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Molecular Techniques for Blood and Blood Product Screening

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Introduction

"Blood banking has become a manufacturing industry, an industry that must conform to high standards and quality control requirements comparable to those of pharmaceutical companies or other regulated industries," said David A. Kessler, M.D., former FDA commissioner (Revelle, 1995). Screening donated blood for infectious diseases that can be transmitted through blood transfusion is very important in ensuring safety. The United States has the safest blood supply in the world (Revelle, 1995), and the Food and Drug Administration (FDA) is striving to keep it safe by decreasing the risk of infectious disease transmission. The regulatory agency is continuously updating its requirements and standards for collecting and processing blood. An important step in ensuring safety is the screening of donated blood for infectious diseases. In the United States, tests for infectious diseases are routinely conducted on each unit of donated blood, and these tests are designed to comply with regulatory requirements (Table 21.1). The field of clinical microbiology and virology is now moving into the focus of molecular technology. Currently, nucleic acid testing techniques have been developed to screen blood and plasma products for evidence of very recent viral infections that could be missed by conventional serologic tests. It is time for all blood safety staffs to use molecular detection techniques. This approach can significantly aid in blood safety to reduce the risk of transmission of serious disease by transfusion. This chapter will review the current antigen/antibody–based technology, molecular biological technology, and published regulatory policy data for blood safety.

Limitations for Current Technologies Used in Blood Safety

Direct detection of viral antigens and virus-specific antibodies have been the common tools for diagnosis of virus infections in the past 10 years. There are some limitations. For direct detection of virus antigens, shortly after virus infection, only a few viruses release antigens in amounts sufficiently detectable in the body by antibody assay. For indirect virus detection by virus specific antibodies [e.g., an

TABLE 21.1. Licensed / Approved Clinical HIV, HTLV and Hepatitis Tests (Source: Center for Biologics Evaluation and Research, US Food and Drug Administration)

Tradename(s)	Format	Sample	Use	Manufacturer	Approval Date
Antibody to Hepatitis B Surface Antigen (HBsAg Assay)					
Auszyme Monoclonal	EIA	Serum/Plasma	Donor Screen & Conf Kit	Abbott Laboratories Abbott Park, IL US License 0043	4/1/1985
Genetic Systems HBsAg EIA 3.0	EIA	Serum/Plasma/ Cadaveric Serum	Donor Screen & Conf Kit	Bio-Rad Laboratories Redmond, WA 98052 US License 1109	1/23/2003
ORTHO Antibody to HBsAg ELISA Test System 3	EIA	Serum/Plasma	Donor Screen / Diagnosis & Conf Kit	Ortho-Clinical Diagnostics, Inc Raritan, NJ 08869 US License 1236	4/23/2003
Antibody to Human Immunodeficiency Virus (HIV-1 Antigen Assay)					
Coulter HIV-1 p24 Ag Assay; HIV-1 p24 Antigen ELISA Test System	EIA	Serum/Plasma	Donor Screen/Prognosis & Neut Kit	Coulter Corporation Miami, FL US License 1185	3/14/1996
Coulter HIV-1 p24 Ag Assay	EIA	Viral Culture Supernatant	Prognosis (Quantitative) & Neut Kit	Coulter Corp	3/14/1996
Anti-HIV-1 Oral Specimen Collection Device					
Epitope OraSure HIV-1 Oral Specimen Collection Device	Oral Specimen Collection Device	Oral Fluid	For Use in Designated Non-Donor Screen and Non-Donor Supplemental Assays	Epitope, Inc Beaverton, OR	12/23/1994
Anti-HIV-1 Testing Service					
Home Access HIV-1 Test System	Dried Blood Spot Collection Device	Dried Blood Spot	Non-Donor Screen	Home Access Health Corp Hoffman Estates, IL	7/22/1996

