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Sensitive detection of genetic variants of HIV-1 and HCV with an HIV-1/HCV assay based on transcription-mediated amplification

Jeffrey M. Linnen *, J. Michael Gilker, Alanna Menez, Alyshia Vaughn, Amy Broulik, Janel Dockter, Kristin Gillotte-Taylor, Kim Greenbaum, Daniel P. Kolk, Larry T. Mimms, Cristina Giachetti

Gen-Probe Incorporated, Research and Development, 10210 Genetic Center Drive, San Diego, CA 92121-4362, USA

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Abstract

This paper describes a comprehensive study of hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) genotype sensitivity of the transcription-mediated amplification (TMA)-based HIV-1/HCV assay, developed and manufactured by Gen-Probe Incorporated (San Diego, CA) for screening human plasma specimens in blood bank settings. The TMA HIV-1/HCV assay is a qualitative, in vitro nucleic acid testing system used for initial screening. HIV-1 and HCV discriminatory assays are used to distinguish between HIV-1 and HCV infection or co-infection. In this study, multiple unique specimens representing HCV genotypes 1–6 were tested at various dilutions. The results show that the TMA HIV-1/HCV assay and the TMA HCV discriminatory assay have similar HCV genotype sensitivity, as both assays detected all six genotypes at 100 copies/ml and nearly all replicates tested at 30 copies/ml. Similarly, numerous unique specimens representing HIV-1 group M subtypes (A–G), HIV-1 group N, and group O specimens were tested at various dilutions. The TMA HIV-1/HCV assay and the TMA HIV-1 discriminatory assay were found to have similar HIV-1 subtype sensitivity; all variants at 100 copies/ml and nearly all at 30 copies/ml were detected. These results indicate that the TMA assays meet the sensitivity requirements for blood screening in blood banks worldwide. © 2002 Published by Elsevier Science B.V.

Keywords: HIV-1; HCV; Nucleic acid testing; Transcription-mediated amplification; Genotype; Sensitivity

1. Introduction

Blood centers use many approaches to prevent transfusion–transmitted infections of human immunodeficiency virus, type 1 (HIV-1) and hepatitis C virus (HCV). Screening of the blood supply has been carried out for the most part with

* Corresponding author. Tel.: +1-858-410-8754; fax: +1-858-410-8871.

E-mail address: jeffl@gen-probe.com (J.M. Linnen).

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methodology relying on the detection of HIV-1 p24 antigen and antibodies to HCV or HIV-1 antigen (Allain, 2000a). Although these serological methods of viral detection have reduced significantly the incidence of post-transfusion infection, HCV- and/or HIV-1 positive donations provided prior to seroconversion cannot be reliably identified. Direct viral detection of HCV and/or HIV-1 by nucleic acid amplification testing is predicted to reduce the pre-seroconversion window period for these viruses and subsequently decrease the risk of transfusion–transmitted infections (Busch and Kleinman, 2000; Allain, 2000b; Gallarda and Dragon, 2000; Kolk et al., 2002).

The large genetic heterogeneity among HCV and HIV-1 isolates creates a challenge for the development of sensitive methods for nucleic acid testing. Sequencing of HCV genomes from around the world has revealed considerable genetic diversity, and has led to the classification of HCV isolates into six major genotypes, referred to as genotypes 1 through 6 (Simmonds et al., 1994). HCV genotypes are further classified into subtypes (e.g. 1a, 1b, 1c, etc.) based on intra-genotype variation. Similarly, HIV-1 has a worldwide distribution and sequence analysis of virus isolates from different geographical locations has allowed classification of HIV-1 into three major groups: M, N and O. Within the HIV-1 group M family of viruses, distinct subtypes designated A through K have been identified. Subtype E is now referred to as CRF01_AE (circulating recombinant form), and the subtype I designation has been dropped based on sequence data showing that subtype I genomes are actually mosaics of subtypes A, G, K, and H (Robertson et al., 1999). The TMA assays target the conserved 5′ non-coding region of HCV and conserved regions in the LTR and pol regions of HIV-1. Although nucleic acid testing procedures for HCV and HIV-1 virus detection typically target the most conserved regions of the genome, considerable effort must still be made to design assays capable of reliably detecting all genetic variants of HCV and HIV-1 with equivalent sensitivity. In this paper, a comprehensive study is described of the analytical sensitivity of the transcription-mediated amplification (TMA)-based assays for the detection of HIV-1 and HCV genetic variants.

The TMA HIV-1/HCV assay is a qualitative, in vitro nucleic acid assay system, developed by Gen-Probe Incorporated (San Diego, CA), for the simultaneous detection of HIV-1 and HCV in human plasma (Giachetti et al., 2002). In initial screening with the TMA HIV-1/HCV assay, a positive result cannot distinguish between the presence of HIV-1, HCV or both viruses, therefore, discriminatory assays for HIV-1 and HCV are needed to identify the virus present. The TMA HCV discriminatory assay and the TMA HIV-1 discriminatory assay are based on chemistry that is identical to the TMA HIV-1/HCV assay except for the specificity of the labeled probes. In addition to its use in blood banks, the discriminatory versions of the assay will be useful in clinical diagnostic labs for sensitive diagnosis of HIV-1 and/or HCV. The TMA HIV-1/HCV and HIV-1 and HCV discriminatory assays are currently used in blood banks in the United States under an Investigative New Drug (IND) approval and the clinical trials for blood screening applications have been completed.

The TMA assays have three main steps which take place in a single tube: (1) sample preparation; (2) RNA target amplification by TMA; and (3) detection of the amplification products with chemiluminescent probes by the hybridization protection assay (HPA) (Giachetti et al., 2002). During sample preparation, viral RNA is isolated from plasma and separated from potentially inhibitory substances by target capture onto paramagnetic microparticles. Nucleic acid amplification of the viral target occurs via TMA, a transcription-based technology that uses two enzymes, MMLV reverse transcriptase and T7 RNA polymerase (Kacian and Fultz, 1995). Reverse transcriptase creates a DNA copy of the viral target, in which a promoter sequence for T7 polymerase is incorporated. T7 polymerase then catalyzes the production of multiple RNA transcripts from the DNA copy template. Unlike the polymerase chain reaction (PCR), TMA is an isothermal process and therefore does not require temperature cycling. Finally, detection of amplicon is achieved using single-stranded nucleic acid probes that contain chemiluminescent acridinium ester labels (Arnold et al., 1989; Majlessi et al.,

1998). Incubation at 60 °C under alkaline conditions inactivates unhybridized probe, allowing the chemiluminescent signal from the hybridized probe to be measured in a luminometer.

