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Performance of the Amplicor Human Immunodeficiency Virus Type 1 PCR and Analysis of Specimens with False-Negative Results

K. L. BARLOW, J. H. C. TOSSWILL, J. V. PARRY, AND J. P. CLEWLEY*

Virus Reference Division, Central Public Health Laboratory, London NW9 5HT, United Kingdom

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Over a 4-year period, the Roche Amplicor kit was used in a United Kingdom reference laboratory for the detection or confirmation of human immunodeficiency virus (HIV) type 1 infection, particularly in infants born to HIV-infected mothers. Of 408 specimens from adults and older children tested, the 122 seronegative specimens were all Amplicor negative. Of the 286 seropositive specimens, 268 were Amplicor positive. On the basis of these results, the Amplicor assay has a specificity of 100% and a sensitivity of 93.7%. In addition, for 247 specimens from infants and young children, serological results may not have been diagnostic because of placental transfer of maternal antibodies. Forty-eight were Amplicor positive, and of the 199 Amplicor-negative specimens, 19 were assumed to be false negative on the basis of clinical data, serological markers (including p24 antigen), and/or results for previous or follow-up specimens. This represents a sensitivity of 75% for the Amplicor test for specimens from patients under 2 years of age. Of these 37 false-negative specimens plus 2 specimens from other laboratories, 31 could be characterized by amplifying extracted material from them by an in-house nested *gag* PCR spanning the Amplicor target region. The amplicons were sequenced and found to represent subtypes A (35.5%), B (22.6%), C (22.6%), D (16.1%), and G (3.2%). False-negative results by the Amplicor assay may be ascribed to low-target copy number, the physical behavior of one primer (SK462), and sequence variation in the target region of the other primer (SK431).

The conventional approach to the diagnosis of human immunodeficiency virus (HIV) infection involves detection of virus-specific antibodies. In contrast, PCR directly amplifies HIV DNA or RNA without reliance on a host response. PCR has a role in confirmatory diagnostic testing when serological results are ambiguous. Ambiguity may arise for specimens from patients who have been infected very recently or whose humoral response is impaired; false-positive serological findings may also occur. However, the most important use for diagnostic PCR of proviral DNA is the investigation of infants born to HIV-infected mothers (8, 12, 17, 20), in whom maternal antibodies may be detected for up to 18 months after birth (12), and therefore seropositivity may not be indicative of HIV infection.

The Roche Amplicor HIV-1 DNA test is the only commercial PCR assay currently available for the diagnosis of HIV type 1 (HIV-1) infection (6). It has three component kits: reagents for preparation of lymphocytes from whole blood, an amplification system for proviral HIV-1 in a single round of PCR, and a nonisotopic, microtiter plate-based detection system for the specific amplicon. The assay also incorporates a uracil *N*-glycosylase (UNG) anticontamination system to prevent false-positive results arising from carryover from previously synthesized amplicons.

Several groups have described the performance of the primers used in the Amplicor assay, both in prototype versions of the kit and in the final commercial product (3, 5, 8, 14, 17, 19, 20, 23, 27, 29). Studies with the commercially available version of the assay have evaluated it with panels of specimens whose serologic status is known rather than reporting its prospective use as a diagnostic test (14, 19, 20, 23, 27). Also, some studies have entirely or predominantly used Ficoll-Hypaque-isolated peripheral blood mononuclear cells in place of cells prepared with the kit reagents (14, 19).

False-negative Amplicor reactions have been reported, but their cause has not been conclusively established. They have been ascribed to inhibitors of amplification (19) and/or to a low copy number of proviral DNA (3, 10, 14, 20, 23). In addition, the diversity of the HIV-1 genome has raised concern about the ability of the Amplicor primers to detect all infections (1, 14, 22, 27).

In the present study, carried out over 4 years of use of the Amplicor test, specimens from infants and others that gave Amplicor results discordant with serological results were investigated by amplification and sequencing with an in-house PCR with primers flanking those in the kit (3). The sequences were analyzed phylogenetically to determine the subtype of the virus. In addition, the sequences of the Amplicor primer and probe target regions were analyzed to determine the extent of base mismatching. The annealing and melting temperatures and relative stability of the primer-template duplex structures were also calculated.

MATERIALS AND METHODS

Patients. EDTA-anticoagulated whole-blood specimens were received from infants born to HIV-infected mothers (45.8% of specimens with a known risk factor) and from older patients with the following risk behaviors: men who had had sex with men (17.3%), patients thought to be exposed as a result of intercourse with someone infected in Africa (14.7%), patients in whom no risk other than heterosexual intercourse was reported (5.4%), and patients who had injected drugs (3.0%). Of the specimens from older patients for whom gender information was known, two-thirds (67.8%) were males, with ages ranging from 2 to 84 years, and the remainder were females (32.2%), with ages ranging from 2 to 82 years.

