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Parotid mycobacteriosis is frequently caused by *Mycobacterium tuberculosis* in advanced AIDS

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BACKGROUND: Tuberculosis is one of the leading infectious diseases in the world, with more than 2 million new cases annually. It is one of the main causes of death of human immunodeficiency virus (HIV)-positive patients, involving multiple organs and particularly the lungs. Nevertheless there are few consistent studies about tuberculosis involving the parotid of HIV patients. The objective of this work was to describe the histological and immunohistochemical characteristics of 10 cases of mycobacteriosis involving the parotid of autopsied patients with advanced acquired immunodeficiency syndrome (AIDS), including identification of the *Mycobacterium* species.

METHODS: Detection of '*M. tuberculosis* complex' was performed by polymerase chain reaction (PCR) and ligase chain reaction (LCR) and *Mycobacterium avium* by PCR.

RESULTS: All cases showed involvement of intraparotid lymph nodes, but the glandular parenchyma was affected in only three cases. Most of the cases (80%) presented a chronic non-caseating granulomatous inflammation, and in two cases predominated foamy macrophages, full of bacteria, and no granuloma formation. In areas of mycobacteriosis, macrophages predominated followed by TCD8, B and TCD4 lymphocytes. All cases were infected by *Mycobacterium* genus and '*M. tuberculosis* complex' was detected in five cases by LCR and in eight by PCR, while *M. avium* was positive in one case only, which was also positive for *M. tuberculosis*.

CONCLUSIONS: Parotid mycobacteriosis in advanced AIDS is characterized by intraparotid lymph node non-caseating inflammatory granulomatous lesion, caused mainly by *M. tuberculosis*.

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Introduction

Tuberculosis resurged during the last 20 years as one of the leading diseases in some parts of the world. Almost half of acquired immunodeficiency syndrome (AIDS) patients develop mycobacteriosis during his lifetime, particularly in the lungs, but it is a common systemic infection (1, 2). Tuberculosis of the salivary glands is uncommon, and there are about 100 cases described in the literature, mainly in immunocompetent patients (3–6). Nevertheless, in most of the reports the *Mycobacterium* species were not identified. Culture is the usual method to identify mycobacteria, but the microorganism can fail to grow or clinical samples were not collected. DNA amplification in paraffin-embedded tissue has been used to improve diagnosis of many diseases, and this also can be the case of tuberculous infections (7). In a postmortem study of 100 patients who died with advanced AIDS, 22 showed infectious lesions in the parotid and of these, 10 had mycobacteriosis (8). The purpose of this work is to describe the histological and immunohistochemical features of 10 cases of mycobacteriosis, identifying the *Mycobacterium* species by polymerase chain reaction (PCR) and ligase chain reaction (LCR).

Material and methods

This work was approved by the Ethics Committee of the Dental School of Piracicaba, UNICAMP, Brazil.

Histology, immunohistochemistry and histomorphometry

Ten cases of formalin-fixed, paraffin-embedded tissues of parotid mycobacteriosis from autopsied patients who died of advanced AIDS were selected from a previous study (8). *Mycobacterium* genus was detected by Ziehl-Neelsen staining and by immunohistochemistry using anti-BCG (Bacillus Calmette-Guérin) antibody.

Table 1 Primary antibodies, sources and protocols of antibodies used for determination of immunological profile of parotid mycobacteriosis

Antibody	*Source/clone*	*Protocol*
Anti-BCG	Dako®*, Rabbit Anti-Mycobacterium bovis, code no. B0124	SHAR, 1:10000; incubation – 30 min at 37°C, StABC
CD8	Dako®*, clone MxH	SHAR, 1:200; incubation – 30 min at 37°C, StABC
CD20	Dako®*, clone L26 (1,2)	SHAR, 1:10000; incubation – 30 min at 37°C, StABC
CD4	CD45RO, Dako®*, clone OPD4	SHAR, 1:200; incubation – 30 min at 37°C, StABC
CD68	Dako®*, clone PG-M1	SHAR, 1:400; incubation – 30 min at 37°C, StABC
CD45	LCA, Dako®*, CD45RO clone UCHL 1 (1)	SHAR, 1:200; incubation – 30 min at 37°C, StABC

SHAR, steam heat antigen retrieval; StABC, streptavidin-biotin standard protocol.
*Dako, Dako Corporation, Carpinteria, CA, USA.