For successful use worldwide, nucleic acid testing must be capable of sensitive detection of all genetic variants of HCV and HIV-1, preferably with equivalent sensitivity. To determine the analytical sensitivity of the assays for detection of genetic variants, we tested multiple examples of each of six HCV genotypes, seven HIV-1 group M subtypes as well as HIV-1 group N and O specimens diluted to 1000, 300, 100, and 30 copies/ml. The studies reported here utilized three separate kit lots of the TMA HIV-1/HCV, HCV discriminatory, and HIV-1 discriminatory assays, two of which were used for the clinical trial study submitted to the U.S. FDA for licensure.

2. Materials and methods

2.1. Viral panels

For preparation of the HCV genotype panel, specimens from patients infected with different HCV genotypes were obtained from Boston Biomedica, Inc. (BBI; West Bridgewater, MA), Intergen Company (Purchase, NY), Millennium Biotech, Inc. (Ft. Lauderdale, FL), ProMedDx LLC (Norton, MA), Dr Ching-Lung Lai (University of Hong Kong, Hong Kong, China), Dr Angelos Hatzakis (University of Athens School of Medicine, Athens, Greece) and Dr Michael Kew (University of the Witwatersrand, Johannesburg, South Africa). The specimens tested are described in Table 1a. The specimens were serotyped using the Murex HCV Serotyping 1–6 Assay or genotyped using the Innogenetics INNO-LiPA HCV II Assay. The viral titers of the specimens were based on value assignments obtained from the vendor's certificates of analysis, which included test results from ROCHE Amplicor (v. 2.0) or Bayer Quantiplex bDNA (v. 2.0). Additional bDNA testing for some samples was performed by Quest Diagnostics (San Juan Capistrano, CA). Other samples were assigned values according to SuperQuant PCR performed by the National Genetics Institute (NGI, Los Angeles, CA).

Specimens from patients infected with different HIV-1 subtypes were obtained from Boston Biomedica (West Bridgewater, MA). Viral samples from Boston Biomedica were genotyped by the vendor based on sequencing in the pol, gag, and env genomic regions. Dilutions of infected tissue culture supernatants of HIV-1 isolates were prepared in-house from samples obtained from the National Institutes of Health's AIDS Research and Reference Reagent Program. Specific information regarding the genotyping of the samples obtained from the AIDS Research and Reference Reagent Program can be found at the web site www.aidsreagent.org. The specimens tested are described in Table 1b. The panel members were made by serial dilution of patient plasma samples or tissue culture supernatants using HIV-1 and HCV RNA negative human serum. The concentrations of HIV-1 Group M (genotypes A through G) plasmas used to make the panels were determined by value assignments obtained from the vendor's Certificates of Analysis, ROCHE Amplicor (ver. 2.0) or Bayer Quantiplex bDNA (ver 2.0) quantitative testing performed by Quest Diagnostics (San Juan Capistrano, CA), SuperQuant quantitative PCR performed by National Genetics Institute (Los Angeles, CA), or an in-house, TMA-based HIV quantitative assay (Gen-Probe quantitative assay). HIV-1 groups N and O plasma specimens were quantitated with the Gen-Probe quantitative assay.

The panel members were made by serial dilution of the patient plasma samples using human serum that was pre-tested and found to be negative for HIV-1 and HCV RNA. The in-house genotype panel members were created at concentrations of 1000, 300, 100 and 30 copies/ml. For specimens in which the initial titer or sample volume was insufficient to create full panels, subsets of the panels were prepared that included at least one panel member at 100 copies/ml. The panel members were stored at or below −65 °C in single use aliquots and were thawed at room temperature immediately prior to testing.

Table 1
Specimen description

	Specimen lot #	Country of origin	Quantitation method
(a) HCV type			
1	E8-1205-0010	USA	ROCHE Amplicor
1	E8-1205-0100	USA	ROCHE Amplicor
1	E8-1205-0090	USA	ROCHE Amplicor
1	E8-1205-0144	USA	ROCHE Amplicor
1	Wt3	USA	ROCHE Amplicor
1a	9992160/169293	USA	Bayer Quantiplex bDNA
1b	9810030/169282	USA	Bayer Quantiplex bDNA
1b	9810049/169283	USA	Bayer Quantiplex bDNA
1b	9990992/169286	USA	Bayer Quantiplex bDNA
1b	9991145/169287	USA	Bayer Quantiplex bDNA
2	JE6-3107-0008^{+}	China	ROCHE Amplicor
2b	9991162/169289	USA	Bayer Quantiplex bDNA
2b	9991164/169290	USA	Bayer Quantiplex bDNA
2b	9992154/169291	USA	Bayer Quantiplex bDNA
2b	9992158/169292	USA	Bayer Quantiplex bDNA
2b	21775	USA	ROCHE Amplicor
2b	22715	USA	Super Quant
2b	22716	USA	Super Quant
2b	21780	USA	Super Quant
2b	21786	USA	Super Quant
2b	23835	USA	ROCHE Amplicor
2b	9810038	USA	ROCHE Amplicor
2a/c	5889	USA	ROCHE Amplicor
2a/c	9810067/169284	USA	Bayer Quantiplex bDNA
3	KJ9-1102-0033	Thailand	ROCHE Amplicor
3	KJ9-1102-0002	Thailand	ROCHE Amplicor
3	KJ9-1102-0007	Thailand	ROCHE Amplicor
3	KJ9-1102-0025	Thailand	ROCHE Amplicor
3	KJ9-1102-0005	Thailand	ROCHE Amplicor
3	KJ9-1102-0014	Thailand	ROCHE Amplicor
3a	KJ9-1102-0028	Thailand	ROCHE Amplicor
3a	9990981/169285	USA	Bayer Quantiplex bDNA
3a	9991151/169288	USA	Bayer Quantiplex bDNA
3e	9990998	USA	Bayer Quantiplex bDNA
3e	11950	USA	Bayer Quantiplex bDNA
4	IH6-2606-0002^{+}	Uganda	ROCHE Amplicor
4	CT7-1504-0003	Egypt	ROCHE Amplicor
4	21776	Egypt	ROCHE Amplicor
4	22717	Egypt	Super Quant
4	22718	Egypt	Super Quant
4	39377	Greece	Bayer Quantiplex bDNA
4	38111	Greece	Bayer Quantiplex bDNA
4a	22848	Egypt	Super Quant
4a	9991692/169294	Egypt	Super Quant
4b/c	22719	USA	Super Quant
4b/c	21779	USA	Super Quant
4b/c	11951	USA	Bayer Quantiplex bDNA
5	C2-JD8081-161	South Africa	Super Quant
5	C4-JD8081-161	South Africa	Super Quant
5	C6-JD8081-161	South Africa	Super Quant
5	C42-JD8081-161	South Africa	Super Quant