Laboratory diagnosis of HIV infection. (i) Specimens from patients 2 years of age and older. Demonstration of the presence of anti-HIV in the serum or plasma of these patients was evidence of HIV infection and was the primary means of diagnosis. Usually, the blood sample received for the Amplicor assay

* Corresponding author. Mailing address: Virus Reference Division, Central Public Health Laboratory, 61 Colindale Ave., London NW9 5HT, United Kingdom. Phone: 44 (0)181 200 4400. Fax: 44 (0)181 200 1569. E-mail: jclewley@hgmp.mrc.ac.uk.

was for confirmation of infection. Serological diagnosis of HIV infection was performed according to the recommendations of the AIDS Diagnosis Working Group of the Public Health Laboratory Service (26) and the World Health Organization (18) by using a combination of screening tests. All specimens were examined by three distinct anti-HIV tests. The first, Biotest Anti-HIV-1/-2 recombinant (Biotest AG), is an indirect enzyme immunoassay (EIA) with recombinant antigens; the second, Innotest HIV-1/HIV-2 Ab s.p. (Innogenetics N.V.), is also an indirect EIA, that is primarily based on synthetic oligopeptide antigens; the third, GACPAT HIV-1+2, is an in-house class-specific antibody (immunoglobulin G [IgG]) capture method with gelatin microparticles coated with native HIV antigens (25). It fulfills the dual roles of distinguishing anti-HIV-1 from anti-HIV-2 and contributing to the overall positive predictive value. The presence of anti-HIV was confirmed only if the two EIAs and GACPAT HIV-1 and/or HIV-2 were each unambiguously positive. Reactivity in GACPAT determined which HIV type was present. Western blot (WB) investigation (Genelabs HIV blot 2.2 [Genelabs Pte Ltd.] and HIV 2 WB [Cambridge Biotech Corporation] to confirm HIV-2 infection) was performed for the small proportion of samples for which the serological investigations were ambiguous. In most cases this was due to dual reactivity in the GACPAT HIV-1+2, in which case the purpose was to determine whether infection was with HIV-1, HIV-2, or both types. Reactivity with the transmembrane gp41 and gp36 antigens was taken to indicate the type(s) of HIV infection. All the specimens from patients 2 years of age and older reported here as giving rise to false-negative Amplicor findings unequivocally contained anti-HIV.

(ii) Specimens from patients younger than 2 years of age. Since no single method is entirely sensitive or specific when undertaking diagnosis of HIV infection in children younger than the age of 2 years, several independent approaches were used. All specimens were examined for IgG anti-HIV (as outlined in the previous section), for HIV proviral DNA (Roche HIV Amplicor), for HIV p24 antigen by the immune complex dissociation protocol with confirmation by a neutralization assay (HIV-1 p24 Antigen Assay and p24 Antigen Neutralisation Kit; Coulter Corporation), and for IgA and IgM anti-HIV (24). In children younger than 3 months of age, only HIV proviral DNA and p24 antigen are meaningful markers of infection. The presence of both of these was taken as clear evidence of HIV in the specimen; the presence of only one was taken to indicate probable infection, suggesting a false-negative diagnostic finding in the other, particularly if the clinical history was also consistent with HIV infection. In children ages 3 months or older the presence of IgA anti-HIV (and IgM anti-HIV) has been shown to be very highly predictive of infection (21, 24, 28, 30). Therefore, its presence was considered significant in these children, as was the persistence of IgG anti-HIV beyond 18 months of age. Results obtained by an in-house nested PCR for HIV DNA, which was applied to specimens thought to be false negative by Roche Amplicor, also contributed to the diagnosis. A definitive diagnosis of HIV infection in young children was reported only if specimens collected on at least two separate occasions were shown to contain two or more of the specific markers of HIV infection outlined here.

Amplicor HIV-1 PCR. Specimens were processed by the Amplicor whole-blood specimen preparation protocol. Up to 100 μl of plasma was removed for serological testing. If there was less than the recommended 0.5 ml of blood required for each cell pellet, the volume available was noted. The leukocyte pellets were stored at below −50°C prior to extraction, which was usually within 1 week. Cell pellets were extracted in 200 μl of extraction reagent or a proportionally reduced amount for smaller blood volumes and were amplified according to the manufacturer's instructions with a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer Cetus).

Sequencing of proviral DNA. Any unexpected Amplicor results or serological ambiguities were investigated by amplification by an in-house, nested PCR. Amplicor-positive controls were chosen from patients matched for the same risk category as the specimens with ambiguous results and included separate specimens from patients who had also provided specimens false negative by the Amplicor assay.

Usually, DNA was extracted from cell pellets prepared for use in the Amplicor assay. When these were not available, cells were prepared by Ficoll extraction of blood or with anti-CD4 cell-labelled Dynabeads (Dynal) (16). In initial experiments, the DNA was purified and concentrated (two to six times) by extraction with phenol-water-chloroform (68:18:14) and chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol and resuspended in water (3). Subsequently, it was found that the majority of specimens could be amplified without this additional extraction, and it was then only performed with those specimens whose HIV-1 DNA failed to be amplified by nested PCR. Five microliters of extracted DNA or lysate was added to 95 μl of the PCR mixture (5 pmol of each primer, 1.5 mM $MgCl_2$, and *Taq* polymerase from Boehringer Mannheim or Gibco) for the first round of a nested PCR as described previously (3). The first-round primers (RB1 and RB5) were sited in conserved regions of the *gag* gene approximately 500 bases on either side of the Amplicor target region. One microliter of the first-round reaction mixture was added to 50 μl of the reaction mixture for the second round of PCR (3). Amplicons were visualized by electrophoresis in a 1% agarose gel and ethidium bromide staining. On occasion, actin gene primers were used to check that the DNA could be amplified from the lysed cell pellets, to exclude total inhibition of PCR.