Hepatitis B Surface Antigen (Anti-HBs Assay)					
Ausab	RIA	Serum/Plasma	Anti-HBs	Abbott Laboratories Abbott Park, IL US License 0043	2/5/1975
Ausab EIA	EIA	Serum/Plasma	Anti-HBs	Abbott Laboratories	11/18/1982
Hepatitis B Virus Core Antigen (Anti-HBc Assay)					
Corzyme	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	3/19/1991
Ortho HBc ELISA Test System	EIA	Serum/Plasma	Donor Screen	Ortho-Clinical Diagnostics, Inc Raritan, NJ US License 1236	4/18/1991
Hepatitis C Virus Encoded Antigen (Anti-HCV Assay)					
Abbott HCV EIA 2.0	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	5/6/1992
Ortho HCV Version 3.0 ELISA Test System	EIA	Serum/Plasma	Donor Screen	Ortho-Clinical Diagnostics, Inc Raritan, NJ US License 1236	5/20/1996
Chiron RIBA HCV 3.0 Strip Immunoblot Assay	SIA	Serum/Plasma	Donor Supplemental	Chiron Corp Emeryville, CA US License 1106	2/11/1999
HIV-1/HCV Nucleic Acid Testing					
Roche Amplicor HIV-1 Monitor Test	PCR	Plasma	Prognosis / Patient Management HIV-1 Viral Load Assay	Roche Molecular Systems, Inc Branchburg Township, NJ	3/2/1999
NucliSens HIV-1 QT	NASBA	Plasma	Prognosis / Patient Management HIV-1 Viral Load Assay	bioMerieux, Inc Durham, NC 27712	11/19/2001
COBAS Ampliscreen HIV-1 Test	PCR	Plasma	Donor Screen	Roche Molecular Systems, Inc Pleasanton, CA 94566	12/20/2002
Procleix	HIV-1/ HCV Nucleic Acid Test (TMA)	Plasma	Donor Screen	Gen-Probe San Diego, CA 92121	2/8/2002

(*continued*)

TABLE 21.1. (*Continued*)

Tradename(s)	Format	Sample	Use	Manufacturer	Approval Date
Trugene HIV-1 Genotyping Kit and Open Gene DNA Sequencing System	HIV-1 Genotyping	Plasma	Patient Monitoring	Visible Genetics, Inc Toronto, CA	4/24/2002
UltraQual HIV-1 RT-PCR Assay	PCR	Plasma	Donor Screen	National Genetics Institute Los Angeles, CA 92121	9/18/2001
UltraQual HCV RT-PCR Assay	PCR	Plasma	Donor Screen	National Genetics Institute Los Angeles, CA 92121	9/18/2001
ViroSeq HIV-1 Genotyping System with the 3700 Genetic Analyzer	HIV-1 Genotyping	Plasma	Detecting HIV genomic mutations that confer resistance to specific types of antiretroviral drugs, such as an aid in monitoring and treating HIV infection	Celera Diagnostics Alameda, CA 94502	6/11/2003
Versant HIV-1 RNA 3.0 (bDNA)	Signal amplification nucleic acid probe	Plasma	Patient Monitoring	Bayer Corp Berkeley, CA 94702	9/11/2002
COBAS AmpliScreen HCV Test	PCR	Plasma	Donor Screen	Roche Molecular Systems, Inc Pleasanton, CA 94566	12/3/2002
		Human Immunodeficiency Virus Type 1 (Anti-HIV-1 Assay)			
HIVAB HIV-1 EIA	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	3/1/1985
Genetic Systems rLAV EIA	EIA	Serum/Plasma	Donor Screen	Bio-Rad Laboratories Blood Virus Division Redmond, WA US License 1109	6/29/1998
Murex SUDS HIV-1 Test	Rapid EIA	Serum/Plasma	Donor Screen	Murex Diagnostics, Inc Norcross, GA US License 1152	5/22/1992

Vironostika HIV-1 Microelisa System	EIA	Serum/Plasma	Donor Screen	bioMerieux, Inc Durham, NC 27712 US License 1624	12/18/1987
Vironostika HIV-1 Plus O Microelisa System	EIA	Plasma/Serum/Dried Blood Spots	Diagnostic Non-Donor Screen	bioMerieux, Inc Durham, NC 27712 US License 1624	6/6/2003
Cambridge Biotech HIV-1 Western Blot Kit	WB	Serum/Plasma	Donor Supplemental	Calypte Biomedical Corp Berkeley, CA US License 1207	1/3/1991
Genetic Systems HIV-1 Western Blot	WB	Serum/Plasma	Donor Supplemental	Bio-Rad Laboratories Blood Virus Division	11/13/1998
Fluorognost HIV-1 IFA	IFA	Serum/Plasma	Donor Supplemental	Waldheim Pharmazeutika GmbH Vienna, Austria US License 1150	2/5/1992
HIVAB HIV-1 EIA	EIA	Dried Blood Spot	Non-Donor Screen	Abbott Laboratories	4/22/1992
HIV-1 Urine EIA	EIA	Urine Screen	Non-Donor Screen	Calypte Biomedical Corp	8/6/1996
Genetic Systems rLAV EIA	EIA	Dried Blood Spot	Non-Donor Screen	Bio-Rad Laboratories Blood Virus Division	6/29/1998
Vironostika HIV-1 Microelisa System	EIA	Dried Blood Spot	Non-Donor Screen	bioMerieux, Inc Durham, NC 27712	4/11/1990
Oral Fluid Vironostika HIV-1 Microelisa System	EIA	Oral Fluid	Non-Donor Screen	bioMerieux, Inc Durham, NC 27712	12/23/1994
Cambridge Biotech HIV-1 Western Blot Kit	WB	Urine	Non-Donor Supplemental	Calypte Biomedical Corp	5/28/1998
Genetic Systems HIV-1 Western Blot	WB	Dried Blood Spot	Non-Donor Supplemental	Bio-Rad Laboratories Blood Virus Division	11/13/1998
OraSure HIV-1 Western Blot Kit	WB	Oral Fluid	Non-Donor Supplemental	Epitope, Inc	6/3/1996
Fluorognost HIV-1 IFA	IFA	Dried Blood Spot	Non-Donor Supplemental	Waldheim Pharmazeutika GmbH	5/14/1996
OraQuick Rapid HIV-1 Antibody Test	Rapid EIA	Fingerstick	Non-Donor Screen	OraSure Technologies, Inc Bethlehem, PA 18015	11/7/2002