Table 1 (*Continued*)

	Specimen lot #	Country of origin	Quantitation method
5	C40	South Africa	Super Quant
5a	21777	South Africa	ROCHE Amplicor
5a	22849	South Africa	Super Quant
5a	10180	South Africa	ROCHE Amplicor
6	720079/7492	China	Super Quant
6	720080/7502	China	Super Quant
6	720081/7440	China	Super Quant
6	720082/7514	China	Super Quant
6a	10027618/207457	Vietnam	Super Quant
6a	20759	China	ROCHE Amplicor
HIV-1 type			
A	JS8-1002-0023*	Cote d'Ivoire	Bayer Quantiplex bDNA
A	JS8-1002-0025*	Cote d'Ivoire	Bayer Quantiplex bDNA
A	JS8-1002-0027*	Cote d'Ivoire	GP Quant Assay
A	JS8-1002-0028*	Cote d'Ivoire	Bayer Quantiplex bDNA
A	GM7-0312-0004*	Ghana	Bayer Quantiplex bDNA
A	GM7-0312-0005*	Ghana	Bayer Quantiplex bDNA
A	GM7-0312-0010*	Ghana	Bayer Quantiplex bDNA
A	GM7-0312-0011*	Ghana	Bayer Quantiplex bDNA
A	GM7-1811-0008*	Ghana	Bayer Quantiplex bDNA
A	92UG029**	Uganda	GP Quant Assay
A	92RW025**	Rwanda	GP Quant Assay
A	92UG037**	Uganda	GP Quant Assay
B	ER8-1404-0031*	USA	ROCHE Amplicor
B	JG8-0501-0217*	USA	ROCHE Amplicor
B	JG8-0501-0223*	USA	ROCHE Amplicor
B	E7-2909-0167^{++}*	USA	ROCHE Amplicor
B	E8-1901-0117*	USA	ROCHE Amplicor
B	E9-1901-0124*	USA	ROCHE Amplicor
B	E8-1702-0125*	USA	ROCHE Amplicor
B	E8-1404-0030*	USA	ROCHE Amplicor
B	E8-2306-0060*	USA	ROCHE Amplicor
B	E8-1702-0127^{++}*	USA	Bayer Quantiplex bDNA
B	92BR014**	Brazil	GP Quant Assay
C	IM6-0304-0007*	Zimbabwe	Bayer Quantiplex bDNA
C	IM6-0304-0010*	Zimbabwe	Bayer Quantiplex bDNA
C	IM6-0304-0011*	Zimbabwe	Bayer Quantiplex bDNA
C	IM6-0304-0012*	Zimbabwe	Bayer Quantiplex bDNA
C	IM6-0304-0014*	Zimbabwe	ROCHE Amplicor
C	IM6-0304-0015*	Zimbabwe	Bayer Quantiplex bDNA
C	CQ8-1702-0002*	Mozambique	Bayer Quantiplex bDNA
C	CQ8-1702-0005*	Mozambique	Bayer Quantiplex bDNA
C	CQ8-1702-0008*	Mozambique	Bayer Quantiplex bDNA
C	CQ8-1702-0009*	Mozambique	Bayer Quantiplex bDNA
D	IH6-2606-0008*	Uganda	Bayer Quantiplex bDNA
D	IH6-2606-0010*	Uganda	Bayer Quantiplex bDNA
D	IH6-2606-0012^{++}*	Uganda	Bayer Quantiplex bDNA
D	IH6-2606-0014*	Uganda	Bayer Quantiplex bDNA
D	IH6-2606-0016^{++}*	Uganda	Bayer Quantiplex bDNA
D	92UG021**	Uganda	GP Quant Assay
D	92UG001**	Uganda	GP Quant Assay
D	92UG046**	Uganda	GP Quant Assay
D	93UG065**	Uganda	GP Quant Assay

Table 1 (*Continued*)

	Specimen lot #	Country of origin	Quantitation method
D	93UG070**	Uganda	GP Quant Assay
E***	GM6-2511-0001*	Ghana	Bayer Quantiplex bDNA
E***	GM6-2511-0006*	Ghana	Bayer Quantiplex bDNA
E***	JE6-3107-0012^{+}$^{+}$*	China	Bayer Quantiplex bDNA
E***	BR4-1310-0005*	Thailand	Bayer Quantiplex bDNA
E***	92TH005**	Thailand	GP Quant Assay
E***	93TH069A**	Thailand	GP Quant Assay
E***	93TH072**	Thailand	GP Quant Assay
E***	93TH073**	Thailand	GP Quant Assay
E***	CMU10**	Thailand	GP Quant Assay
F	JE6-3107-0009*	China	Bayer Quantiplex bDNA
F	JE6-3107-0004^{+}$^{+}$*	China	Bayer Quantiplex bDNA
F	BZ6-1511-0002*	Argentina	Bayer Quantiplex bDNA
F	93BR020**	Brazil	GP Quant Assay
F	93BR029**	Brazil	GP Quant Assay
F	93BR019**	Brazil	GP Quant Assay
G	GM7-0312-0002*	Ghana	Bayer Quantiplex bDNA
G	GM7-1109-0003*	Ghana	Bayer Quantiplex bDNA
G	JS8-1002-0022*	Cote d'Ivoire	Bayer Quantiplex bDNA
Group N	CBER-IF**	Cameroon	GP Quant Assay
Group O	B7-2705-0001*	USA	GP Quant Assay
Group O	JP8-2707-0001*	Spain	GP Quant Assay
Group O	BCF01**	Cameroon	GP Quant Assay
Group O	BCF02**	Cameroon	GP Quant Assay
Group O	BCF03**	Cameroon	GP Quant Assay
Group O	CA9 (W)**	N/A	GP Quant Assay
Group O	V1686**	N/A	GP Quant Assay
Group O	MVP5180**	Cameroon	GP Quant Assay
Group O	2899P**	USA	GP Quant Assay

$^{+}$Co-infected with HIV-1; Specimen origin: *Patient specimen; **Supernatant of tissue culture specimen; $^{+}$$^{+}$Co-infected with HCV; ***Now referred to as CRF01_AE (circulating recombinant form).