Lymphocytes and macrophages in areas of mycobacteriosis were also studied by immunohistochemistry (Table 1). The immunoreactivity of inflammatory cells present in the parotid mycobacteriosis was evaluated by counting of positive cells in 10 fields of 21 208.57 μm^2 around the necrotic areas.

DNA extraction for PCR

From each case it was used ten 5-μm-thick sections. A new microtome blade was used for each paraffin-embedded tissue bloc, and the microtome was cleaned with 70% ethanol after each sample. Genomic DNA extracted from the MT14323 strain of *Mycobacterium tuberculosis* and IWGMT49 strain of *Mycobacterium avium* (kindly provided by Marcelo de Carvalho Ramos, Microbiology Department of Medical School, UNICAMP, Brazil) and a formalin-fixed, paraffin-embedded mesenteric lymph node infected with *M. avium* (kindly provided by Dr. Nèlson Morés, Brazilian Agricultural Research Corporation, Embrapa Pig and Poultry, Brazil) were used as positive controls. Paraffin was removed by vortexing the sections in 1 ml of xylene, followed by centrifugation at 16 000 *g* for 2 min. Supernatant was discarded, and the same procedure repeated. One milliliter of pure ethanol was added, and samples were quickly vortexed and centrifuged at 16 000 *g* for 5 min. The pellets were air dried for approximately 10 min and resuspended in a solution containing 450 μl of Tris-EDTA (pH 8.0), 50 μl of 10% SDS and 15 μl of proteinase K (20 mg/ml). After incubation at 50–55°C for 16 h, DNA was extracted with 500 μl of phenol-chloroform-isoamyl alcohol (25:24:1), the mixtures vortexed for 2 min, incubated at room temperature for 5 min and centrifuged at 6610 *g* for 15 min. The supernatants were removed, transferred into new tubes and the procedure repeated. After the last extration with 500 μl of chloroform, the DNA in the supernatants was precipitated which 0.02 volume of 5 M NaCl and 2.5 volume of absolute ethanol. After 16 h at −20°C, samples were centrifuged at 8160 *g* for 30 min at 0°C, the supernatants were discarded and pellets washed twice with 1 ml of 75% ethanol. The final pellets containing DNA was air dried for 10 min, resuspended in 50 μl of Tris-EDTA (pH 8.0), and the concentration of DNA in each sample was determined by spectrophotometry at 260 nm (Spectronic Genesys 2, Spectronic Instruments, Rochester, NY, USA).

Amplification of M. tuberculosis *and* M. avium *sequences by PCR*

In order to identify the *Mycobacterium* genus, was detected a small region of the gene encoding the highly conserved 65-kDa antigen in a variety of *Mycobacterium* species by nested PCR (PCRG). Nested PCR reactions were also made to amplify a region of IS*6110* (PCRMtb), an insertion sequence usually represented in multiple copies within the '*M. tuberculosis* complex' genome (7). The IS*1245* region which is usually represented in multiple copies within the *M. avium* genome, was detected by one step PCR (PCRMa) and the using primers were designed by the software 'Amplify 1.2' (Wisconsin University, Madison, WI, USA) with the complete IS*1245* sequence obtained from the 'Gene Bank' [National Center for Biotechnology Information (NCBI) – NIH, USA, access number L33879]. The integrity of DNA samples was checked by β-actin gene

Table 2 Primer sequences and predicted product sizes

Primers	*Forward (5′-3′)*	*Reverse (5′-3′)*	*Predicted product size (bp)*
PCRG			
Outer primers	AGGCGTTGGTTCGCGAGGG	TGATGACGCCCTCGTTGCC	234
Inner primers	CCAACCCGCTCGGTCTCAA	CCGATGGACTGGTCACCC	142
PCRMtb			
Outer primers	CGGGACCACCCGCGGCAAAGCCCGCAGGAC	CATCGTGGAAGCGACCCGCCAGCCCAGGAT	220
Inner primers	CCTGCGAGCGTAGGCGTCGG	CTCGTCCAGCGCCGCTTCGG	123
PCRMa	TGGACCAGTCTGCCTT	CGTCGATCAAGGCTTGGTAG	106
β-actin	TCAGAAGGACTCCTATGTGG	TCTCTTTGATGTCACGCACG	506

