Retrospective determination of HIV-1 status by a PCR method on paraffin wax embedded sections

T Slavik, M Wolfaardt, H van Zyl, I W Simson

Abstract

Aim—To develop a simple but reliable polymerase chain reaction (PCR) method to determine the HIV-1 status of patients on formalin fixed, paraffin wax embedded lymph node tissue.

Methods—Fifty lymph node specimens, 20 from HIV-1 seropositive and 30 from HIV-1 seronegative patients, were analysed. Lymph nodes with a variety of disease conditions were included in the study. Tissue sections were treated with a DNA extraction buffer containing proteinase K and the crude cell lysate was used in PCR analysis. Nested primers were used to amplify HIV-1 DNA sequences coding for gag, pol and env proteins. PCR products were demonstrated by polyacrylamide gel electrophoresis. Results were then compared with HIV-1 serology of the patients from whom the tissue was obtained.

Results—The PCR method yielded a specificity of 100%, a sensitivity of 95%, a positive predictive value of 100%, and a negative predictive value of 97% when compared with HIV-1 serology. The κ statistic (0.958) showed an excellent agreement between the PCR method and serology. Furthermore, HIV-1 DNA was demonstrated in lymph node tissue from a serologically unconfirmed acquired immunodeficiency syndrome case necropsied in 1982.

Conclusion—This PCR method is a simple and reliable means of retrospectively determining the HIV-1 status of patients using formalin fixed, paraffin wax embedded lymph node tissue.

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Keywords: HIV, PCR, paraffin wax section, lymph node.

The HIV-1 pandemic has led to increasing reports of various opportunistic infections and malignancies constituting the acquired immunodeficiency syndrome (AIDS).^{1,2} Studies of the pathology of these diseases and their precise association with HIV-1 infection is often limited by the lack of fresh material. Many laboratories have ample archival tissue in the form of formalin fixed, paraffin wax embedded blocks, but retrospective studies are hampered because the HIV-1 status of the patients is often unknown.

The polymerase chain reaction (PCR) amplifies viral genetic material and has been widely used for detecting HIV-1 infection.³ A potential problem with the sensitivity of PCR is contamination of the sample or reagents which can lead to false positive results. Such false positive results can, however, be avoided by simple protocols for sample preparation and strict laboratory routines. Various pre-treatment techniques for processing formalin fixed, paraffin wax embedded specimens have been described,⁴⁻⁸ but they are relatively complicated, expensive or cause significant DNA loss due to many manipulations.

This study was conducted to develop and evaluate a simple but reliable method to determine the HIV-1 status of patients using formalin fixed, paraffin wax embedded tissue. Paraffin wax sections of lymph nodes were used for detection of viral DNA as they carry a high viral load in HIV-1 infection.⁹

Methods

Fifty formalin fixed, paraffin wax embedded lymph node specimens, including 20 from HIV-1 seropositive and 30 from HIV-1 seronegative patients, were selected. Both the seropositive and control seronegative groups included lymph nodes with reactive change, as well as inflammatory and neoplastic lesions (table 1). Thirty eight of the specimens were obtained antemortem and 12 postmortem. The age of the paraffin blocks varied from one to 137 months.

Between sectioning of the different specimens, the microtome knife was decontaminated with 4N HCl solution. Further precautions taken to minimise possible contamination included aliquoting of all reagents, physical separation of pre- and post-PCR reactions, irradiation of reagents with ultraviolet light, the inclusion of multiple negative controls, and the use of nested PCR primers. Gloves were worn during all procedures when the specimens were being handled and processed.

After transferral into sterile 1 ml tubes, the coded 10 μ m sections were treated with 400 μ l DNA extraction buffer¹⁰ containing 50 mM KCl, 10 mM Tris, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween-20, and 20 μ l 10 mg/ml proteinase K for eight hours at 56°C. The reaction was stopped by boiling for eight minutes. Five microlitres of this crude cell lysate were used for the PCR.

Department of
Anatomical Pathology,
Faculty of Medicine,
University of Pretoria,
P.O. Box 2034,
Pretoria, South Africa
T Slavik
I W Simson

Department of
Medical Virology
M Wolfaardt
H van Zyl

Correspondence to:
Dr T Slavik.