2.2. Assay run calibrators and cutoff determination

Three negative calibrators, three HIV-1 positive calibrators, and three HCV positive calibrators were included in each assay run which consists of a maximum of 100 reactions. The negative calibrator is defibrinated normal human plasma, the HIV-1 positive calibrator consists of heat-inactivated, HIV-1 positive plasma in defibrinated normal human plasma, and the HCV positive calibrator consists of heat-inactivated HCV positive plasma in defibrinated normal human plasma. The results obtained from these calibrators were used to determine the validity of the run and to establish the run cutoffs (Giachetti et al., 2002). As an additional control for specificity, 2–10 additional negative specimens were included in each assay run ($N = 398$ negative control samples for the entire study).

2.3. Specimen processing

For this study, 400 μl of target capture reagent (HEPES-buffered detergent solution containing capture oligonucleotides and magnetic particles) containing internal control and 500 μl of each specimen or panel member were pipetted manually into reaction tubes. In most blood bank laboratories, this step is carried out using a Tecan Genesis RSP 150/8 automated pipetting system. After addition of target capture reagent and specimen, the reaction tubes were vortexed and incubated in a 60 °C water bath for 20 min, followed

by room temperature incubation (15–30 °C) for 14–20 min. The rack of tubes was then placed in the magnetic separation bay for 9–20 min, after which the liquid was aspirated from each tube and 1 ml of wash solution was added. After vortexing, the rack was again placed on the separation bay for 4–10 min. The wash solution was aspirated from each tube and the wash and separation steps were repeated, finishing with a final aspiration.

2.4. Amplification

After the final aspiration step, 75 μl of amplification reagent (Primers, dNTPs, NTPs and cofactors in Tris-buffered solution) was added to each tube. To prevent evaporation during the amplification step, 200 μl of oil was added to each tube and the rack of tubes was vortexed to resuspend the microparticles. Prior to addition of 25 μl of enzyme reagent (MMLV reverse transcriptase and T7 RNA polymerase in HEPES/TRIS buffered solution), the rack was incubated in a water bath at 60 °C for 10 min, followed by equilibration at 41.5 °C for 9–20 min. Immediately after adding the enzyme reagent, the rack of tubes was quickly removed from the incubator and shaken to mix. The rack was then incubated in the water bath at 41.5 °C for 60 min.

2.5. Detection

After amplification, 100 μl of probe reagent (acridinium ester labeled oligonucleotide probes in succinate buffered detergent solution) was added to each tube, vortexed, and incubated in a water bath at 60 °C for 15 min. Following the completion of the probe hybridization step, 250 μl of selection reagent (borate-buffered solution with surfactant) was added to each tube, vortexed, and then incubated at 60 °C for 10 min. After removal from the 60 °C water bath, the rack of tubes was cooled in a water bath at 19–27 °C for 10–75 min and then placed in a HC+ Luminometer which performs automatic injection of 200 μl of Auto Detect 1 (0.1% hydrogen peroxide, 1 mM nitric acid) and 200 μl of Auto Detect 2 (1 N NaOH). To determine reactivity, the resulting chemiluminescence, measured in relative light units (RLU), was compared to a cutoff value generated from the positive and negative calibrators included in each 100 tube run using the Chiron Procleix System Software. A sample with an analyte signal (RLU)/analyte cutoff (S/CO) ratio ≥ 1 was considered reactive.

2.6. Internal control

To monitor assay performance for each specimen reaction, an internal control contained in the target capture reagent is added to each test specimen and assay calibrator reaction. The internal control consists of an in vitro synthesized transcript containing a portion of HIV-1 and a unique sequence targeted by the internal control probe. The internal control signal in each reaction was discriminated from the HIV-1 and/or HCV signal by the differential kinetics of light emission from probes with different labels (Nelson et al., 1996). The internal control amplification product was detected using a probe with rapid emission of light while the amplicon specific to HIV-1/HCV was detected using probes with slower kinetics of light emission; the Procleix software differentiates between these two signals. For a reaction with an analyte signal less than the analyte cutoff (i.e. analyte $S/CO < 1$), the internal control signal must be equal to or greater than the internal control cutoff for the result to be valid and non-reactive. For a sample with an analyte signal less than the analyte cutoff and internal control less than the internal control cutoff, the result is reported as invalid. If the analyte signal is above the cutoff, the internal control result is not used and the sample is reported as reactive.

3. Results

3.1. Testing of HCV genotypes

For analysis of the sensitivity of detection of HCV genotypes by the TMA assays, specimens from patients infected with HCV genotypes were obtained and serially diluted to make analytical sensitivity panels. Ten type 1 specimens, 14 type 2 specimens, 11 type 3 specimens, 12 type 4 speci-

Table 2
Detection of HCV genotypes with the TMA HIV-1/HCV assay

HCV type	Concentration (copies/ml)	# Unique specimens[a]	#Reactive/ # tested	% Positive	S/CO[b]
1	1000	10	60/60	100	9.0
	300	10	59/60	98.3	8.7
	100	10	60/60	100	8.3
	30	10	54/60	90.0	7.0
2	1000	13	78/78	100	7.3
	300	13	75/78	96.2	6.0
	100	13	70/78	89.7	4.5
	30	13	47/78	60.3	3.2
3	1000	11	66/66	100	8.1
	300	11	66/66	100	8.0
	100	11	64/66	97.0	7.2
	30	11	58/66	87.9	6.7
4	1000	11	65/66	98.5	8.6
	300	11	64/66	97.0	8.0
	100	11	64/66	97.0	7.7
	30	11	60/66	90.9	6.7
5	1000	4	24/24	100	8.7
	300	7	40/40	100	8.1
	100	8	48/48	100	7.8
	30	8	46/48	95.8	6.8
6	1000	4	24/24	100	8.4
	300	5	30/30	100	7.4
	100	6	35/36	97.2	5.9
	30	5	28/30	93.3	5.0