PCRG, polymerase chain reaction for *Mycobacterium* genus detection; PCRMtb, polymerase chain reaction for '*M. tuberculosis* complex' detection; PCRMa, polymerase chain reaction for *M. avium* detection.

amplification. The primer sequences and their predicted product sizes are shown in Table 2.

The total reaction volume in each round was 50 μl and contained a mixture of 5 μl of 10X PCR buffer, 3 μl of 50 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 1 μl of each primer (from 10 μM solutions) and 0.5 μl of 5 U/μl Taq DNA polimerase (Invitrogen, Life Technologies, Brazil). The amount of DNA used for the first round of PCRG and PCRMtb was 1 and 3 μg, respectively, and PCRMa was performed with 3 μg of DNA. For the second round of PCRG and PCRMtb were used 15 μl of each first round. The amount of DNA used from the control strains was 10 ng and from the paraffin-embedded control tissue was 2 μg. The PCRG conditions were: initial 4 min denaturation step at 94°C coupled to 35 cycles of 1 min at 94°C, 2 min at 57°C, and 2 min at 72°C, followed by a final extension at 72°C for 7 min (first round); and initial 4 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C, with a final extension at 72°C for 7 min (second round). The PCRMtb conditions were: initial 4 min denaturation step at 94°C coupled to 20 cycles of 1.30 min at 94°C, 1.30 min at 63°C, and 1.30 min at 72°C, followed by a final extension at 72°C for 7 min (first round); and initial 4 min denaturation at 94°C followed by 40 cycles of 1.30 min at 94°C, 1.30 min at 63°C, and 1.30 min at 72°C, with a final extension of 7 min at 72°C (second round) (7). PCRMa was performed with an initial 5 min denaturation step at 94°C coupled to 45 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C, followed by a final extension at 72°C for 4 min. Ten microliters of the second round of amplification (PCRG and PCRMtb) or from the single round of PCRMa were resolved on 8% non-denaturating polyacrylamide gel and detected by silver staining as described (9).

Ligase chain reaction

Paraffin was removed from the sections as described above and the samples were resuspended in 350 μl of milli-Q water.

Ligase chain reaction (LCx® *M. tuberculosis* Assay; Abbott, Chicago, IL, USA) also allows identify the '*M. tuberculosis* complex' and consists of three steps – specimen preparation, amplification and detection – and was performed according to the manufacturer's recommendations. A 500 μl of each sample was added to LCx Respiratory Specimen Tube and then were centrifuged at 1500 *g* for 10 min. The supernatant was aspirated, and 1 ml of LCx Respiratory Specimen Resuspension Buffer was added and the procedure repeated. After the supernatant removal, 0.5 ml of the same buffer was added and vortexed for 2–5 s. The suspension was then placed into LCx Covered Dry Bath for 20 min at 95°C. Finally, mycobacterial DNA was released by mechanical lysis in the LCx lysor for 10 min.

For the amplification reaction, 100 μl of the specimen lysed was added to the appropriately labeled LCx Tuberculosis Amplification Vial containing 100 μl of LCR mixture. The specimens and controls were placed in the LCx Thermal Cycler and amplified for 37 cycles of incubation for 1 s at 94°C, 1 s at 55°C, and 40 s at 69°C.

Amplified tubes were transferred unopened to the carousel of the LCx Analyser, which directly detects the amplification products and displays the results as fluorescence rates, which are compared with the calibrator rate. A sample rate/cutoff value ratio of >1.0 indicates an LCx *M. tuberculosis* Assay – positive result.