The genome diversity of HIV-1 was investigated by sequencing to determine its contribution to the false-negative results. PCR products were purified by gel purification (QIAquick [Qiagen] or Geneclean II [Bio 101]) or by ultrafiltration (Centricon-100; Amicon) prior to cycle sequencing with *Taq* DyeDeoxy Terminator chemistry (Applied Biosystems). Between two and four sequencing primers were used to give reliable sequence data in both directions (3). Sequencing reactions were run on an Applied Biosystems 373A DNA sequencer, and the data were analyzed by using SeqEd (Applied Biosystems) and MegAlign (Lasergene; DNAStar). No two unrelated specimens gave identical sequences during this work, showing that there were no false-positive results from the in-house PCR. All PCRs were performed in a dedicated suite of rooms, with appropriate precautions taken (use of dedicated equipment, use of plugged pipette tips, etc.) to prevent contamination events.

Sequence analysis: primers and probe regions. The reasons for amplification failure were investigated by PrimerSelect (Lasergene; DNAStar), which allows for comparison of a fixed-sequence primer with a variable-sequence template. PrimerSelect implements the algorithms of Freier et al. (15) and Breslauer et al. (4) and calculates the stability and temperature behavior of DNA duplexes from their base sequences. It was, however, impossible to analyze several of the sequences due to limitations in the program code on mismatches positioned within 5 bases of the 5′ end. This excluded six specimens with false-negative results and six controls from the analysis. The sequence differences and data on the properties of the primers and probes were analyzed statistically by chi-square and difference-between-means tests.

The relative stability (indicated by free energy [ΔG_0]) and melting temperature (T_m) of the actual primer-template duplex structures were calculated for each primer for the false-negative and control sequences. These were also calculated for primer sequences changed to complement the template exactly (the ideal sequence duplex structure). In addition, the relative stability (indicated by the lowest ΔG_0) of both primer duplex structures was calculated for a subtype B sequence (SF2) modified such that there was a perfect match with the primers in the kit. The optimal annealing temperatures (T_a), the T_m, the difference between the product T_m and the primer T_m, and the G+C content of the products were calculated for the actual primer-template duplex structures, the ideal duplex structures (the primer sequence changed to complement that of the template), and the perfect duplex structure (the modified SF2 sequence).

TABLE 1. Performance of Amplicor PCR with HIV-1-seropositive and -seronegative specimens from patients 2 years of age or older

Amplicor result[a]	No. of specimens with the following HIV-1 serology result:		
	Positive	Negative[b]	Total
Positive	268[c]	0	268
Negative	18	122[d]	140
Total	286	122	408

[a] Specificity, 100%; sensitivity, 93.7%.
[b] The majority of the anti-HIV-negative specimens were examined by PCR because of reactivity in one or more screening tests.
[c] Data include 13 samples from patients of unknown age but who were assumed to be adults.
[d] Includes six samples from patients of unknown age.

RESULTS

Specimens from adults and older children. Of the 408 specimens from 397 patients 2 years of age or older, 268 were positive both by the serological tests and by the Amplicor PCR (Table 1). A further 18 specimens were from seropositive patients who were believed to be infected but who were negative by the Amplicor PCR. One hundred twenty-two specimens were negative by both approaches. The 18 false-negative specimens (17 patients) were from two patients under 5 years of age, 14 patients between the ages of 24 and 41 years, and one person who was 59 years of age. Their risk factors included homosexual contact, heterosexual contact, and injection drug use. Only one specimen is known to have been from a woman. The specimens were characterized by in-house nested *gag* PCR and sequencing. HIV-1 DNA could be amplified from 14 of the 18 specimens and all positive control specimens.

Children born to HIV-1-infected mothers. There were 247 specimens from 146 infants and children less than 2 years of age. Forty-eight specimens were Amplicor positive and 199

were Amplicor negative. Nineteen specimens from 16 infants were assumed to be false negative by the Amplicor assay because of clinical data, serological evidence (e.g., neutralizable p24 antigen reactivity, or anti-HIV persisting beyond 18 months of age) or because other specimens from the same patient were PCR positive, or both (Table 2). A further two infant specimens referred from other laboratories were also investigated. The in-house PCR amplified HIV-1 DNA from 18 of these 21 false-negative specimens. One of the specimens, from an infant who was 13 months of age, was negative both by Amplicor and by serology (false-negative specimen 18 in Table 2). The ages at which either a positive or a negative result was obtained from the infants with conflicting Amplicor results are presented in Table 2. The optical density readings in the Amplicor test, the viral genome subtype, and markers of infection are given in Table 2, together with the equivalent data for controls.