(*continued*)

TABLE 21.1. (*Continued*)

Tradename(s)	Format	Sample	Use	Manufacturer	Approval Date
Reveal Rapid HIV-1 Antibody Test	Rapid Immunoassay	Serum/Plasma	Non-Donor Screen	MedMira Laboratories, Inc Halifax, Nova Scotia Canada B3S 1B3	4/16/2003
Uni-Gold Recombigen HIV	Rapid Immunoassay	Serum/Plasma/Whole Blood	Non-Donor Screen	Trinity Biotech, plc Bray Co., Wicklow Ireland	12/23/2003
Human Immunodeficiency Virus Types 1 & 2 (Anti-HIV-1/2 Assay)					
Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	2/14/1992
Genetic Systems HIV-1/HIV-2 Peptide EIA	EIA	Serum/Plasma/ Cadaveric Serum	Donor Screen	Bio-Rad Laboratories Blood Virus Division Redmond, WA US License 1109	12/9/2000
Genetic Systems HIV-1/HIV-2 Plus O EIA	EIA	Serum/Plasma	Donor Screen	Bio-Rad Laboratories Inc Hercules, CA US License 1109	8/5/2003
Human Immunodeficiency Virus Type 2 (Anti-HIV-2 Assay)					
Genetic Systems HIV-2 EIA	EIA	Serum/Plasma	Donor Screen	Bio-Rad Laboratories Blood Virus Division Redmond, WA US License 1109	4/25/1990
Human T-Lymphotropic Virus Types I & II (Anti-HTLV-I/II Assay)					
Abbott HTLV-I/HTLV-II EIA	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	8/15/1997
Vironostika HTLV-I/II Microelisa System	EIA	Serum/Plasma	Donor Screen	bioMerieux, Inc Durham, NC 27712 US License 1624	1/17/1998

immunofluorescence assay or enzyme immunoassay (EIA), etc.], there is a problem in that shortly after infection by a pathogenic virus, there is a window period in which antibody generation is insufficient for detection (Chamberland, 2001). To reduce this window period, direct nucleic acid tests are needed.

Application of Advanced Molecular Techniques in Blood Safety Applications

Through the application of molecular biology, biological and biochemical analyses have been revolutionized, and nucleic acid, gene-based techniques have been developed to screen blood and plasma donations for evidence of very recent and earlier viral infections that could be missed by conventional serologic tests. The nucleic acid tests can also provide evidence for genetic variation in viruses. Molecular methods include the use of nucleic acid probes as well as amplification based and DNA sequence–based techniques. More and more molecular diagnostic methods are now available commercially. In comparison to classical methods, molecular biological methods are superior in terms of rapidness, specificity, and sensitivity. The current nucleic acid detection methods in the field may be grouped into two major classes: amplifying techniques such as PCR and nonamplifying techniques such as Southern blot hybridization. Amplifying techniques are more sensitive than nonamplifying techniques. There are two different amplifying methods (Hayden, 2005), target amplification methods and signal amplification methods. Target amplifying techniques include PCR, nucleic acid sequence–based amplification (NASBA) (Guichon et al., 2004, Starkey et al., 2004), self-sustaining sequence amplification (3SR), transcription-based amplification (TAS), transcription-mediated amplification (TMA), strand displacement amplification (SDA), and ligase chain reaction (LCR). Signal amplification methods include branched DNA signal amplification (bDNA) (Peter and Sevall, 2004), cleavage-based signal amplification (cycling probe technologies and invader assay), Qß replicase, hybrid capture, cycling probe technologies (CPT), and rolling-circle amplification (RCA) (Tang et al., 1997). To further insure the safety of blood products, it is of importance to further develop nucleic acid testing.