Results

Intraparotid lymph nodes were affected in all cases, while the glandular parenchyma in three cases only (Fig. 1). Eight of 10 cases showed poorly organized granulomas, with large amounts of mycobacteria easily visualized by Ziehl-Neelsen staining and anti-BCG antibody (Fig. 2), but without multinucleated giant cells. In these cases the predominant cells were TCD8 lymphocytes, followed by macrophages, B and TCD4 lymphocytes (Fig. 3). The other two cases showed a predominant infiltrate of foamy macrophages, full of mycobacteria, scant areas of necrosis and no granuloma

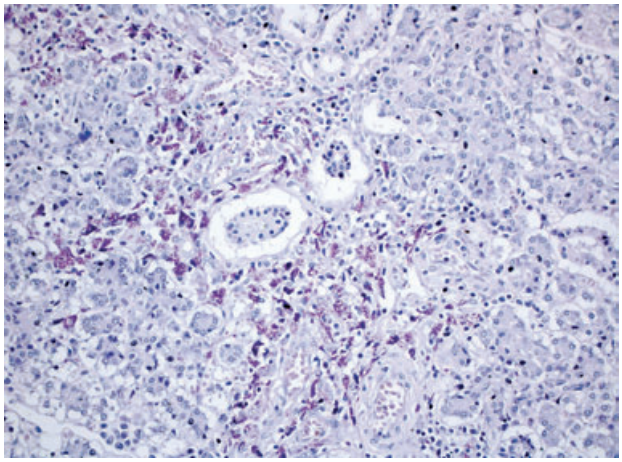

Figure 1 Mycobacteriosis affecting the parotid parenchyma (Ziehl-Neelsen, original magnification 200×).

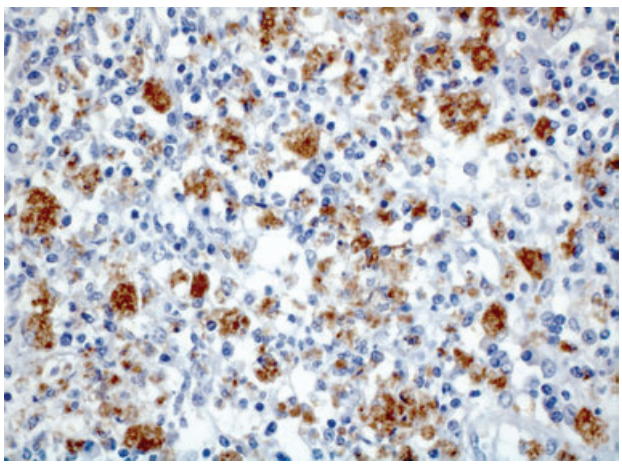

Figure 2 Macrophages replete of bacilli in intraparotid lymph node (immunohistochemistry for BCG, original magnification ×400).

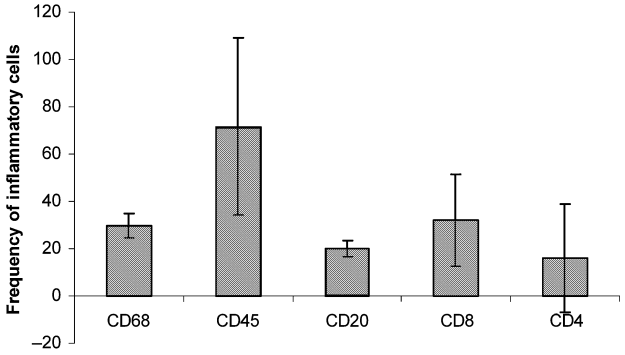

Figure 3 Average comparison of immune cells involved in poorly organized granulomas. The TCD8 lymphocytes were the most prevalent cells, followed by macrophages, B and TCD4 lymphocytes.