Sequence analysis: primer and probe regions. All specimens amplified by in-house PCR were sequenced. The sequences obtained, which encompass the Amplicor target primer and probe regions, were aligned and compared with the sequences of the forward primer (SK462), the reverse primer (SK431), and the probe (SK102) (Fig. 1). The number and distribution of base mismatches are shown as a proportion of the number of specimens in Fig. 2.

For the SK462 target region (Fig. 2), the sequence differences for false-negative specimens ranged from one sample with one base difference to one sample with six base differences. In comparison, the controls had two to six base differences. The differences between specimens false negative by the Amplicor assay and control specimens were not statistically significant by the chi-square test ($P = 0.74$).

For the SK431 target region, the false-negative specimens had from one base difference (17 specimens) to six base differences (1 sample). In comparison, the controls had from one to three base differences. This was not a statistically significant difference whether or not the subtype G with 6 of 27 base differences (false-negative specimen 1 in Table 2) was included in the analysis ($P = 0.75$ or $P = 0.73$, respectively).

For the SK102 target region, the false-negative specimens had from zero to four base differences and the controls had from zero to three base differences (borderline statistically significant; $P = 0.09$).

The relative stability (ΔG_0), T_m, T_a, difference between the product T_m and primer T_m, and the G+C content of the products were calculated for each primer for the false-negative and control sequences, for the actual primer-template combinations, and for the primer whose sequence was changed to complement that of the template exactly (the ideal sequence). Also, the lowest ΔG_0 of both primers was calculated for SF2 modified to perfectly match the primer sequences. These results (Table 3) can be summarized as follows. If there was an exact complement between SK462 and its template, the T_m of the primer-template duplex structure would be 72°C (mean) for both the controls and the false-negative specimens. In practice, the T_m was calculated to be 56.5°C. There was a difference of 12.9 kcal/mol (mean) between the ΔG_0 of the exactly complementary SK462 primer-template duplex structure and the observed ones for both the controls ($P = 0.001$; calculated by the difference-between-means test) and the false-negative specimens ($P = 0.002$). However, there was no significant difference between the ΔG_0 of the controls and false-negative specimens when their actual sequences were compared ($P = 0.226$).

Similarly, if there were an exact complement between SK431 and its template, the T_m of the primer-template duplex structure would be 60.4°C (mean) for both the controls and the false-negative specimens. In practice, the T_m was calculated to be 54.6°C. There was a significant difference of 4.4 kcal/mol (mean) between the ΔG_0 of the exactly complementary SK431 primer-template duplex structure and the observed ones for both the controls ($P = 0.02$) and the false-negative specimens ($P = 0.01$). However, there was no significant difference between the ΔG_0 of the controls and the false-negative specimens when their actual sequences were compared ($P = 0.89$).

Sequence analysis: HIV subtypes. The total *gag* p24 sequences obtained were aligned with the equivalent region from representative subtypes from the database and were analyzed phylogenetically. On the basis of this comparison the subtypes of the specimens were clearly apparent. The specimens false negative by the Amplicor assay were subtypes A ($n = 11$), B ($n = 7$), C ($n = 7$), D ($n = 5$), and G ($n = 1$). The control Amplicor-positive specimens were similarly distributed, being subtypes A ($n = 7$), B ($n = 6$), C ($n = 5$), D ($n = 7$), and G ($n = 1$).

DISCUSSION

In this study of the diagnosis of HIV infection by PCR, the Roche Amplicor assay was found to be easy to use. It allowed results to be generated within a day of receiving the blood sample. No false-positive results were observed, as judged by serological findings and clinical information. However, the Amplicor assay failed to detect HIV DNA in 18 of 286 specimens from seropositive infected patients 2 years of age or older, giving a sensitivity of 93.7%. HIV-1 DNA was not detected in a further 19 specimens from infants and young children with evidence of HIV infection. Sixteen of these 19 specimens were positive in the in-house PCR. The sensitivity of the Amplicor assay with specimens from infants was calculated by age group. For 43 specimens taken from birth to 4 weeks of age, only 1 was Amplicor positive. One of 42 negative specimens contained detectable proviral DNA by the in-house PCR, and another 3 were shown to be HIV-1 infected by serological criteria, thus giving a sensitivity estimate of 20% (1 of 5) for the Amplicor assay in this age group. Of 36 specimens taken at 1 to 2 months of age, six were Amplicor positive and a further four were in-house PCR positive, giving a sensitivity estimate of 60% (6 of 10). Of 70 specimens taken at 3 to 6 months of age, 20 were Amplicor positive and a further 2 were in-house PCR positive, giving a sensitivity estimate of 91% (20 of 22). Of 117 specimens taken at 6 to 24 months of age, 21 were Amplicor positive and a further 9 were in-house PCR positive, giving a sensitivity of 70% (21 of 30). Overall, of the 266 specimens taken from infants up to 2 years of age, 48 were Amplicor positive and a further 16 were in-house PCR positive, giving an overall sensitivity of 75% (48 of 64) for the Amplicor assay. If the three early specimens negative by both the Amplicor and the in-house assays are also considered, the overall sensitivity of Amplicor was 72% (48 of 67).