[a] Specimens were tested in duplicate with three lots of reagents except where noted in Section 3.
[b] Average of reactive replicates.

mens, eight type 5 specimens and six type 6 specimens were available for testing (Table 1a). Specimens were diluted with HIV-1 and HCV RNA-negative human serum to specific concentrations. The method of quantitation and the country of origin for each specimen are also shown in Table 1a. Most panel members were tested at 1000, 300, 100, and 30 copies/ml. Two replicates at each copy level were tested with each of three reagent kit lots in the TMA HIV-1/HCV assay and the TMA HCV discriminatory assay. Similar results were obtained regardless of kit lot used, therefore the data shown are a compilation of that obtained with the three reagent lots. The S/CO values were expected to be higher in the TMA HCV discriminatory assay as compared to testing in the TMA HIV-1/HCV assay, because the HCV-specific labeled oligonucleotides are present at a higher concentration in the discriminatory assay than in the TMA HIV-1/HCV assay.

The results from testing the genotype panels in the TMA HIV-1/HCV assay are shown in Table 2 and the TMA HCV discriminatory assay results are presented in Table 3; the results from the individual specimens are compiled according to genotype. Each HCV genotype was detected in the assays, even at a concentration of 30 copies/ml. Furthermore, each individual specimen was detected at each concentration tested (data not shown). The lowest S/CO values observed were with specimens tested at 30 copies/ml.

The average analyte signal/analyte cutoff (S/CO) values for detection of HCV type 1 ranged from 7.0 to 9.0 in the TMA HIV-1/HCV assay (Table 2) and from 14.4 to 21.2 in the HCV discriminatory assay (Table 3), demonstrating

good separation from the cutoff of S/CO ≥ 1. Seven of the 10 HCV type 1 panels were detected in the TMA HIV-1/HCV assay with 100% reactive rates at 1000, 300, 100, and 30 copies/ml and eight of the panels were similarly detected in the TMA HCV discriminatory assay (data not shown).

The S/CO ratios in testing HCV type 2 panels ranged from 3.2 to 7.3 for the TMA HIV-1/HCV assay (Table 2) and from 6.2 to 18.5 for the HCV discriminatory assay (Table 3). One sample, JE6-3107-0008, was co-infected with HIV-1 and was therefore, only tested in the TMA HCV discriminatory assay, with which 100% positivity was achieved at all concentrations. Of the HCV type 2 specimens tested, 10 of 11 specimens were detected in the TMA HIV-1/HCV assay with 100% positivity at 100 copies/ml. Two HCV type 2a/c specimens were tested; the first, sample 5889, had variable detection rates at the copy levels tested but the second, 9810067/169284 had 100% positivity at all concentrations in both assays (data not shown).

Overall, the S/CO values for detection of HCV type 3 panels ranged from 6.7 to 8.1 in the TMA HIV-1/HCV assay (Table 2) and from 16.7 to 21.2 in the TMA HCV discriminatory assay (Table 3). All 11 HCV type 3 specimens were detected with 100% positivity in both assays when tested at 1000 and 300 copies/ml, and six of the panel members had 100% reactive rates at all concentrations (1000, 300, 100 and 30 copies/ml).

The range of S/CO values obtained for detection of HCV type 4 specimens was similar to those of the other genotype panels (Tables 2 and 3). One HCV type 4 specimen, IH6-2606-0002,

Table 3
Detection of HCV genotypes with the TMA HCV discriminatory assay

HCV type	Concentration (copies/ml)	# Unique specimens[a]	#Reactive/ # tested	% Positive	S/CO[b]
1	1000	10	60/60	100	21.2
	300	10	59/60	98.3	20.4
	100	10	60/60	100	19.4
	30	10	59/60	98.3	14.4
2	1000	14	84/84	100	18.5
	300	14	84/84	100	14.4
	100	14	79/84	94.0	10.3
	30	14	68/84	81.0	6.2
3	1000	11	66/66	100	21.2
	300	11	66/66	100	20.6
	100	11	66/66	100	19.0
	30	11	61/66	92.4	16.7
4	1000	12	72/72	100	22.1
	300	12	72/72	100	21.5
	100	12	70/72	97.2	20.6
	30	12	66/72	91.7	18.4
5	1000	4	24/24	100	21.6
	300	7	38/38	100	21.1
	100	8	43/44	97.7	19.9
	30	8	42/44	95.5	18.5
6	1000	4	24/24	100	21.8
	300	5	30/30	100	20.5
	100	6	33/34	97.1	17.3
	30	5	29/30	96.7	14.6

[a] Specimens were tested in duplicate with three lots of reagents except where noted in Section 3.
[b] Average of reactive replicates.

was co-infected with HIV-1 and was therefore tested only in the TMA HCV discriminatory both assays. This specimen was 100% positive at all concentrations tested. Five specimens were detected with 100% positivity at all concentrations in both assays (data not shown).

Some of the HCV type 5 specimens could not be tested at all four diluted concentrations due to limited availability (C4-JD8081-161, C6-JD8081-161, C42-JD8081-161, and C40). All samples were tested at 300, 100, and 30 copies/ml except for C40, which was only tested at 100 and 30 copies/ml. Average S/CO ratios ranged from 6.8 to 8.7 in the TMA HIV-1/HCV assay (Table 2) and from 18.5 to 21.6 in the TMA HCV discriminatory assay (Table 3). All samples tested at 1000 and 300, copies/ml were reactive in the HCV discriminatory assay. At 100 and 30 copies/ml, 43/44 and 42/44 samples, respectively, were reactive in the discriminatory assay.

Average S/CO ranges of 5.0–8.4 and 14.6–21.8 were obtained for detection of HCV type 6 in the TMA HIV-1/HCV assay (Table 2) and the TMA HCV discriminatory assay (Table 3), respectively. Of the HCV type 6 panels, specimen 720079/7492 was tested only at 300, 100 and 30 copies/ml and specimen 720080/7502 was tested only at 100 copies/ml. One hundred percent reactivity was seen at 1000 and 300 copies/ml for both the TMA HIV-1/HCV assay and the HCV discriminatory assay. Both assay achieved greater than 97% detection at 100 copies/ml and greater than 93% at 30 copies/ml.