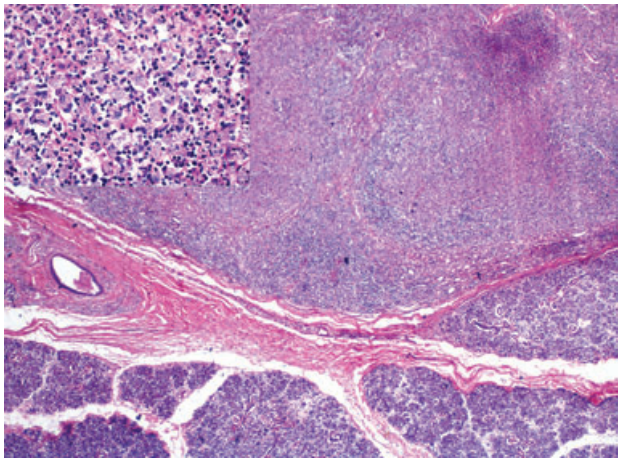

Figure 4 Diffuse macrophagic pattern observed in the intraparotid lymph node (H&E, ×100). Note the macrophages rounded by lymphocytes (H&E, original magnification ×400).

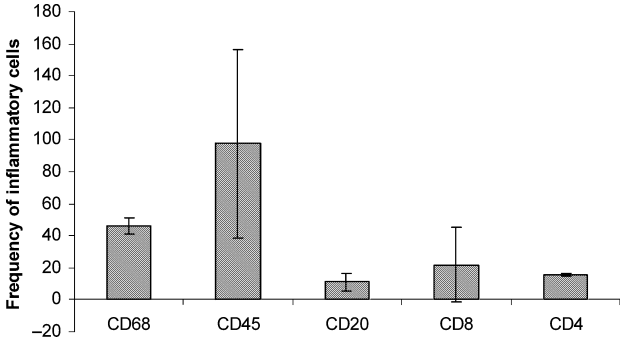

Figure 5 Average comparison of immune cells involved in diffuse macrophagic pattern of parotid mycobacteriosis. The macrophages were the most prevalent cells, followed by TCD8, TCD4 and B lymphocytes.

formation (Fig. 4). In these two cases the predominant cells were macrophages, followed by TCD8, TCD4 and B lymphocytes (Figs 5 and 6).

All cases were infected by *Mycobacterium* genus and positive for Ziehl-Neelsen staining, anti-BCG immunohistochemistry and PCR (Fig. 7). '*M. tuberculosis* complex' was detected in five cases by LCR (case 6–10; Table 3) and in eight cases by PCR (cases 2, 3 and 5–10; Fig. 7). *Mycobacterium avium* was detected in only one case, which was also positive for '*M. tuberculosis* complex' (Fig. 7). Results obtained from all tissues studied are shown in Table 4.

Discussion

There are few reports of mycobacteriosis involving the salivary glands of AIDS patients (4, 5, 8). The present study describes the morphological and molecular characteristics of 10 cases of mycobacteriosis involving the parotid of human immunodeficiency virus (HIV)-positive patients with advanced AIDS. All our cases showed involvement of the intraparotid lymph nodes, similar to report of tuberculosis in HIV-negative patients (10). This probably reflects the lymphatic dissemination of mycobacteria, rather than a predilection for the parotid gland.

The predominant morphological pattern observed of macrophages full of mycobacteria, and poorly organized granulomas, reflects low cellular immune response of these patients (11). This also was confirmed by the low number of TCD4 lymphocytes, were the least prevalent cells in the inflammatory infiltrate.

Identification of mycobacterium in paraffin embedded tissue is usually carried out by Ziehl-Neelsen staining, but immunohistochemistry reaction for BCG also can be used. According to Ohtomo *et al.* (12), these two methods have similar sensitivity, while PCR showed better results. In this study a comparison of sensitivity was not possible, as all cases were full of mycobacteria and strongly positive for the three methods.

The LCR was positive in only 50% of the cases, discouraging the use of this technique, as three more cases were detected by PCR. This technique seems to be the best at the moment, but its efficacy in paraffin embedded tissue can be impaired by multiple factors, including type and time of fixation and embedding. Number of copies of the fragment to be amplified in the *Mycobacterium* genoma seems to be an important factor for the efficacy of the PCR, and insertion sequences have been widely used. IS*6110* and IS*1245* are mobile genetic elements usually present in multiple copies within the genomes of '*M. tuberculosis* complex' and *M. avium* respectively, and have been used successfully for amplification (7, 13–16). Using the amplification of these fragments, positive PCR results for '*M. tuberculosis* complex' occurred in eight of 10 cases, one case being concomitantly positive for *M. avium*. Two cases were negative, although positive by Ziehl-Neelsen staining, immunohistochemistry for BCG and PCR for detection of *Mycobacterium* genus. They can be false-negative, or caused by other species of mycobacterias. It is well accepted at the moment that failure of DNA amplification in paraffin-embedded tissue is relatively common. Improvement of the results could be obtained decreasing the amount of DNA, but this can lead also to decrease sensitivity of the technique (17, 18).