Although only the specimens false negative by the Amplicor assay and some controls were investigated by the in-house nested PCR, it can be assumed to be more sensitive than the Amplicor assay, because it amplified 31 of the 39 specimens false negative by the Amplicor assay. Sequence data from all specimens positive by the in-house PCR confirmed that in no instance was positivity due to contamination. Of the eight specimens for which no amplification of HIV-1 DNA was possible, one was from a patient of unknown age, four were from adults, and the other three were from infants ages 4, 5, and 13 days. The possibility that later transmission occurred in the infants, perhaps through breast-feeding, cannot be excluded,

TABLE 2. Age, subtype, optical density, and markers of infection of the false-negative and control specimens from patients 2 years of age or younger

Reference no.	Specimen type[a]	Age	Amplicor result or OD[b]	Subtype	Markers of infection			
					p24	IgA	IgM	IgG
1	FN	Neonate	NA[c]	G	NA	NA	NA	NA
	C	Adult, NK[d]	NA	G	NA	NA	NA	NA
2	FN	4 days	N, N	NK	−	+	+	+
	FN	2.5 mo	N, N	A	−	−	−	+
	C	7 mo	1.684, 1.056	A	−	+	+	+
	C	16 mo	P, P	A	−	+	+	+
3	FN	5 days	0.112, N	NK	−	+	+	+
	FN	2.5 mo	N, N	D	−	−	+	+
	C	6 mo	P, P	D	−	+	+	+
	FN	9 mo	N, N	D	+	+	+	+
	C	16 mo	P, P	ND[e]	ND	+	−	+
4	FN	13 days	N, N	NK	−	−	−	+
	C	2/4/11 mo	All P, P	ND	−/+/+	−/−/+	−/−/−	+
5	FN	17 days	N, N	A	ND	+	+	+
	C	4 mo	P, P	A	+	+	−	+
6	Equiv	6 wk	1.117, N, N	C	ND	−	−	+
	C	4 mo	P, 0.364	C	−	−	−	+
	Equiv	5 mo	P, N	C	−	−	−	+
	FN	10 mo	0.111, 0.222	C	ND	+	−	+
7	FN	2 mo	N, N	C	+	−	−	+
	C	3.5 mo	P, P	C	+	−	−	+
8	FN	3 mo	N, N, 0.215	A	+	−	−	+
9	C	4 mo	P, P	A	ND	+	+	+
10	C	2 mo	1.048, 0.764	B	+	−	−	+
	C	5 mo	0.867, 0.612	B	+	−	−	+
11	FN	5 mo	N, N	A	+	−	−	+
12	C	6 mo	P, P	D	−	−	−	+
	C	13 mo	0.717, 0.524	D	−	+	−	+
	C	15/17 mo	P, P/P, P	D	−	+/+	−/−	+
13	FN	7.5 mo	N, N	D	+	+	+	+
14	C	9 mo	P, P	A	ND	+	+	+
15	C	9.5 mo	P, P	C	+	+	+−	+
16	FN	10 mo	N, N	A	ND	+	+	+
	FN	13 mo	N, N	A	−	+	+	+
	FN	16 mo	NA	A	NA	NA	NA	NA
17	FN	13 mo	0.158, 0.130	C	+	+	+	+
18	C	8 mo	1.266, 0.765	ND	+	−	−	+
	FN	13 mo	0.226, 0.125	D	ND	−	−	−
	C	14 mo	P, P	D	+	−	−	+
19	FN	10 mo	N, N	C	+	+	+	+
	C	10 mo	ND	C	ND	−	−	+
20	FN	19 mo	N, N	C	+	+	+	+

[a] FN, false negative; C, control; Equiv, equivocal; specimens from the same patient or from linked infections (e.g., mother and child) are grouped.
[b] OD, optical density at 450 nm. The exact value is given if it is between 0.1 and 2; N, OD value of <0.1; P, OD value of >2.0 (the assay cutoff was 0.35).
[c] NA, not available.
[d] NK, not known; no amplification in in-house PCR.
[e] ND, not done.