Major Different Generations of Nucleic Acid Detection Techniques

Southern Blot Hybridization (1970s)

Southern blotting (Mornet, 2004) was named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s. This technique is used to detect specific sequences within mixtures of DNA, which is size-fractionated by gel electrophoresis and then transferred by capillary action to a suitable membrane. After blocking of nonspecific binding sites, the nitrocellulose replica of the original

gel electrophoresis experiment is then allowed to hybridize with an oligonucleotide probe representing the specific DNA sequence of interest. Should specific DNA be present on the blot, it will combine with the labeled probe and be detectable. By co-electrophoresing DNA fragments of known molecular weight, the size(s) of the hybridizing band(s) can then be determined. Southern blotting hybridization technology is one of the major tools that have already helped clinical staffs world-wide interpret genomic information. Other competing methodologies include *in situ* hybridization and solution hybridization. Important clinical examples of the use of this technology are DNA fingerprinting and the ability to detect DNA gene rearrangements.

Polymerase Chain Reaction (1980s)

In 1983, Dr. Kary Mullis at Cetus Corporation conceived of polymerase chain reaction (Hersing, 1993). There is not any technique that has had a greater impact on the practice of molecular biology than PCR. With this technique, we can detect infectious disease agents at an extremely low level. It is based on the ability of sense and anti-sense DNA primers to hybridize to a DNA of interest. After extension from the primers on the DNA template by DNA polymerase, the reaction is heat-denatured and allowed to anneal with the primers once again. Another round of extension leads to a multiplicative increase in DNA products. Therefore, a minute amount of DNA can be efficiently amplified in an exponential fashion to result in easily manipulable amounts of DNA. By including critical controls, the technique can be made quantitative. The current level of the sensitivity and detection limit is as low as 10–50 copies per mL in HIV testing (Ginocchio et al., 2003). Important clinical examples of the use of PCR are detection of HIV and HCV (Abbott, 2003; Roche, 2003; Katsoulidou, 2004). PCR techniques have evolved into different branches. Some of them are now widely in use for virus detection in clinical diagnostics. These are real-time PCR by Taqman (Roche), Light Cycler (Roche), and Smart Cycler (Cepheid), and *in situ* PCR, nested-PCR, broad-range PCR, multiplex PCR, RT-PCR, arbitrarily primer PCR, long PCR, and quantitative PCR. Real-time sequence technology will be coming soon for more detailed detection. In the past, identification of viral serotypes was restricted to investigative methods using antibody detection and restriction fragment length polymorphism (RFLP). With real-time sequences technology, we will be able to detect a virus early as well as obtain the viral sequence.

Microarrays (1990s)

Microarrays were developed at Stanford University by Schena and co-workers in the early 1990s (Schena, 2002). For medical applications, a microarray analysis offers a very accurate screening technology. It allows hundreds or thousands of nucleic acid hybridization reaction to be performed on a solid substrate. It will be a fast and accurate diagnostic tool in the field of clinical microbiology and virology.

Applied to infection safety for blood and blood products, it will be able to screen for the presence of viral pathogens by matching genetic sequences. Compared with existing technologies, it allows for a wider variety of specific tests to be carried out simultaneously to determine the quality of the blood and will provide consumers with extra safety. With the use of molecular biology protocols, the microarray will permit the detection of lower concentrations of microorganisms in the blood and the accurate identification of many types of pathogenic contaminants. In the future, progress can be expected in the application of microarray technology for screening of donated blood for infectious agents. It can provide vast information about the identity of bloodborne pathogens as well as their gene expression profiles (yu et al., 2004).

Screening of Donor Blood for Infectious Agents

To ensure a safe blood supply for everyone who may need a transfusion, an important step in ensuring safety is the screening of donated blood for infectious agents. After donation, each unit of donated blood undergoes a series of tests for bloodborne agents such as human immunodeficiency virus (HIV)-1, HIV-2, hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell lymphotropic virus (HTLV)-1 and HTLV-II, West Nile virus (WNV), and the agent of syphilis.

Confirmatory Testing of Donor Blood for Infectious Agents

All of the above tests are referred to as screening tests and are designed to detect as many infectious agents as possible. Because these tests are so sensitive, some donors may have a false-positive result, even when the donor was never exposed to the particular infection. In order to sort out true infections from false-positive test results, screening tests that are reactive may be followed up with more specific tests called confirmatory tests. Thus, confirmatory tests help determine whether a donor is truly infected. If any one of these tests fails, affected blood products are considered unsuitable for transfusion (U.S. FDA, 2004).