3.2. Testing of HIV-1 subtypes

For a complete assessment of the sensitivity of detection of HIV-1 subtypes by the TMA assays, patient plasma samples or tissue culture supernatants containing HIV-1 group M (genotype A–G), N or O were serially diluted to make analytical sensitivity panels. There were 12 type A specimens, 11 type B specimens, 10 type C specimens, 10 type D specimens, nine type E (CRF01_AE) specimens, six type F specimens, three type G specimens, one group N specimen, and nine group O specimens available for testing (Table 1b), which were diluted with HIV-1 and HCV RNA-negative human serum to specific concentrations. The method of quantitation and the country of origin for each specimen are also shown in Table 1b. Most panel members were tested at 1000, 300, 100, and 30 copies/ml.

The results from testing the HIV-1 subtype panels in the TMA HIV-1/HCV assay are shown in Table 4 and the TMA HIV-1 discriminatory assay results are presented in Table 5; the data from the individual specimens are compiled according to genotype. As shown in the tables, each HIV-1 subtype was detected in the assays, even at a concentration of 30 copies/ml. Furthermore, each individual specimen was detected at each concentration tested (data not shown). The lowest S/CO ratios observed were with testing at 30 copies/ml.

The average analyte signal/analyte cutoff (S/CO) values for detection of HIV-1, group M, type A ranged from 16.6 to 20.5 in the TMA HIV-1/HCV assay (Table 4) and from 15.8 to 19.3 in the TMA HIV-1 discriminatory assay (Table 5), demonstrating good separation from the cutoff of $S/CO \geq 1$. HIV-1 type A specimens JS8-1002-0023, JS8-1002-0027 and JS8-1002-0028 were present in quantities that did not allow testing at all four proposed concentrations. Eleven of the 12 panel members had 100% reactive rates in the TMA HIV-1/HCV assay when tested at 100 copies/ml; the 12th member was detected in five out of six tests. Sensitivity of detection in the TMA HIV-1 discriminatory assay was somewhat better, as all 11 panels tested had 100% detection rates at 30 copies/ml.

The overall average S/CO ratios for detection of HIV-1, group M, type B specimens ranged from 11.6 to 17.4 in the TMA HIV-1/HCV assay (Table 4) and from 9.8 to 17.5 in the TMA HIV-1 discriminatory assay (Table 5). Two specimens, E7-2909-0167 and E8-1702-0127, were not tested in the TMA HIV-1/HCV assay as they were co-infected with HCV. All samples had 100% positive rates when concentrations of 1000 or 300 copies/ml were tested. The sensitivity of detection was even greater in the TMA HIV-1 discriminatory assay, as 11 of the 12 specimens were detected with 100% positive rates at 100 copies/ml and four specimens had 100% positive rates at all concentrations tested (data not shown).

Table 4
Detection of HIV-1 subtypes with the TMA HIV-1/HCV assay

HIV-1 type	Concentration (copies/ml)	# Unique specimens[a]	#Reactive/#tested	% Positive	S/CO[b]
A	1000	9	54/54	100	20.5
	300	11	65/66	98.5	19.8
	100	12	71/72	98.6	18.4
	30	11	63/66	95.5	16.6
B	1000	9	54/54	100	17.4
	300	9	54/54	100	14.8
	100	9	48/54	88.9	13.4
	30	9	37/54	68.5	11.6
C	1000	9	54/54	100	18.4
	300	10	60/60	100	16.0
	100	10	55/60	91.7	13.2
	30	10	51/60	85.0	11.6
D	1000	7	42/42	100	20.0
	300	7	42/42	100	17.8
	100	8	46/48	95.8	13.8
	30	8	44/48	91.7	11.3
E (CRF01_AE)	1000	8	48/48	100	20.7
	300	8	48/48	100	19.7
	100	8	48/48	100	17.7
	30	8	45/48	93.8	14.9
F	1000	5	30/30	100	22.0
	300	5	29/30	96.7	20.3
	100	5	30/30	100	17.1
	30	5	26/30	86.7	14.1
G	1000	3	18/18	100	18.1
	300	3	18/18	100	17.5
	100	3	18/18	100	17.4
	30	3	18/18	100	15.6
Group N	1000	1	6/6	100	16.2
	300	1	6/6	100	15.9
	100	1	6/6	100	12.9
	30	1	6/6	100	8.0
Group O	1000	8	48/48	100	17.7
	300	9	54/54	100	16.6
	100	9	54/54	100	12.4
	30	9	48/54	88.9	8.7

[a] Specimens were tested in duplicate with three lots of reagents.
[b] Average of reactive replicates.

The S/CO ranges for detection of HIV-1, group M, type C were similar to those observed with the other subtypes (Tables 4 and 5). Six of the 10 HIV-1 type C panel members were detected with 100% positive rates in the TMA HIV-1/HCV assay at all concentrations. Similarly, seven of the specimens were detected with 100% reactivity when tested at 30 copies/ml in the TMA HIV-1 discriminatory assay (data not shown).

As with the other subtypes, all concentrations of each HIV-1 group M, type D specimen were detected with both assays with S/CO values ranging from 11.3 to 20.0 in the TMA HIV-1/HCV assay (Table 4) and from 11.7 to 19.7 in the

HIV-1 discriminatory assay (Table 5). Two specimens, IH6-2606-0012 and IH6-2606-0016, were co-infected with HCV and were therefore tested only in the TMA HIV-1 discriminatory assay. Additionally, two specimens, IH6-2606-0010 and IH6-2606-0012, were present in quantities that allowed only 100 and 30 copies/ml testing. Five of the panel members were detected with 100% reactivity at all concentrations tested in the TMA HIV-1/HCV assay. Nine of the 10 samples assessed at 100 copies/ml were detected with 100% reactive rates in the TMA HIV-1 discriminatory assay (data not shown).

