Histopathology and Genotyping in Infectious Spondylitis of HIV− and HIV+ Patients

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Each author certifies that his or her institution has approved the human protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research, and that informed consent for participation in the study was obtained.

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Abstract

The recent resurgence in the incidence of spinal tuberculosis (TB) in both developed and developing countries has been attributed to the HIV pandemic. In addition, spinal infections occur two to 10 times more frequently in HIV-infected versus HIV-negative patients. Developing countries, in particular, have noted significant escalations in dual HIV/TB infections. In South Africa, dual infections including all forms of TB account for approximately 80% of general admissions at public hospitals. An escalation of 30% in the incidence of spinal TB was noted in the last 5 years at a referral hospital in the KwaZulu-Natal region. Of the nine provinces, KwaZulu-Natal displayed the highest incidence of HIV/TB coinfection and was among the three highest HIV incidence regions. The primary reason for these parallel escalations is their impact on the host immune response. Impaired immunity may arise from HIV coinfection, malnutrition, intravenous drug abuse, alcoholism, cirrhosis, diabetes mellitus, and pharmacological factors. Anti-TB therapy is often initiated at the primary referral hospital on clinical and radiographic findings. Apart from the paucibacillary nature of the disease, a definitive diagnosis is further complicated by the occurrence of nontuberculous mycobacterial (NTM) infections in regions carrying a high index of exposure to opportunistic pathogens. NTM infections, including spondylitis, have been reported in severely immune-suppressed HIV-negative patients in South Africa and India using conventional diagnostic and identification assays as well as the more complex polymerase chain reaction (PCR) genotyping. Newer diagnostic approaches exploit molecular techniques, which target the genetic material or DNA of the organism. Specific genes in the bacterial genome are amplified by polymerase chain reaction (PCR), which facilitates detection and identification of the isolate. A single assay is required with molecular technology, for accurate and rapid identification, and may include restriction fragment-length polymorphisms (RFLP), fluorescence-amplified fragment length polymorphisms (FAFLP), molecular beacon assays, and sequencing. These techniques may
also provide an understanding of infectivity, pathogenicity, and drug-resistance patterns. Genotyping, which identifies an organism by sequencing a specific gene is particularly useful if a broad-range target such as the 16S rDNA (ribosomal DNA) gene is assessed. In the majority of cases, TB infection precedes HIV infection and manifests only when immune function becomes compromised. In adults, coinfection is likely a reactivation event and proceeds much more rapidly than in HIV-negative patients. Children, similarly, experience a more disseminated disease particularly in association with HIV coinfection. While some believe the impact of coinfection on clinical presentation and disease progression plays a minor role, others relate disease progression and the rapid progression to death directly to HIV coinfection. Because these observations relate primarily to pulmonary TB, this study was undertaken to compare the hematologic and histopathologic presentation of spinal TB among HIV-positive and -negative patients and to investigate whether the occurrence of atypical opportunistic infection is HIV-mediated. We explored the hypothesis that HIV coinfection manifests in more severe clinical and histopathological features, and increases susceptibility to atypical opportunistic infections.

MATERIALS AND METHODS

We recruited 60 consecutive patients diagnosed with TB spondylitis diagnosed by clinical and radiographic evaluation from June 2002 to November 2003. All patients presented with an extradural granuloma and vertebral collapse with progressive deformity and neural deterioration despite 8 weeks (range, 5-24 weeks) of anti-TB therapy. HIV/TB coinfection accounted for 37% (22 of 60 patients) of the population in an equal number of male and female patients (Table 1). Granulomatous tissue biopsies and peripheral blood samples were collected from all patients during spinal decompression. Tissue specimens were submitted for confirmatory diagnosis including staining for acid-fast bacilli (AFB) (Ziehl-Neelsen and Auromine stains) and culture assays (BACTEC MGiT™; Beckton Dickenson, San Jose, CA). Routine pathological investigations to determine the full blood count (FBC), CD4+/CD8+ T cell counts, HIV-1 serology, and HIV-1 RNA quantification (plasma and tissue) were conducted. Tissue viral loads were stratified using the input mass of the tissue to correspond to an input volume of 1 mL of plasma.