Application of Nucleic Acid Testing for Infectious Agents

Nucleic acid testing (NAT) employs testing technology that directly detects the genomes of viruses. Because NAT detects a virus's genetic material instead of waiting for the body's response— the formation of antibodies, as with many current tests—it offers the opportunity to reduce the window period during which an infecting agent is undetectable by traditional tests (Stramer et al., 2004), thus further improving blood safety. Nucleic acid testing will become the gold standard because of greater sensitivity compared to antibody tests.

Since 1999, NAT has been approved by the FDA and used to detect HIV-1 and HCV, and this technology is under investigation for detecting other infectious disease agents. We know that viral RNA appears very early in the infection, in 1 to 2 weeks, but the antibody doesn't appear until 10–12 weeks (e.g., HIV and HCV) (CDC, 1997). In order to virtually prevent infection by all the transfusion associated viruses, we need to detect the viruses in their window period, and a NAT or gene-based testing method is needed. NAT also provides an opportunity for the viral (e.g. HIV or HCV) infected donor to seek early treatment. On the other hand, NAT is not only a sensitive method but also a rapid method that is suitable for a blood bank laboratory because the turn-around time for maintaining blood donations is extremely critical.

Hepatitis B Virus

The hepatitis B virus (HBV) is a highly infectious and often nonsymptomatic virus that is transmitted primarily through blood and blood-derived fluids and is a leading cause of liver infection worldwide. The World Health Organization (WHO) estimates that 2 billion people worldwide have been infected with HBV and 350,000,000 people are chronically infected. Chronic infection results in a high risk for liver cancer and cirrhosis of the liver, which cause about 1,000,000 deaths each year. Each year up to 200,000 people become newly infected in the United States alone. Since screening for HBV began in 1969, the rate of infection through blood transfusions has greatly decreased. However, as of 2000, HBV is still transmitted through blood transfusions in 1 out of 137,000 units of blood. One reason for this is that currently available blood screening technologies detect core antibodies or surface antigens, which appear up to 8 weeks after infection. Serologic tests for hepatitis B virus include hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (HBcAb).

Hepatitis B Surface Antigen

HBV, which mainly infects the liver, has an inner core and an outer envelope (the surface). The HBsAg test detects the outer envelope, identifying an individual infected with the hepatitis B virus. This virus can cause inflammation of the liver, and in the earliest stage of the disease, infected people may feel ill or even have yellow discoloration of the skin or eyes, a condition known as jaundice. Fortunately, most patients recover completely and test negative for HBsAg within a few months after the illness. A small percentage of people become chronic carriers of the virus, and in these cases, the test may remain positive for years. Chronically infected people can develop severe liver disease as time passes and need to be followed carefully by an experienced doctor. To reduce the occurrence of post-transfusion hepatitis, it is essential to screen all blood donations for hepatitis B surface antigen by the most sensitive and specific assays. Blood donations that are found to be reactive in the HBsAg test are automatically confirmed by the HBsAg confirmatory assay. If the specimen is neutralizable in the confirmatory test, the specimen is

considered positive for HBsAg. Hepatitis B surface antigen testing of donated blood was begun in 1975 (Table 21.1).

Currently, all blood donors are screened for HBsAg, but occasional transmission of HBV still occurs due to the inclusion of window period donations. (i.e., blood from recently infected donors who are antibody negative but still viremic). Detection of early HBV infection of blood donors is still a major problem of blood transfusion. Using the current third-generation licensed HBsAg tests (mostly radioimmunoassay and enzyme immunoassays) cannot detect HBV in the window period for HBV infection. This is a strong motivation for introducing molecular detection techniques to the field. There are some commercially available test methods for detecting HBV DNA in the market now, such as Chiron's Quantiplex HBV DNA (Krajden et al., 1998), Digene's Hybrid Capture, Abbott's HBV DNA assay, and Roche's Amplicor HBV Monitor. Using these commercial hybridization or PCR-based assays, HBV DNA can be detected 1 to 3 weeks before the appearance of HBsAg (Hollinger and Jake, 2001). All HBsAg positive patients (blood donors) have detectable HBV DNA in their serum. Some chronically infected patients who have lost their HBsAg remain HBV DNA positive but are disqualified as potential blood donors. Molecular detection of HBV DNA is more sensitive than current methods employed for HBsAg screening.