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Table 1 Study cases with histological diagnosis, specimen information and results

Specimen number	Histological diagnosis	Tissue obtained ante/postmortem	Age of paraffin wax block (in months)	HIV serology	PCR result
1	Reactive change	Post	12	—	—
2	Reactive change	Post	12	—	—
3	Reactive change	Post	11	—	—
4	Reactive change	Ante	4	—	—
5	Reactive change	Ante	4	—	—
6	Reactive change	Ante	3	—	—
7	Reactive change	Ante	3	—	—
8	Reactive change	Ante	2	—	—
9	Reactive change	Ante	2	—	—
10	Reactive change	Ante	1	—	—
11	Tuberculous lymphadenitis	Ante	12	—	—
12	Tuberculous lymphadenitis	Ante	15	—	—
13	Tuberculous lymphadenitis	Ante	11	—	—
14	Tuberculous lymphadenitis	Ante	20	—	—
15	Tuberculous lymphadenitis	Ante	17	—	—
16	Tuberculous lymphadenitis	Ante	22	—	—
17	Tuberculous lymphadenitis	Ante	20	—	—
18	Tuberculous lymphadenitis	Ante	22	—	—
19	Tuberculous lymphadenitis	Ante	26	—	—
20	Tuberculous lymphadenitis	Ante	20	—	—
21	Sarcoidosis	Ante	40	—	—
22	Sarcoidosis	Ante	29	—	—
23	Sarcoidosis	Ante	27	—	—
24	Sarcoidosis	Ante	7	—	—
25	Metastatic ganglioneuroblastoma	Ante	4	—	—
26	Metastatic breast carcinoma	Ante	2	—	—
27	Metastatic neuroendocrine carcinoma	Ante	5	—	—
28	Hodgkin's lymphoma	Ante	2	—	—
29	Hodgkin's lymphoma	Ante	1	—	—
30	T cell lymphoblastic lymphoma	Ante	1	—	—
31	Reactive change	Post	22	+	+
32	Reactive change	Post	40	+	+
33	Reactive change	Post	116	+	+
34	Reactive change	Post	137	+	+
35	Reactive change	Ante	6	+	+
36	Reactive change	Ante	5	+	+
37	Reactive change	Ante	49	+	+
38	Reactive change	Ante	3	+	+
39	Reactive change	Ante	3	+	+
40	Histoplasmosis	Post	31	+	+
41	Histoplasmosis	Post	118	+	+
42	Measles	Post	61	+	+
43	Cryptococcosis	Post	59	+	+
44	Tuberculous lymphadenitis	Ante	6	+	—
45	Tuberculous lymphadenitis	Ante	10	+	+
46	Tuberculous lymphadenitis	Ante	10	+	+
47	Tuberculous lymphadenitis	Ante	2	+	+
48	Tuberculous lymphadenitis	Ante	4	+	+
49	Tuberculous lymphadenitis	Ante	2	+	+
50	Kaposi's sarcoma	Post	79	+	+

The PCR was performed using oligonucleotide primer pairs JA9/12 (nested JA10/11), JA17/20 (nested JA18/19) and GAG1/2 (nested GAG3/4) (synthesised by the Department of Biochemistry, University of Cape Town, Cape Town, South Africa) to amplify fragments of DNA from the envelope, polymerase and p24 regions of HIV-1.^{11,12} The PCR was performed in a two-step reaction, first with a pair of outer primers and then with a pair of inner nested primers. The reaction mixtures contained 100 mM Tris-HCl (pH 8.3), 40 mM KCl, MgCl₂ (3 mM for primer pair JA10/11, JA18/19, GAG1/2, and GAG3/4, 4 mM for primer pair JA9/12 and 7 mM for JA17/20) (final concentrations), each primer at 0.1 µM, 0.5 mM dNTPs, and 1 unit Taq polymerase per 50 µl (Boehringer Mannheim, Mannheim, Germany). In addition, lysates were amplified with human β-globin primers PCO3/PCO4 as an internal control.¹³

The samples were denatured for five minutes at 94°C, then cycled 30 times at 95°C for two minutes, annealed at 40°C for 30 seconds (gag and pol primer pairs) or 50°C for 30 seconds (env primer pairs), and extended at 72°C for two minutes. After the first PCR, 5 µl of the product was amplified for 30 cycles with the corresponding inner primers. The final PCR product was visualised by polyacrylamide gel electrophoresis and ethidium bromide staining.

The HIV-1 PCR result was taken as positive if DNA from two of the three regions was amplified. PCR results were then compared with the HIV-1 serology determined at the time that the lymph node specimen was taken. Two enzyme linked immunosorbent assay (ELISA) HIV antibody tests (Vironostika Mixt, Organon Teknika Corp., Durham, North Carolina, USA, and Wellcozyme HIV 1 + 2, Wellcome Diagnostics, Dartford, UK) were used and positive results were confirmed by means of a western blot (HIV Blot 2.2, Diagnostic Biotechnology Pty Ltd., Singapore) and recombinant immunoblot assay (Pepti-Lav, Diagnostics Pasteur, Marnes-La-Coquette, France).