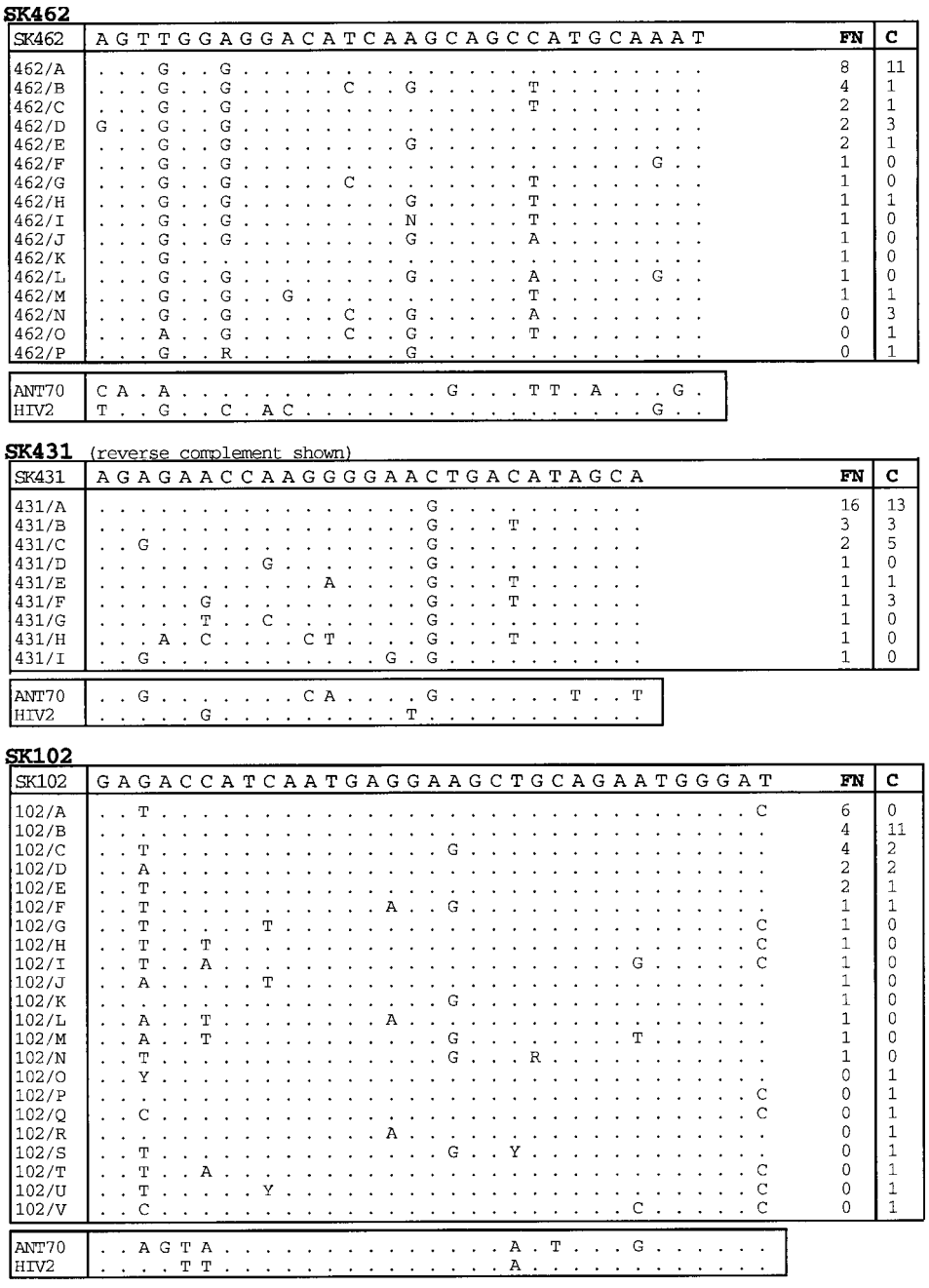

FIG. 1. Sequence variation in the primer and probe regions. FN indicates the number of false-negative specimens with each pattern; C indicates the number of control specimens with each pattern; multiple specimens with false-negative results by the Amplicor assay or control specimens from the same patient have identical mismatches and are included as single datum points. Representative sequences of subtype O (strain ANT70) and HIV-2 (strain ROD) are also shown for comparison.

even though most mother-child transmission occurs perinatally. In some perinatal transmissions virus replication does not reach detectable levels during the first month after birth (12).

Several other groups have reported on the performance of the Amplicor assay. Poussin et al. (27) evaluated the assay with 122 anti-HIV-seropositive subjects and 44 seronegative blood specimens. They reported a sensitivity of 99.1% and a specificity of 100%. Fransen et al. (14) tested both blood processed with the kit reagents and stored specimens prepared by a Ficoll-Hypaque separation protocol and found a sensitivity of 93.2% and a specificity of 100%. Khadir et al. (19) analyzed a panel of 208 well-characterized specimens and found a sensitivity of 98.7% and a specificity of 100%. However, they prepared the cell pellets by Ficoll-Hypaque centrifugation, so their results are not directly comparable to those obtained with the Amplicor kit in which the Amplicor kit reagents were used to prepare the cell pellets. Marin et al. (23) evaluated 401 specimens and reported a 100% correlation of Amplicor PCR and serology but found 1 among 138 seropositive specimens that was only just reactive in the Amplicor assay. The results of Kovacs et al. (20) and a review by Dunn et al. (12) suggest that slightly more than one-third of neonatal HIV-1 infections