Antibodies to the Hepatitis B Core Antigen (Anti-HBc)

Determination of anti-HBc (total) is also used to monitor the progress of the hepatitis B viral infection. Determination of anti-HBc (IgM) is employed to distinguish an acute hepatitis B infection from a chronic infection. The anti-HBc test developed in 1987 detects an antibody to the hepatitis B virus that is produced during and after infection. If an individual has a positive anti-HBc test, but the HBsAg test is negative, it may mean that the person once had hepatitis B but has recovered from the infection. Of the individuals with a positive test for anti-HBc, many have not been exposed to the hepatitis B virus; thus, there is a frequent problem of false positives. Although the individual may be permanently deferred from donating blood, it is unlikely that the person's health will be negatively affected. (Note: This antibody is not produced following vaccination against hepatitis B.)

Hepatitis C Virus

The hepatitis C virus (HCV) is a member of the Flaviviridae family of viruses, which are associated with both human and animal diseases. Hepatitis caused by HCV is the most common chronic bloodborne infection in the United States. Over 4 million Americans are believed to be infected. HCV can also be transmitted through blood transfusion. HCV causes inflammation of the liver, and up to 80% of those exposed to the virus develop a chronic infection, which can lead to liver inflammation, cirrhosis, cancer, and death. Eventually, up to 20% of people with HCV may develop cirrhosis of the liver or other severe liver diseases. As in other forms of hepatitis, individuals may be infected with the virus but may not realize

they are carriers because they do not have any symptoms. Because of the risk of serious illness, people with HCV need to be followed closely by a physician with experience evaluating this infection. Since the full length HCV cDNA was first cloned in 1989, significant progress has been made in characterizing its molecular biology (Lamballerie, 1996). But, the natural history of HCV infection is still largely unclear and current treatment options for patients are limited. There is no vaccine for HCV, and the only available treatment, a combination of alpha-interferon and ribavirin, is efficacious in only a minority of patients (Wang and Heinz, 2001). The life cycle of the HCV is poorly understood due to the lack of an efficient cell culture system (Cohen, 1999). There is an urgent need to develop a highly sensitive detection method for studying possible extrahepatic sites for the replication of hepatitis C virus. We recently established a cell culture system for the replication of HCV by using human T and B leukemia cell lines. (Hu et al., 2003) This model should represent a valuable tool for the detailed study of the initial steps of the HCV replication cycle and for the evaluation of antiviral molecules. Currently, appropriate therapeutic and vaccine strategies for HCV have not been developed. Early detection and prevention of HCV infection are most important for blood safety.

It is a formidable task to design primers and probes for sensitive nucleic acid level diagnostic assays throughout the open reading frame of the HCV genome because of a high mutation rate in this genomic region. However, the untranslated region of about 341 nucleotides contains highly conserved domains, which allows for stable primer design and sensitive diagnostic tests, both qualitative and quantitative, which have equivalent sensitivity against the known six various genotypes of HCV.

Antibodies to the Hepatitis C Virus (Anti-HCV)

In 1990, the first specific test for HCV, the major cause of "non-A, non-B" hepatitis was introduced. Now, a third-generation ELISA kit is available to detect antibodies to HCV, and screening blood for HCV antibodies is recommended. These assays are based on detection of serum antibody to various HCV antigens because these antibodies are nearly universally present in patients who are chronically infected with HCV (Major et al., 2001). The HCV screening tests are known to have significant limitations, and positive samples should be further tested by HCV confirmatory tests.

HCV Confirmatory Tests

Guidelines provided by the CDC recommend that HCV antibody screening test positive samples should be confirmed with serologic or nucleic acid supplemental testing. HCV confirmatory tests include the recombinant immunoblot assay in which several recombinant peptide antigens are applied on a strip that is then probed with the patient's serum. In this way, the response to individual antigens can be recognized, and some false-positive ELISA results can be eliminated

(e.g., RIBA, Chiron HCV 3.0 and PCR assay) (e.g., Roche COBAS AMPLICOR HCV Test, version 2.0). Laboratories can choose to perform this testing on all positive specimens or based on screening test positive (signal to cutoff) ratios. The positive predictive values (s/co) can vary depending on the prevalence of infection in the population being screened.