The granulomatous tissue of a randomly selected subset of 35 patients (20 HIV- and 15 HIV+) was evaluated histopathologically following fixation, wax embedding, sectioning, and staining (hematoxylin and eosin). In addition, Ziehl-Neelsen (ZN) and periodic acid-Schiff (PAS) stains were administered to tissue sections to investigate the presence of acid fast bacilli and fungal infections, respectively. All analyses were confirmed independently by a qualified pathologist (AB) not associated with the study. The pathologist was blinded to the HIV status of the specimens to reduce the potential risk of observer bias. The presence of granulomas, caseation/necrosis, lymphocytes, epithelioid cells, and plasma cells were subjectively scored according to their relative prevalence in the infected tissue (undetectable to moderate = 0 to 45%; moderate to profuse = > 45%). Macrophage and macrophage-derived cells were immunolocalized using the CD68+ antibody in a modified immunoperoxidase method. DNA was extracted (QiaAmp DNA minikit, Hilden, Germany) from all positive BACTEC
MGiT bacterial cultures grown in an accredited pathology laboratory where drug sensitivity and resistance was assessed by conventional means. Cultures were heat inactivated (95°C for 10 minutes), amplified by polymerase chain reaction and a fragment of 500 base pairs (bp) (MicroSeq 500 16S rDNA kit, Applied Biosystems, Foster City, CA) of the 16S gene were sequenced on an ABI PRISM 3100® Genetic Analyser (ABI Big Dye platform; Applied Biosystems, Foster City, CA). Positive (E. coli) and negative (sterile water) were processed and analyzed concurrently. Five of these isolates were selected at random and submitted to an independent laboratory for confirmatory genotyping. Resulting sequences that were analyzable were compared with the published reference strains of three sequence databases (at > 90% homology for positivity): National Centre of Biotechnology Information (NCBI), Ribosomal Differentiation of Medical Microorganisms (RIDOM), and DNA Data Bank of Japan (DDBJ). 37

Nonparametric statistical analyses were conducted following evaluation of the respective distribution curves of the data. Comparisons between HIV-positive and -negative patients included: incidence of paralysis; CD4/CD8 counts; full blood counts (FBC); granuloma formation; caseation, lymphocyte, epithelioid and plasma cell and CD68+ macrophage immunoreactivity in tissue biopsies. In addition, the occurrence of Mtb and NTM infections identified by conventional culture (MGiT) and genotyping were compared between the groups. Nonparametric Pearson's correlation was also performed to identify associations between these markers for all data and stratified according to HIV status or age category (pediatric versus adult). The level of statistical significance was accepted at 5% (p < 0.05). Statistical analysis was performed using the SPSS version 11.5 software package (SPSS Inc, Chicago, IL).

RESULTS
We observed clinical recovery in 55 of 60 (86%) patients; recovery was full in 46 of 60 patients, partial in 10 of the 60 patients, and four had no recovery. There were no differences in the clinical recovery patterns between the HIV-infected and -uninfected groups. Plasma viral loads were associated with patients coinfected with Mtb, where elevated plasma viral loads and Mtb infection was age-specific. Tissue viral load correlated negatively with (r = -0.458; p < 0.05) age of patient. The tissue viral loads were greater (p < 0.05) in children (6.63 log_{10} copies/mL) than in adults (3.94 log_{10} copies/mL) (Table 1) and were correlated with greater (r = 0.600; p < 0.05) platelet counts. In 40% of patients (Table 1), the CD4+ T cell counts were in the normal range (> 500 cells/µL). The CD4/CD8 ratio correlated with (p < 0.01) HIV status where higher ratios were associated with a negative status and lower ratios were typical of the HIV-positive group.

Histopathological investigation of the excised granulomas of the randomly selected 35-patient subset (20 HIV- and 15 HIV+) revealed features diagnostic of TB in 92% of patients (Table 2). The HIV-positive group tended to display greater caseation but this observation (and our hypothesis) could not be confirmed with the limited power of the study (Table 2). We observed acid-fast bacilli (ZN stain) in only one HIV-positive adult female patient. In addition, none of these 35 patients displayed evidence of a fungal infection. Greater caseation was associated with greater granuloma formation (r = 0.791; p < 0.05), lymphocyte (r = 0.799; p < 0.05), epithelioid cell (r = 0.783; p < 0.05), and plasma cell (r = 0.804; p < 0.05)
infiltration. Immunolocalization of CD68-reactive cells demonstrated the relative distribution of macrophages and macrophage-derived cells in the granulomas. When comparing the HIV-positive and -negative specimens, the minimum area of CD68 immune-reactivity was greater (p < 0.05) in infected than in uninfected specimens (Table 3). There were no differences in the intensity of immunoreactivity between HIV-infected and -uninfected groups.