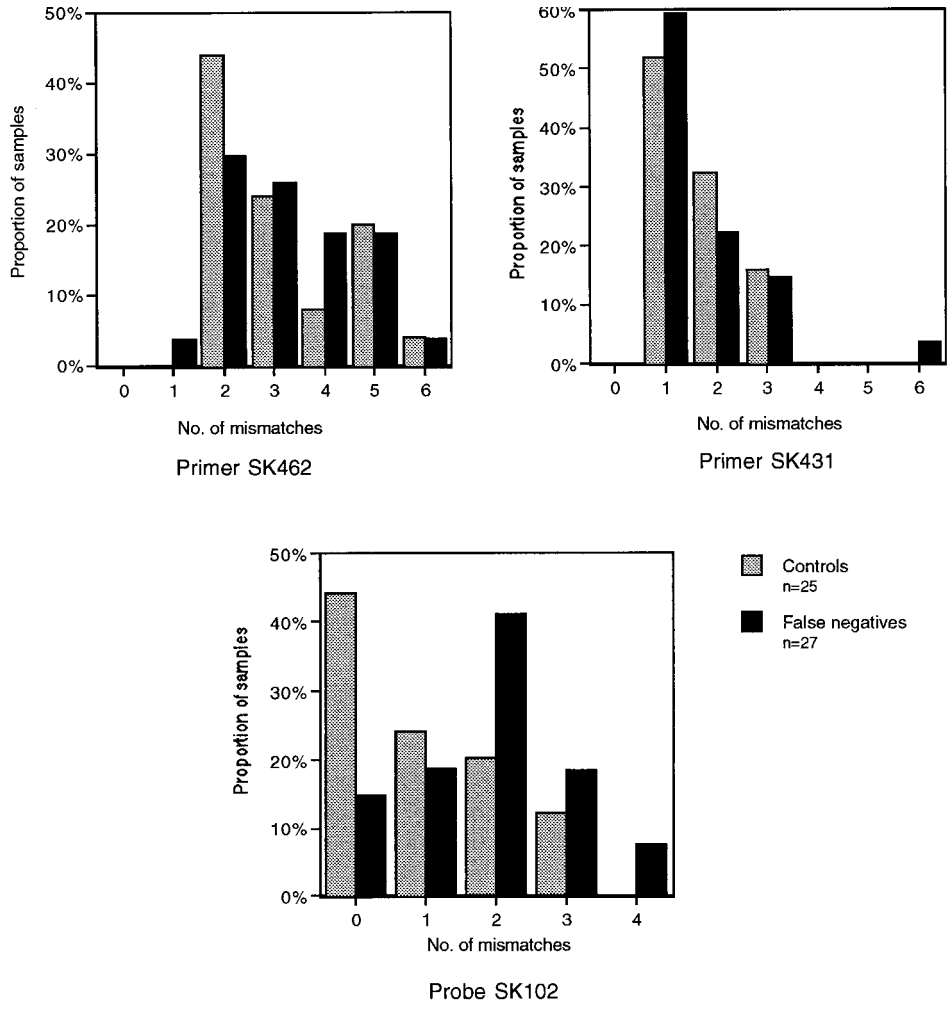

FIG. 2. Number of base mismatches in the primer and probe regions as a proportion of the number of specimens. Multiple samples with false-negative results by the Amplicor assay or control samples from the same patient have identical mismatches and are included as single datum points.

could be detected by PCR 0 to 2 days after birth, rising to two-thirds at 2 weeks and almost all of them by 1 month. However, our results (detailed above) suggest that most infections (90%) can be detected by 3 to 6 months of age rather than 1 month of age (sensitivity at 1 to 2 months, 40%).

False-negative PCR results can be due to one or a combination of the following: (i) the presence of inhibitors of *Taq* polymerase or other components of the reaction (2, 11, 14, 19), (ii) a low number of copies of the starting target sequence (3, 14, 17, 23, 31), or (iii) sequence variability in the amplicon primer and probe region (7, 14, 22, 27). Resolution of the causes of false-negative PCR results is not straightforward. For instance, for some patients (e.g., patient 6 in Table 2) both positive and negative results were obtained in duplicate tests with the same specimen. This may be due to sampling bias as a result of low proviral DNA copy number. The copy number can be investigated by quantitative DNA PCR, but this is also prone to error (13). Amplification of housekeeping genes can help rule out the presence of inhibitors of PCR. Viral coculture can also be used to overcome the problem of the presence of PCR inhibitors in a specimen, but the extent to which infected cells (i.e., those containing proviral DNA) contribute to the culture product compared with the extent to which free virus contributes to the culture product may not be clear. Chemical extraction of DNA may overcome the problem of inhibitors and is likely to include a concentration step (e.g., ethanol precipitation) which may also resolve the problem of low proviral DNA load. However, the approach to investigating the false-negative results by the Amplicor assay that we have adopted is to design nested PCR primers flanking those in the Amplicor kit, amplify the product from purified and concentrated DNA, and sequence the region of p24 containing the primer (SK431 and SK462) and probe (SK102) sequences of the Amplicor target (3). Thirty-one of the 39 false-negative specimens could be amplified and the sequences of these and of 33 controls were compared. There were no significant sequence differences between the false-negative specimens and the controls in the primer SK462 ($P = 0.74$), SK431 ($P = 0.73$), or probe SK102 ($P = 0.09$) region. Seven specimens that were negative both by the Amplicor assay and by the in-house PCR were positive with actin housekeeping primers, suggesting the absence of nonspecific inhibitors. Presumably, these specimens had either a very low amount of proviral DNA or virus with a very diverse genomic sequence.