HCV antibodies are not generally detectable for at least 6 weeks and may not appear for several months. Acute HCV infections are relatively rare among blood donors, but the antibody tests often fail to detect these patients in the window period between the time of infection and the time of appearance of antibody detectable by the above assays. High-sensitivity detection of HCV during the window period is a long-term technical challenge in the field. Tests for HCV RNA genome detection based on the PCR or other highly sensitive RNA detection systems have been used for the diagnosis of acute hepatitis (Major et al., 2001). Sensitive detection of HCV RNA based on RT-PCR or other nucleic acid amplification techniques can be readily accomplished with kits that are now available commercially. For example, in 1999 the FDA approved Roche's Amplicor HIV-1 Monitor Ultra Sensitive quantitative assay. It can measure HIV levels at as few as 50 copies/mL and another commercial kit, the LCx HIV RNA Quantitative Assay from Abbott Laboratories, also has a detection limit at 50 copies/mL. Some research papers even showed a sensitivity limit at 1 copy (Palmer et al., 2003). In fact, a qualitative assay should be much more sensitive than a quantitative assay for HIV/HCV screening. A sensitive qualitative HCV molecular detection assay will possibly interdict and virtually prevent all transfusion-associated HIV/HCV. The current sensitivity standard for clinical diagnostics is 100 copies per mL, but since there has been an improvement in technology, this would be the time to change sensitivity standard to 50 copies per mL.

Human Retroviruses

Antibodies to the Human Immunodeficiency Virus, Types 1 and 2 (Anti-HIV-1, -2)

HIV-1 and/or HIV-2 virus cause acquired immunodeficiency syndrome, or AIDS. The test is designed to detect antibodies directed against antigens of the HIV-1 or HIV-2 viruses. HIV-1 is much more common in the United States, whereas HIV-2 is prevalent in Western Africa. Donors are tested for both viruses because both are transmitted by infected blood, and a few cases of HIV-2 have been identified in U.S. residents. In 1985, the first blood-screening EIA test to detect HIV was licensed and quickly implemented by blood banks to protect the blood supply. And, in 1992, testing of donor blood for both HIV-1 and HIV-2 antibodies (anti-HIV-1 and anti-HIV-2) was implemented. In 1996, HIV p24 antigen testing of donated blood was mandated although the test does not completely close the HIV window. Now, the p24 antigen testing is going to be compared with a PCR-based test for their ability to detect HIV in the window period.

Antibodies to Human T-Lymphotropic Virus, Types I and II (Anti-HTLV-I, -II)

HTLV retroviruses are endemic in Japan and the Caribbean but relatively uncommon in the United States. They cause adult T-cell leukemia/lymphoma and a neurological disorder similar to multiple sclerosis. The infection can persist for a lifetime but rarely causes major illnesses in most people who are infected. In rare instances, the virus may, after many years of infection, cause nervous system disease or an unusual type of leukemia. HTLV-II infections are usually associated with intravenous drug usage, especially among people who share needles or syringes. Disease associations with HTLV-II have been hard to confirm, but the virus may cause subtle abnormalities of immunity that lead to frequent infections, or rare cases of neurological disease.

In 1989, human T-lymphotropic virus antibody testing of donated blood was begun. Blood is now routinely screened for antibodies to HTLV-I, II. These tests screen for antibodies directed against epitopes of the HTLV-I and HTLV-II viruses. Several commercial assays based on the enzyme-linked immunosorbent assay (ELISA) or particle agglutination formats are used for screening of HTLV antibodies, followed by confirmatory assays using Western blotting. In some infected individuals, the serologic response to HTLV infection is very low. These problems have been solved by the application of PCR amplification of specific sequences in the virus genome. PCR can be used to detect HTLV-I, II provirus and is now the method of choice for detection of HTLV DNA directly from blood and many other tissues. Commercial PCR kits for HTLV are available (Green and Chen, 2001).

West Nile Virus

West Nile virus (WNV) is a single-stranded RNA virus of the Flaviviridae family and is the most recent emerging infectious disease threat to public health and, potentially, to the safety of our blood supply. In 2002, WNV was identified as transfusion transmissible. It is transmitted by mosquitoes to birds and other animals through a mosquito bite. The virus can infect people, horses, many types of birds, and some other animals. WNV was shown in 2002 to be transmissible by blood (Biggerstaff and Peterson, 2003), with an estimated mean risk of 2/10,000 to 5/10,000 in outbreak regions in the United States. The most common symptoms of transfusion-transmitted cases of WNV were fever and headache. Detection of WNV includes either a measurement of WNV antibodies or of WNV nucleic acid (detecting genetic material from the virus itself). There are two types of WNV antibody testing: IgM and IgG. In most individuals, IgM antibodies will be present within 8 days after the initial exposure to WNV, followed by IgG production several weeks later. But, the antibodies tested to detect WNV are not expedient for donor blood screening. Nucleic acid testing involves amplifying and measuring the West Nile virus's genetic material to detect the presence of the virus in blood or tissue. WNV NAT will be negative in the blood once clinical illness has occurred. In this situation, both NAT and IgM antibody testing may be needed. Nucleic

acid tests to screen blood for WNV are commercially available and in current use. But, the viral yield for WNV infection is much lower than other viruses. Consequently, a more sensitive WNV NAT system for donor blood screening will be required, which could further reduce the risks of transfusion transmitted WNV.