Table 5
Detection of HIV-1 subtypes with the TMA HIV-1 discriminatory assay

HIV-1 type	Concentration (copies/ml)	# Unique specimens[a]	#Reactive/#tested	% Positive	S/CO[b]
A	1000	9	54/54	100	19.3
	300	11	65/66	98.5	18.9
	100	12	70/72	97.2	18.3
	30	11	66/66	100	15.8
B	1000	11	66/66	100	17.5
	300	11	64/66	97.0	15.8
	100	11	64/66	97.0	13.1
	30	11	49/66	74.2	9.8
C	1000	9	54/54	100	18.8
	300	10	60/60	100	15.7
	100	10	58/60	96.7	13.4
	30	10	52/60	86.7	10.6
D	1000	8	48/48	100	19.7
	300	8	48/48	100	18.4
	100	10	58/60	96.7	15.4
	30	10	50/60	83.3	11.7
E (CRF01_AE)	1000	9	54/54	100	18.9
	300	9	54/54	100	18.5
	100	9	54/54	100	16.3
	30	9	53/54	98.1	13.0
F	1000	6	36/36	100	18.0
	300	6	36/36	100	16.3
	100	6	35/36	97.2	14.2
	30	6	31/36	86.1	11.8
G	1000	3	18/18	100	19.2
	300	3	18/18	100	18.8
	100	3	18/18	100	18.5
	30	3	17/18	94.4	15.6
Group N	1000	1	6/6	100	16.1
	300	1	6/6	100	15.0
	100	1	6/6	100	12.4
	30	1	5/6	83.3	7.5
Group O	1000	8	48/48	100	18.1
	300	9	54/54	100	16.3
	100	9	54/54	100	12.4
	30	9	48/54	88.9	9.6

[a] Specimens were tested in duplicate with three lots of reagents.
[b] Average of reactive replicates.

The assays demonstrated marked sensitivity in detection of HIV-1, group M, type CRF01_AE in the nine unique panel members available. The overall average S/CO ratios ranged from 14.9 to 20.7 in the TMA HIV-1/HCV assay (Table 4) and from 13.0 to 18.9 in the TMA HIV-1 discriminatory assay (Table 5), demonstrating good separation from the cutoff S/CO of ≥ 1. One specimen, JE6-3107-0012, was co-infected with HCV and therefore tested only in the TMA HIV-1 discriminatory assay.

The S/CO values for detection of HIV-1, group M, type F were similar to that observed for the other HIV-1 subtypes (Tables 4 and 5). One of the specimens, JE6-3107-0004, was co-infected with HCV and was therefore only tested in the TMA HIV-1 discriminatory assay. The remaining five type F specimens were detected with 100% reactivity in all cases when tested at 100 copies/ml. Furthermore, five of the six HIV-1, type F specimens tested in the HIV-1 discriminatory assay had 100% detection rates at all concentrations tested, including 30 copies/ml (data not shown).

The HIV-1 group M, type G specimens were detected with 100% reactive rates in the TMA HIV-1/HCV assay at all concentrations tested (Table 4). Similar results were seen when these samples were tested in the TMA HIV-1 discriminatory assay with the exception that one panel tested at 30 copies/ml had a reactive rate of 5/6, resulting in overall detection of 17/18 (Table 5). The overall average S/CO ratios ranged from 15.6 to 18.1 in the TMA HIV-1/HCV assay (Table 4) and from 15.6 to 19.2 in the TMA HIV-1 discriminatory assay (Table 5).

One sample was available for testing of HIV-1, group N. At each concentration tested in the TMA HIV-1/HCV assay, 100% positive rates were attained (Table 4). Similarly, six out of six replicates were reactive in the TMA HIV-1 discriminatory assay when the HIV-1, group N panel was tested at 1000, 300, or 100 copies/ml and the specimen was detected 5/6 when tested at 30 copies/ml (Table 5). The average S/CO values were similar to those observed for the HIV-1 group M subtypes, ranging from 8.0 to 16.2 in the TMA HIV-1/HCV assay and from 7.5 to 16.1 in the HIV-1 discriminatory assay.

The HIV-1 group O specimen JP8-2707-0001 was present in quantities that allowed testing at only 300, 100, and 30 copies/ml. All nine panels were detected with 100% positive rates in the TMA HIV-1/HCV assay at 100 copies/ml (Table 4) and five of the panels also had detection rates of 100% in the assay when tested at 30 copies/ml. Four of the specimens were detected with 100% positive rates at all concentrations in the TMA HIV-1 discriminatory assay and the specimens were detected 54/54 at 100 copies/ml (Table 5). The range of S/CO values for the TMA HIV-1/HCV assay and the TMA HIV-1 discriminatory assay were comparable to those observed for the group M and group N panels (Tables 4 and 5).

3.3. Assay specificity

To control for assay specificity, 2–10 additional negative control samples were included in each run (in addition to the three negative calibrators included in each run). A total of 398 negative control samples were tested along with the positive, genotyped HIV-1 and HCV specimens included in this study. Two false reactive results were obtained during the course of the study (2/398 or 0.5%). This specificity is comparable to results obtained previously with the TMA HIV-1/HCV and discriminatory assays (Giachetti et al., 2002).

4. Discussion

4.1. Assay sensitivity for HCV genotypes

Isolates of the same HCV genotype display average sequence identity of about 95%, whereas isolates of different genotypes have sequence identity of about 65% (Simmonds et al., 1994; Davis, 1999). Studies examining the worldwide geographic distribution of genotypes have shown that certain genotypes are characteristic of particular geographic regions (Simmonds, 1999). Genotypes 1, 2, and 3 are the most widely distributed and the most common in the U.S., Europe, and Japan; genotype 4 predominates in Egypt; genotype 5 is found almost exclusively in South Africa and

HCV genotype 6 has been identified primarily in Hong Kong and other parts of Asia (Zein and Persing, 1996; Simmonds, 1995). The results presented here show that the TMA HIV-1/HCV and HCV discriminatory assays have excellent sensitivity for a wide range of HCV genetic variants obtained from around the world. The TMA HIV-1/HCV assay, used for initial blood bank screening, and the TMA HCV discriminatory assay, used for detection of the presence of HCV after an initial positive result is obtained, achieved similar detection rates with the panels tested. Each representative of genotypes 1 through 6 was detected in the TMA assays.