Sequence analysis allows for the identification of the mismatches between the DNA target in the specimen and the primers in the kit (Fig. 1 and 2). These data may explain why certain primer-template combinations allow amplification and

TABLE 3. Properties of actual, ideal, and perfect template-primer combinations

Primer or product, analysis, and specimen	ΔG_0 (kcal/mol)	T_m (°C)	T_a (°C)	Pro-Pri T_m (°C)[a]	G+C (%)
SK462					
Actual[b]					
C[c]	−48.24 (±4.39)	56.5[d]			
FN[e]	−49.98 (±5.04)	56.5[d]			
Ideal[f]					
C	−62.62 (±1.11)	72.20 (±1.50)			
FN	−61.38 (±1.25)	71.97 (±1.53)			
Perfect[g]	−58	68.4			
SK431					
Actual					
C	−44.81 (±2.86)	54.6			
FN	−44.92 (±2.69)	54.6			
Ideal					
C	−49.06 (±0.92)	60.38 (±1.70)			
FN	−49.24 (±0.89)	60.50 (±1.64)			
Perfect	−49.5	61.3			
Product					
Actual					
C		75.69 (±0.60)[h]	54.46 (±0.45)	21.06 (±0.61)	50.14 (±1.46)
FN		75.53 (±5.04)[h]	54.33 (±0.32)	20.89 (±0.45)	49.73 (±1.09)
Ideal					
C			54.04 (0.92)	15.29 (±1.62)	
FN			56.10 (±0.52)	14.97 (1.82)	
Perfect		74.8[h]	55.8	13.5	47.9

[a] Pro-Pri T_m, The optimal T_a between the primers and the product.
[b] Calculated with the observed mismatches between the primer and the template.
[c] C, control sequences (both primers: actual, $n = 20$; ideal, $n = 18$).
[d] The T_m of the actual primer sequence, irrespective of the target.
[e] FN, false negative sequences (SK462 actual and ideal: $n = 26$; SK431: actual, $n = 26$; ideal, $n = 25$).
[f] Calculated with the primer sequence changed to complement the target sequence exactly.
[g] Calculated for an example target (SF2) changed to complement the primer sequences exactly.
[h] The T_m of the product.

others do not. To investigate this we sought computer algorithms that would predict the T_a and ΔG_0 of the primers and their corresponding mismatched templates. However, most primer analysis programs have been written to predict primers or analyze the behavior of existing perfectly matched ones, and PrimerSelect was the only program which met our needs. Moreover, because of limitations in the program code, it could not accept mismatches positioned within five bases of the 5′ end, and this precluded analysis of the false-negative subtype G specimen with six mismatches in the target region of primer SK431. For the specimens which could be analyzed with PrimerSelect it was apparent that the behavior of SK462 in the amplification conditions used was not optimal (Table 3). The manufacturer's recommended thermal cycling conditions of five cycles with a T_a of 55°C followed by 30 cycles at 60°C were used in this study. Thus, the T_a of 60°C may have been too high for efficient amplification of all specimens with SK462, for which the optimal T_a was calculated to be 54 to 55°C. Since this work was completed the manufacturer has recommended lowering the T_a to 50°C. Therefore, the physical behavior of SK462 may have contributed to the false-negative Amplicor results. Since the diagnoses of the infections in the patients involved in this study were confirmed by the in-house PCR, they were not retested by using the manufacturer's revised protocol.

On the basis of the sequence data obtained in this study, the Amplicor assay can detect *gag* subtypes A, B, C, D, and G, although one of the two subtype G viruses sequenced was more diverse and failed to be amplified. We found no evidence to support a link between subtype A and failure of the assay to detect HIV-1 infections, as proposed by Loussert-Ajaka et al. (22). Previous studies also reported amplification of subtype H, but failed to amplify the diverse subtype O and HIV-2 (14, 22). The failure to amplify subtype O can be predicted from comparison of the primers and subtype O *gag* sequences (Fig. 2) (9). There is less divergence between the primer sequences and HIV-2 (Fig. 2). During the course of this study two specimens from patients infected with HIV-2 were assayed by Amplicor: one was negative and the other gave an equivocal result. When tested in duplicate, one sample gave a marginal negative result and the other gave a low-positive result. Subsequent to the completion of this work a further HIV-2 specimen was examined and was found to be weakly positive by Amplicor.

The variability of the HIV-1 genome is likely to impair the sensitivity of the Amplicor assay, which is a single-round PCR.

For the different subtypes sequenced there were between zero and six mismatches in each of the Amplicor primer and probe regions, and this can be expected to affect the sensitivity of the assay, especially when the proviral DNA copy number may be low, as, for example, in some HIV-infected infants.

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