HIV coinfection did not impact susceptibility to opportunistic infections. Nine (47.4%) of the culture-positive isolates, which were identified as Mycobacterium tuberculosis (Mtbb) by conventional Bactec MGIT typing, yielded nonanalyzable 16S rDNA sequences. However, the remaining 10 isolates (52.6%) produced high-quality 16S rDNA sequences. Of the six isolates identified as Mtb by culture (BACTECT MGIT), three were confirmed by 16S rDNA genotyping. A fourth isolate identified as Mtb by culture was genotyped as Mycobacterium intracellulare. A further isolate was genotyped as Mycobacterium fortuitum. Three sequences were environmental pathogens clustering with Stenotrophomonas maltophilia, Brevibacterium sanguinis, and Serratia marcescens. The remaining two isolates displayed no homology with published reference sequences and remained unclassified. No atypical TB drug sensitivity or resistance patterns were noted.

DISCUSSION

We explored the hypothesis that HIV coinfection manifested, firstly, in depleted CD4/CD8 counts and selected FBC markers, poor clinical status, more severe histopathology of infected tissue; and, secondly, a greater incidence of NTMs compared with HIV-negative patients. Previous reports have compared the histopathology and disease progression of pulmonary TB. Similar studies of spinal TB are limited thus prompting this investigation in an HIV/TB endemic region that is also afflicted with a high incidence of opportunistic infections. We observed a 37% incidence of spinal TB was demonstrated among HIV-positive patients in our cohort. Given that worldwide one-third of all HIV-positive people are coinfected with either pulmonary or extrapulmonary TB, the high incidence of spinal TB/HIV coinfection in this setting is not unexpected.

A CD4+ T cell count of less than 200 cells/µL has been used as a preoperative indicator of the level of risk of postoperative wound infection as it reflects both the vigor of the host immune response and the stage of HIV pathogenesis in coinfected patients. Despite approximately 10% of the present study population displaying CD4+ T cell counts less than 200 cells/µL, postoperative outcomes in the present study were favorable. Extrapulmonary TB is AIDS-defining and heralds late-stage disease and together with a CD4+ T cell count of below 200 cell/µL indicates initiation of ARVs. Patients were ARV-naive and reliant, solely, on nutritional support, surgery and anti-TB chemotherapy for disease resolution. There were no differences in either CD4+ T cell counts or patient outcomes between HIV-infected and -uninfected patients in the present study indicating other mediating factors.

HIV viral loads and CD4+ T cell counts are closely associated where the former predisposes subjects to active TB infection. Wolday et al showed high viral loads persisted following anti-TB chemotherapy even though the TB infection had resolved clinically. Similarly, in the present study plasma and tissue viral loads were in excess of 4 log_{10} copies/mL despite preoperative anti-TB therapy (mean, 8 weeks) and substantially reduced bacterial counts. High viral load at the site of infection, as demonstrated in this study, may manifest in more advanced
pathology and greater caseation. In addition, it may indicate sitespecific immune or drug mediators, which amplify viral replication. As a result of greater viral loads overall patient prognosis is poor. Since HIV may lie sequestered in anatomical and cellular compartments, the anatomically isolated TB granuloma provides a suitable environment for latent infection. This leads to unrestrained viral replication at the site of infection, the emergence of viral quasispecies and drug resistant variants, as well as severe histologic features. Others have investigated viral loads in various anatomical sites such as the brain and lymph nodes. However, this is the first study to investigate viral loads in granulomas of infective spondylitis. HIV-1-associated thrombocytopenia, in which platelet counts are reduced, commonly afflicts infected patients. In contrast, the present study demonstrated a positive correlation between platelet counts and tissue viral loads in HIV/spinal TB coinfected patients. Antiretroviral (ARV) therapy generally increases platelet counts, but this cannot account for the findings of the present study since ARV was not administered to the patients in this cohort. Thus coinfection at this anatomical site may contribute to these findings and warrants further investigation of both specific immune responses at the site of HIV/TB coinfection and viral kinetics.