Quantitation of the samples tested within a genotype was determined by one of several different methods, therefore the sensitivity determined in this study was not biased by single quantitative method. Although the quantitative HCV results from the Bayer Quantiplex and Roche Monitor version 2.0 appear to show reasonable correlation (Martinot-Peignoux et al., 2000), we chose to use data from these tests and other quantitative tests (NGI SuperQuant and Gen-Probe Quantitative TMA) to gain more confidence of the true genotype sensitivity of the TMA assays. Ideally, it is preferable to study the analytical sensitivity of the assay with respect to an international standard, such as the World Health Organization (WHO) International Standard for HCV. However, currently WHO international standards for genotypes other than HCV genotype 1 are not available. Due to the lack of an international unit-based reference, the sensitivity of the TMA assay for HCV genotypes was measured by testing panel members quantitated with the Roche Amplicor Test, Bayer bDNA Assay, or NGI SuperQuant Assay. Exceptions to this testing were two of the HCV type 4 specimens, 39377 and 38111, which were quantitated by a Gen-Probe quantitative HCV assay. Regardless of the method used to assign the original titers of the specimens, the TMA assays achieved sensitivity in most cases of approximately 30 copies/ml. The S/CO values were generally higher in the TMA HCV discriminatory assay as compared to testing in the TMA HIV-1/HCV assay, because the HCV-specific labeled probe oligonucleotides (used for chemiluminescent detection) are present at a higher concentration in the discriminatory assay than in the TMA HIV-1/HCV assay.

There has been a tendency to optimize nucleic acid testing assays for detection of genotypes that are common in the U.S. and Europe. Reports have shown nucleic acid testing assay sensitivity problems with the detection of the less common HCV genetic variants (Hawkins et al., 1997). This study shows, however, that the TMA HIV-1/HCV assay and the TMA HCV discriminatory assay can consistently detect all HCV genotypes at very low copy levels. The assays showed a trend of less sensitivity for HCV genotype 2 detection, but detection at 100 copies/ml was observed in nearly all cases. The assay performance demonstrated in this study meets the sensitivity requirements of ≥ 100 copies/ml established by the U.S. FDA for detection of HCV and the European requirement of sensitivity of 5000 IU/ml in plasma pool testing (Busch and Kleinman, 2000).

4.2. Assay sensitivity for HIV-1 subtypes

Virtually all HIV-1 subtypes, including group O, can be found in Africa but they are otherwise distributed according to specific geographical regions. The primary HIV-1 subtype in the U.S. and Europe is HIV-1 group M, subtype B, while subtype C predominates in India, subtype CRF01_AE in Thailand and subtype F in Romania (de Oliveira et al., 2000). The appearance of different HIV-1 group M subtypes and group O in Europe has been increasing and there have been a few instances of non-B group M subtypes and group O infections in the U.S. (Brodine et al., 1995; Rayfield et al., 1996; Alaeus et al., 1997; Barin et al., 1997). As HIV-1 subtypes are rapidly integrating into new geographical locations, it is essential that the blood screening assays are able to detect each HIV-1 variant. There have been reports of difficulties in consistent detection of non-B subtypes with nucleic acid and serological testing (Koch et al., 2001). This is probably a result of mismatches in the primer or probe-binding regions of the RNA or amplicon, as HIV-1 group M and group O viruses can differ by as much as 50% in their overall sequences (Triques et al.,

1999). However, the data shown here indicate that the TMA HIV-1/HCV assay and the TMA HIV-1 discriminatory assay, due to the appropriate choice of primers and probes in the most conservative HIV-1 genomic regions, are capable of sensitive detection of all of the major HIV-1 genetic variants, including groups N and O.

Although the performance of HIV-1 viral RNA quantitative tests have improved considerably since their introduction, significantly different quantitation results can be obtained with currently available tests (Jagodzinski et al., 2000). To avoid the quantiation bias of any single test method, the quantitation of the HIV-1 samples used in this study was not limited to one test method.

The TMA HIV-1/HCV assay, used for initial blood bank screening, and the HIV-1 discriminatory assay, for verification of the presence of HIV-1 after an initial positive result is obtained, achieved similar results with the panels tested. All panels tested were detected at least once at 30 copies/ml and most achieved 100% positive rates at 100 copies/ml. Even at 30 copies/ml, the average reactive S/CO values were well separated from the cutoff of 1.0, as the lowest S/CO mean in the TMA HIV-1/HCV assay was 8.0 and the lowest in the TMA HIV-1 discriminatory assay was 7.5. Six specimens were co-infected with HCV and were therefore tested only in the TMA HIV-1 discriminatory assay. The detection of HIV-1 in these samples was not hindered by the presence of HCV, as all concentrations of these samples had 100% reactive rates when tested in the TMA HIV-1 discriminatory assay. Thus co-infection does not cause interference in the TMA HIV-1 discriminatory assay, consistent with other TMA HIV-1/HCV assay data (Giachetti et al., 2002).

4.3. Further applications

The exquisite sensitivity of the TMA assays for detection of genetic variants of HCV and HIV-1 suggests a wide range of diagnostic and therapeutic applications for the technology. Whereas serological testing provides information about the patient's immune response, RNA assays, like the TMA HIV-1/HCV assay, can reveal the outcome of HCV infection (Urdea et al., 1997). About 15% of confirmed HCV antibody reactive patients have resolved infections and are HCV RNA negative (Allain, 2000a). In several cases, patients have exhibited chronic HCV infection without detectable antibodies; these patients do not seroconvert but remain HCV RNA positive (Allain, 2000a). These infections go undetected by serological testing but proper diagnosis for these patients can be achieved with a sensitive RNA assay like the TMA HIV-1/HCV assay. The low copy level detection limits and ability to recognize a wide range of variants of HCV and HIV-1 make the TMA assays attractive tools for evaluation of the efficacy of treatment and for identification of therapeutic end-points. Accurate identification of the true elimination of the virus during therapy may prevent the occurrence of relapse due to premature cessation of treatment (Urdea et al., 1997). Furthermore, the high throughput capability of the assays can increase the ease of conducting large-scale epidemiological studies.

In conclusion, this study shows that the Gen-Probe assays were capable of the sensitive detection of a wide range of HCV and HIV-1 variants. The data presented here, along with the performance demonstrated in blood bank testing under IND approval in the U.S. (Stramer et al., 2000) and testing carried out in other countries, show that the TMA HIV-1/HCV assay, the TMA HCV discriminatory assay, and the TMA HIV-1 discriminatory assay meet the performance requirements for successful use in blood bank or clinical laboratory settings worldwide.

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