Results

The comparison between PCR results and serology is shown in table 2. There was excellent agreement between these two methods (κ statistic of 0.958). When compared with HIV-1

Table 2 Correlation of HIV-1 serology and PCR results

Serology	PCR positive	PCR negative	Total
Positive	19	1	20
Negative	0	30	30
Total	19	31	50

serology, the PCR technique had a specificity of 100% (30/30), a sensitivity of 95% (19/20), a negative predictive value of 97% (30/31) and a positive predictive value of 100% (19/19).

Table 1 illustrates the age of the paraffin wax blocks and whether the specimen was obtained ante- or postmortem.

Discussion

Of the 30 specimens obtained from HIV-1 seronegative patients, all proved to be negative by the PCR technique. Two of these specimens demonstrated a single HIV-1 DNA sequence (one *gag* and one *pol*) considered to be due to possible cross contamination with other samples.^{14,15} Extensive precautions were taken to prevent pre-PCR contamination, but post-PCR carry-over remains a possibility. This could have been eliminated had a uracil N glycosylase prevention strategy been used.¹⁶

Nineteen of the 20 specimens obtained from HIV-1 seropositive patients were positive by PCR. Fifteen demonstrated all three HIV-1 DNA sequences. Of the remaining four specimens, three lacked *pol* and one lacked *env* DNA sequences. One of the specimens (specimen no. 44), an extremely small lymph node fragment obtained by needle biopsy from an HIV-1 seropositive patient, did not demonstrate any HIV-1 DNA products by PCR. A recent report has documented the occurrence of false positive HIV-1 serology in leprosy patients, possibly due to cross reactivity between HIV-1 proteins and mycobacterial cell wall antigens.¹⁷ Although the histological diagnosis of this specimen was tuberculous lymphadenitis, the presence of antibodies to all three HIV-1 *env* proteins on the patient's western blot makes this an unlikely explanation, as cross reactivity with mycobacterial antigens appears to be limited to HIV-1 *gag*, *pol* and *gp41* proteins.

In practice, a critical quantity of DNA is needed for successful amplification by PCR^{11,14,18} which could explain the false negative PCR result, as the specimen was extremely small. This was confirmed by a negative result after DNA precipitation of the proteinase K digest prior to PCR. Subsequent testing with larger quantities of tissue was not possible as there was no more tissue in the block.

The superior analytical sensitivity of the PCR technique over HIV-1 serology is well documented.¹⁹ This was observed in our study in a patient (specimen no. 39) who was undergoing seroconversion at the time of lymph node biopsy. An HIV-1 ELISA test done on this patient eight days prior to biopsy was negative. HIV-1 serology repeated seven days later yielded a positive ELISA test and an indeterminate western blot (only gp160 present). PCR carried out on the lymph node biopsy performed the following day demonstrated all three HIV-1 DNA products. Serology done 11 days after biopsy revealed a positive ELISA test with p24, p31, gp120, and gp160 bands present on the western blot. Retrospective determination of p24 antigen yielded positive results on both serum specimens drawn prior to biopsy.

Although DNA fragmentation in specimens increases with time and this can lead to false negative PCR results,²⁰ we did not experience this problem in our study. PCR demonstration of all three HIV-1 DNA products was possible in a lymph node specimen more than 11 years (137 months) old. All three DNA products were also demonstrated in lymph node tissue 141 months old obtained from a case diagnosed as AIDS but never confirmed serologically, which was necropsied in our department in August 1982.

In spite of the low number of cases this study shows that this PCR technique is a reliable and relatively simple method of retrospectively determining HIV-1 status using formalin fixed, paraffin wax embedded tissue. Furthermore, using this method it is possible to detect early cases of HIV-1 infection before seroconversion occurs, as the method detects proviral DNA rather than an antibody response.

With techniques such as immunocytochemistry, in situ hybridisation and PCR being used in many laboratories, much study has been undertaken of the pathogenesis and histopathology of HIV-1 infection using tissue sections. The correlation of various pathological changes and HIV-1 positivity has provided important information with regard to the progression of HIV-1 infection to AIDS.

The shortage of tissue specimens, however, remains a problem. Fresh tissue is scarce and retrospective analysis of more abundant archival material is hampered by the fact that many of these specimens come from patients whose HIV-1 status is unknown. Using this PCR method, we have been able to determine retrospectively the HIV-1 status of archival tissue for research purposes.

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