Our data suggest age was an important contributor to the histopathology and immune response at the anatomical site of infection. The pediatric group displayed a failing localized immune response as indicated by significantly greater tissue viral loads compared with the adult group. These findings were confirmed by an inverse correlation between tissue viral loads and age. Physiologic and developmental differences between adult and pediatric patients, particularly in their immune responses, may account for this finding. Histopatholog tended to exhibit greater caseation and related abnormalities in the HIV-infected group but the limited power of the study precluded confirming this finding. However, we interpret this trend together with the clinical status of HIV-infected and -uninfected patients as suggesting of the occurrence of greater pathological and clinical disease manifestations in coin-fected individuals.

However, outcomes were comparable following surgery and pre- and postoperative anti-TB treatment. Related studies have demonstrated the importance of enhanced pre- and postoperative nutritional support to patient outcomes. Nutritional deficiency may severely disrupt both the cellular and humoral immune responses by impacting the basic biochemistry, function, and abundance of immune cells. In developing countries, using antiretroviral therapy to elevate the host immune response of coinfected patients may prove inaccessible, impractical, or economically nonviable. Therefore, enhancing the nutritional status may prove valuable in these settings particularly since its efficacy has been demonstrated previously in this region.

Worldwide, the HIV pandemic coupled with severe immunosuppression as a result of malnutrition has culminated in an increase in atypical and/or opportunistic spinal infections which presently rival the incidence of related Mtb infections. Similarly, our study confirmed the presence of opportunistic pathogens, nontuberculous mycobacterial infections, and infectious spondylitis in the local setting in HIV-uninfected patients. While CD4+ T cell counts less than 50 cells/μL were common to all patients in a study of Mycobacterium avium complex (MAC)-infected patients, of whom 8 of 11 were HIV-coinfected, CD4+ T cell counts in the present study exceeded 200 cells/μL in both the HIV-negative and HIV-positive groups. This is despite the identification of atypical organisms by bacterial genotyping suggesting factors other than CD4+ T cell counts may contribute to susceptibility to NTMs particularly in
regions with a high index of exposure. Several authors recommend anti-TB therapy be initiated promptly in patients with a high suspicion for spinal TB and in endemic regions, even prior to a laboratory confirmation of the disease despite its impact on positive diagnoses.\textsuperscript{25,26} The use of a broad range genotyping target such as 16S rDNA to characterize the opportunistic pathogens in spinal granulomas in the present study was novel. The characterization of environmental bacteria such as S. marcescens, S. maltophilia, B. sanguinis, and NTMs was significant because it may indicate an increase in the incidence of atypical spinal infections. These organisms are usually associated with depressed immunity as a result of cancer, surgical complications, pneumonia, urinary tract infection, trauma, and septicemia.\textsuperscript{8,9,14,20,21,23,32-34,36,38,39,49} In the present study however, they were more commonly associated with the HIV-uninfected. These findings illustrated susceptibility to atypical opportunistic infections in the local setting may not be associated with HIV-infection thus discounting our second hypothesis of a correlation.

Accurate and timely diagnosis effectively directs individualized treatment strategies preventing disease progression to deformity, neurological involvement and the need for surgery. Strategies to enhance diagnosis, such as genomic targeting, must be considered as adjuncts to conventional assays to resolve these infections particularly in high-risk populations and regions with a high index of exposure such as Asia and sub-Saharan Africa.