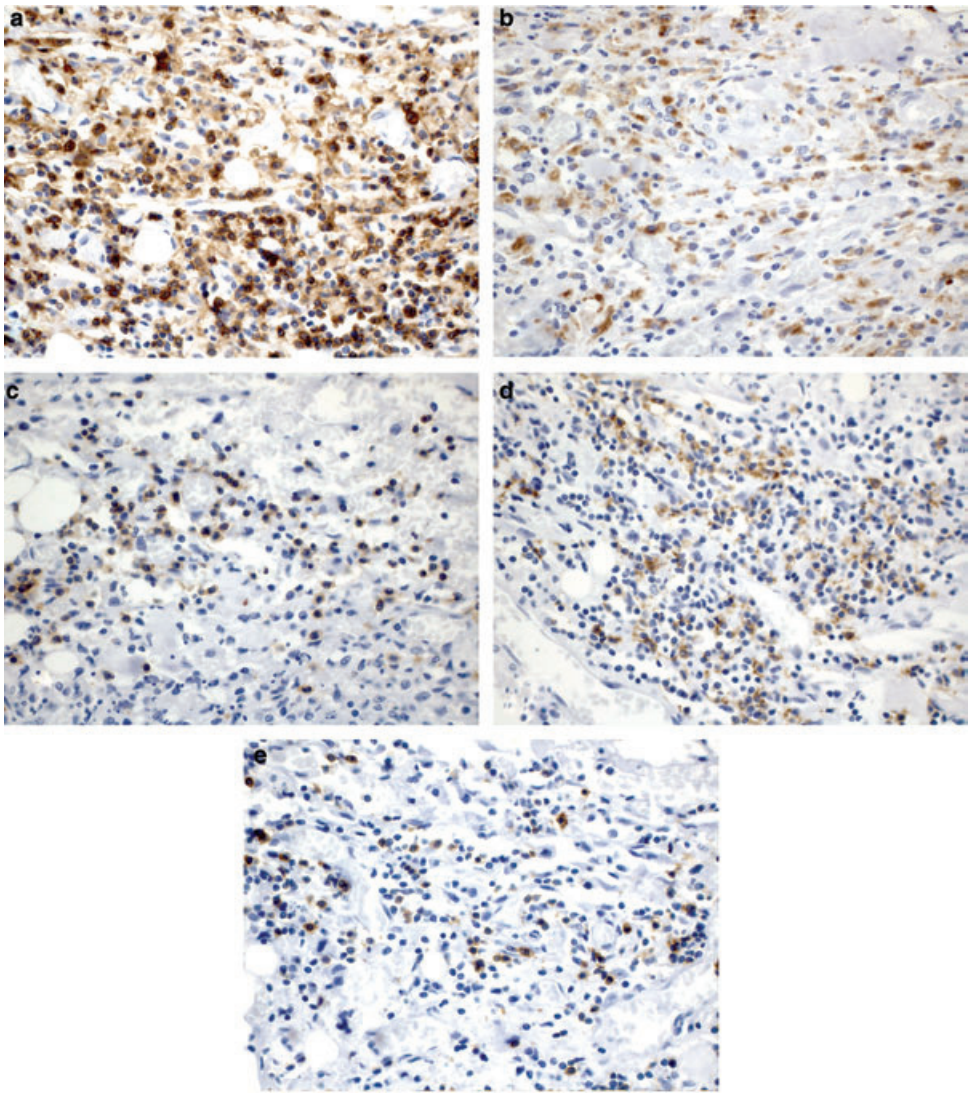

Figure 6 Immunohistochemical profile of parotid mycobacteriosis. (a) Intense and diffuse mark by CD45 antibody. Moderate mark by CD68 (b) and CD8 (c). (d) Focal area of CD20 antibody. (e) Rare cells marked by CD4 (immunohistochemistry, ×400).

