmol/μl). The total volume of the reaction was made up to 25 μl with water. The reaction was allowed to run for 15 min at 95 °C, followed by 45 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min. After the last cycle, the samples were incubated at 72 °C for 10 min. Products were visualized by electrophoresis on 3% agarose gels.

The positive controls included a known *M. bovis* isolate (AN5) and a known *M. tuberculosis* isolate provided by the Medical Research Council, Center for Molecular and Cellular Biology, Stellenbosch University, Cape Town, South Africa, whilst the negative control was water. The resulting gel images were analyzed on the basis of their alignment on the gel (i.e., same band size with either of the controls). The RD9 deletion analysis was done to discriminate *M. tuberculosis* from other *Mycobacterium tuberculosis* complex (MTC). Those with a deletion at this region were further investigated with primers targeting the RD4 region and this discriminated *M. bovis* from the other members of the MTC. Primers targeting the RD1^{mic} and RD2^{seal} regions were later used to confirm the presence of *M. africanum* relative to *Mycobacterium pinnipedii* and *Mycobacterium microti* as described by Warren and colleagues.[19]

Results

One hundred and ninety-two children were recruited into the study, comprising 95 males and 97 females, aged 3 days to 3 years. Thirty children had AFB present in their stool specimens. Mycobacterial culture of stool samples from the 30 children yielded a growth in nine (30%). Spoligotyping and deletion analysis confirmed these isolates as *M. tuberculosis* complex (eight *M. tuberculosis* and one *M. africanum*; Table 1 and Figure 1). The patients with the positive stool mycobacterial cultures included three males and six females, with ages ranging from 1 week to 15 months (Table 1).

Discussion

The diagnosis of TB in pediatric patients is often based on case definitions that incorporate signs and symptoms of TB, suggestive chest radiograph, positive tuberculin skin test, and contact with an active TB patient. It is less frequently based on laboratory isolation of *M. tuberculosis*. Existing algorithms, however, have serious shortcomings, and the development of reliable and widely applicable algorithms is a high research priority.

In this study, we diagnosed *M. tuberculosis* in 27% (8/30) and *M. africanum* in 3% (1/30) of children who had AFB-positive stool specimens. Other investigators have diagnosed tuberculosis based on isolation of *M. tuberculosis* from stool specimens, but in different patient populations. Mwachari et al.[20] cultured *M. tuberculosis* from the stools of 10 (13%) HIV-infected adults with chronic diarrhea in Kenya. Manatsathit et al.[21] also found *M. tuberculosis* in the stools of eight (18%) adult AIDS patients in Thailand. In South Africa, 8% and 5% of 66 children with suspected PTB had stool specimens that were AFB-positive and *M. tuberculosis* culture-positive, respectively.[9] In that study, AFB were identified only in the stools of children who had PTB that was confirmed with positive gastric aspirates, but stool testing was less sensitive than gastric aspirates overall. Our study is unique because the study population included apparently well children who were brought to the clinic for routine immunization. None of

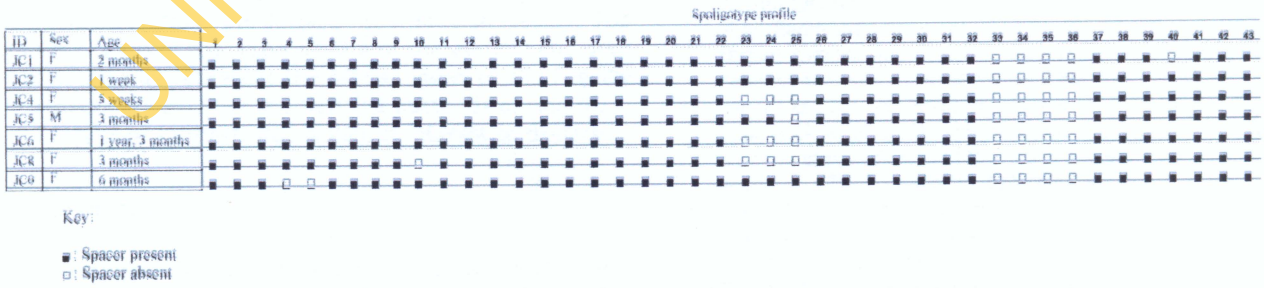

Figure 1 Spoligotype profile of pediatric patients with a positive culture result for *Mycobacterium tuberculosis* complex.

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the children had diarrhea, but four had cough, although clinical data were not collected prospectively in all children. The yield might be higher in children clinically suspected of having TB.

Some findings of this study provide potentially important epidemiological information. Firstly, *M. tuberculosis* has been implicated in most cases of TB in children; however, we found a case of *M. africanum*. This is, to our knowledge, the first published isolation of *M. africanum* in the stools of a Nigerian child. Cases of TB caused by *M. africanum* have been previously reported in adults from Nigeria[22,23] and other African countries.[24,25] In Cameroon, there has been a decline in the prevalence of *M. africanum*;[24] however more cases have recently been observed in HIV/AIDS patients in The Gambia[25] and Nigeria.[23] Secondly, one of the *M. tuberculosis* strains cultured in this study (isolate from patient JC8) has not been previously reported in the SpolDB4 database, which contains a comprehensive listing of the *M. tuberculosis* strains around the world (Figure 1). This implies the possible circulation of poorly characterized or emerging *M. tuberculosis* strains in Nigeria.

This study should be interpreted in the context of its limitations. Since we did not have records of HIV testing in the mothers or children, we were unable to correlate our findings with the HIV infection status of the subjects. Also, full contact tracing was possible only in some of the children with positive stool tests, seriously constricting our ability to comment on the public health significance of the results. Our data cannot be extrapolated to older children because the oldest subject in this study was 3 years old. Finally, we do not have complete data on the subsequent clinical course of the patients. Despite these limitations, our findings add to the evidence that directed stool studies may be useful in pediatric TB diagnosis.

In conclusion, we have described the isolation of *M. tuberculosis* from the stools of a significant proportion of apparently well children attending the University of Ibadan Health Services Clinic. We have also described the first isolation of *M. africanum* from the stools of a Nigerian child. The problematic nature of diagnosing TB disease in this age group justifies further investigation of the diagnostic potential of stool specimens and other readily obtainable specimens, perhaps using more sensitive techniques. The limitations of such testing and the population for which it would be applicable are also fertile areas for clinical and laboratory studies.

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Conflict of interest: No conflict of interest to declare.

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