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**References**


6. Beggs ML, Stevanova R, Eisenach KD. Species identification of Mycobacterium avium


# TABLE 1. Demographic and Laboratory Findings Comparing HIV-infected and -uninfected Patients

<table>
<thead>
<tr>
<th>Finding</th>
<th>All Patients (n = 60)</th>
<th>HIV− (n = 38)</th>
<th>HIV+ (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
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<tr>
<td>Mean age (%) (range)</td>
<td>32 ± 19 (2–71)</td>
<td>34 ± 21 (2–71)</td>
<td>29 ± 15 (2–65)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>17 (28)</td>
<td>12 (63)</td>
<td>17 (77)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>41 (68)</td>
<td>24 (32)</td>
<td>5 (23)</td>
</tr>
<tr>
<td>Missing data (%)</td>
<td>2 (3)</td>
<td>2 (5)</td>
<td>0</td>
</tr>
<tr>
<td>Pediatric (%)</td>
<td>13 (22)</td>
<td>10 (26)</td>
<td>3 (14)</td>
</tr>
<tr>
<td>Adult (%)</td>
<td>43 (72)</td>
<td>24 (63)</td>
<td>19 (86)</td>
</tr>
<tr>
<td>Missing data (%)</td>
<td>4 (7)</td>
<td>4 (11)</td>
<td>0</td>
</tr>
<tr>
<td>Pathology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log plasma viral load (copies/mL)</td>
<td></td>
<td>4.14 ± 1.59 (&lt; LDL–6.40)</td>
<td></td>
</tr>
<tr>
<td>Log tissue viral load (copies/mL)</td>
<td></td>
<td>4.20 ± 1.85 (1.00–7.18)</td>
<td></td>
</tr>
<tr>
<td>Absolute CD4+ T-cells (cells/µL)</td>
<td>720 ± 465 (100–2254)</td>
<td>849 ± 526 (281–2254)</td>
<td>553 ± 311 (100–1081)</td>
</tr>
<tr>
<td>Absolute CD8+ T-cells (cells/µL)</td>
<td>953 ± 616 (194–2512)</td>
<td>830 ± 583 (194–2469)</td>
<td>1112 ± 639 (314–2512)</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>0.89 ± 0.51 (0.10–2.23)</td>
<td>1.16 ± 0.46 (0.34–2.23)</td>
<td>0.50 ± 0.28 (0.10–1.29)</td>
</tr>
<tr>
<td>CD4 distribution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 500 cells/µL</td>
<td>15 (25)</td>
<td>7 (18)</td>
<td>8 (36)</td>
</tr>
<tr>
<td>&gt; 500 cells/µL</td>
<td>24 (40)</td>
<td>15 (40)</td>
<td>9 (41)</td>
</tr>
<tr>
<td>No result available</td>
<td>21 (35)</td>
<td>16 (42)</td>
<td>5 (23)</td>
</tr>
<tr>
<td>Diagnostics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture identification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No result</td>
<td>41 (68)</td>
<td>27 (71)</td>
<td>14 (64)</td>
</tr>
<tr>
<td>MTB</td>
<td>16 (27)</td>
<td>8 (21)</td>
<td>8 (36)</td>
</tr>
<tr>
<td>MOTT</td>
<td>3 (5)</td>
<td>3 (8)</td>
<td>0</td>
</tr>
</tbody>
</table>

< LDL = below detectable levels; MTB = Mycobacterium tuberculosis; MOTT = mycobacteria other than tuberculosis
### TABLE 2. Histopathological Features of Granuloma Biopsies of a 35-patient Subset Comparing HIV-infected and HIV-uninfected Specimens

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>All (n = 35)</th>
<th>HIV− (n = 20)</th>
<th>HIV+ (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number diagnostic of TB (%)</td>
<td>32 (92%)</td>
<td>17 (85%)</td>
<td>15 (100%)</td>
</tr>
<tr>
<td>Occurrence of histological features (%†)</td>
<td>&lt; 45%</td>
<td>&gt; 45%</td>
<td>&lt; 45%</td>
</tr>
<tr>
<td>Granulomas</td>
<td>20 (33)</td>
<td>15 (25)</td>
<td>9 (24)</td>
</tr>
<tr>
<td>Caseation</td>
<td>20 (33)</td>
<td>15 (25)</td>
<td>11 (29)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>16 (27)</td>
<td>19 (32)</td>
<td>11 (29)</td>
</tr>
<tr>
<td>Epithelioid cells</td>
<td>25 (42)</td>
<td>10 (17)</td>
<td>17 (45)</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>33 (55)</td>
<td>2 (3)</td>
<td>19 (50)</td>
</tr>
</tbody>
</table>

†Refers to the percentage of the area of interest that displayed granuloma formation, caseation, and infiltration of lymphocytes, plasma cells and epithelioid cells.

### TABLE 3. Comparison of CD68 Immunolocalization Between HIV-infected and -uninfected Specimens

<table>
<thead>
<tr>
<th>Immunolocalization</th>
<th>All Patients (n = 35)</th>
<th>HIV− (n = 20)</th>
<th>HIV+ (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute area of CD68+ immunoprecipitation per field (μm²/field)</td>
<td>900 ± 557 (152–2294)</td>
<td>1066 ± 542 (415–2294)</td>
<td>776 ± 548 (152–1849)</td>
</tr>
<tr>
<td>Intensity of CD68+ immunoprecipitation (mean intensity/field)*</td>
<td>178 ± 24 (144–222)</td>
<td>174 ± 24 (144–215)</td>
<td>181 ± 25 (144–222)</td>
</tr>
</tbody>
</table>

*Intensity is measured according to an intensity scale of 0–255 where 0 = no reactivity and 255 = maximum reactivity.