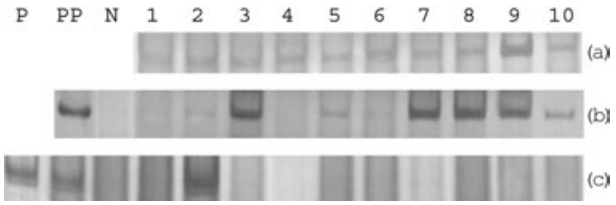

Figure 7 Gel electrophoresis showing: (a) PCR-amplified products of Nested PCR for amplification of small region of gene responsible for conserved protein in the genus *Mycobacterium*; (b) PCR-amplified products of Nested PCR for amplification of IS*6110*, specific for '*M. tuberculosis* complex'; (c) PCR-amplified products of single PCR for amplification of IS*1245*, present in *M. avium* genome. Lanes 1 to 10, cases 1 to 10; P, positive control (MT14323 strain of *M. tuberculosis* and IWGMT49 strain of *M. avium*); PP, positive control (formalin-fixed, paraffin-embedded mesenteric lymph node infected with *M. avium*); N, negative control (formalin-fixed, paraffin-embedded squamous cell carcinoma).

Table 3 LCx® *Mycobacterium tuberculosis* Assay result (Abbott, USA) from 10 cases of parotid mycobacteriosis

Specifications	*Rate*	*Rate/cut off*	*Result*
Case 1	16.3	0.04	Negative
Case 2	12.8	0.03	Negative
Case 3	12.0	0.03	Negative
Case 4	12.2	0.03	Negative
Case 5	15.5	0.04	Negative
Case 6	981.3	2.64	Positive
Case 7	1935.4	5.22	Positive
Case 8	1594.5	4.30	Positive
Case 9	2530.1	6.82	Positive
Case 10	2389.6	6.44	Positive

Negative controls average 12.10.
Positive controls average 1237.05.
Cut off 371.12

In summary, mycobacteriosis involving the parotid of the patients with advanced AIDS is characterized by accumulation of macrophages full of mycobacteria, low number of TCD4 lymphocytes, and poorly organized granulomas. In all cases, infections were predominantly in the intraparotid lymph nodes, and in 30% of the cases

Table 4 Clinical, microscopic, immunohistochemistry and molecular data of 10 parotid mycobacteriosis cases evaluated on this study

Case	*Age (years)*	*Gender*	*Histopathologic pattern*	*Lymph node*	*Glandular parenchyma*	*ZN*	*IH*	*LCR*	*PCRG*	*PCRMtb*	*PCRMa*
1	46	M	DM	Yes	Yes	+	+	–	+	–	–
2	46	M	DM	Yes	Yes	+	+	–	+	+	+
3	69	M	PG	Yes	No	+	+	–	+	+	–
4	27	M	PG	Yes	No	+	+	–	+	–	–
5	52	M	PG	Yes	No	+	+	–	+	+	–
6	46	M	PG	Yes	No	+	+	+	+	+	–
7	43	M	PG	Yes	No	+	+	+	+	+	–
8	24	M	PG	Yes	Yes	+	+	+	+	+	–
9	29	F	PG	Yes	No	+	+	+	+	+	–
10	22	F	PG	Yes	No	+	+	+	+	+	–

M, male; F, female; DM, diffuse macrophagic pattern; PG, poorly organized granulomas; ZN, Ziehl-Neelsen staining; IH, BCG immunohistochemistry; LCR, ligase chain reaction; PCRG, polymerase chain reaction for *Mycobacterium* genus detection; PCRMtb, polymerase chain reaction for '*M. tuberculosis* complex' detection; PCRMa, polymerase chain reaction for *M. avium* detection.

the parenchyma was also involved. '*M. tuberculosis* complex' was detected in most of the cases by PCR, and *M. avium* in only one case. Therefore most cases of parotid infection by *Mycobacterium* in advanced AIDS are tuberculosis.

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