Syphilis

Serum samples from all blood units should be subjected to either the VDRL (Venereal Disease Research Laboratory) test or a treponemal test, such as the *Treponema pallidum* hemagglutination (TPHA) test before transfusion. Any unit found positive should be discarded as per standard safety procedures. This test is done to detect evidence of infection with the spirochete that causes syphilis. Blood centers began testing for this shortly after World War II, when syphilis rates in the general population were much higher. The risk of transmitting syphilis through a blood transfusion is exceedingly small (no cases have been recognized in this country for many years) because the infection is very rare in blood donors, and because the spirochete is fragile and unlikely to survive blood storage conditions. Sensitivity and specificity of serologic tests vary depending on the type of test performed and the stage of the disease. If the donor has spirochetemia, their serologic tests are usually negative, and if the donors are antibody positive, their blood is not infectious. Syphilis serological tests for donors have less clinical significance. A nucleic acid test for accurately detecting syphilis is needed. It can be used to determine whether a blood donor is currently or has recently been infected with the spirochete.

Other Concerns

Hepatitis Viruses

In recent years, numerous infectious agents found worldwide have been identified as potential threats to the blood supply and among these are several newly discovered hepatitis viruses that present unique challenges in assessing possible risks. Even if the hepatitis virus test is negative for all known A–E hepatitis agents, there are some unidentified hepatitis viruses, called non A–E hepatitis viruses that can still be transmitted by blood transfusion. In the future, advances in NAT may allow rapid discovery of the unknown hepatitis viruses.

TT Virus

TT virus (TTV) (Cossart, 2000), named for the patient from whom it was first isolated with non A–E and G post-transfusion hepatitis in Japan in 1997, is a newly discovered transfusion transmitted, single-stranded and circular DNA virus (Okamoto et al., 1999). TTV is non-enveloped and its entire sequence of ~3.9 kb has been determined. It is also often interpreted as a transfusion-transmitted virus (Cossart, 2000). At least 16 genotypes have been identified, and TTV is now found

all over the world. TTV infection was sought by detection of TTV DNA in serum by polymerase chain reaction using primers generated from a conserved region of the TTV genome (e.g., the UTR region) (Das et al., 2004). Donor blood and blood product can be screened for TTV DNA by using PCR or real-time PCR. The significance of positive findings is still unclear, because high-level TTV carriers in healthy populations are currently found (Nishizawa et al., 1997; Blut, 2000). Whether TTV actually causes hepatitis remains to be determined.

Cytomegalovirus

Cytomegalovirus (CMV) is a virus belonging to the herpes group that is rarely transmitted by blood transfusion. Donor blood is not routinely tested for CMV, and the prevalence of CMV antibody ranges from 50% to 80% of the population. But, blood contaminated with CMV can cause problems in neonates or immunocompromised patients. It also remains a major pathogen for solid-organ transplant recipients causing febrile syndromes, hepatitis, pneumonitis, retinitis, and colitis. Potential problems in selected patient populations can be prevented by transfusing CMV negative blood or frozen, deglycerolized red blood cells. Serologic tests for antibody to CMV are useful for determining whether a patient had CMV infection in the past, a determination of great clinical importance for organ and blood donors, and in the pretransplant evaluation of prospective transplant recipients (Pass, 2001). Commercial NAT kits are available for CMV (Hayden, 2004), these include the Amplicor PCR CMV Monitor test and Hybrid capture system CMV DNA test.

Malaria

Sensitive screening tests for malaria are neither commercially available nor officially approved yet. The most effective way of screening donors is to take a proper history of malaria or of fever that could be due to malaria. Donor selection criteria should be designed to exclude potentially infectious individuals from donating red blood cells for transfusion. Because there are no practical laboratory tests available to test donor blood, donors traveling to high-risk malaria areas are excluded from donating blood for 6 months. However, there is a need to develop suitable screening tests, especially for use in an endemic area. A number of clinical research approaches have been developed for the extraction, amplification, and detection of malaria parasite DNA from blood products (Pass, 2001).

Severe Acute Respiratory Syndrome

Coronavirus is an RNA virus known to be associated with respiratory disease. Severe acute respiratory syndrome (SARS) virus is a newly recognized coronavirus whose genome sequence does not belong to any of the known coronavirus groups and which quickly spread all over the world from Asia in 2003. There has been no evidence that this infection is transmitted from blood donors

to transfusion recipients, but the virus associated with SARS is present in the blood of people who are sick, and it is possible that the virus could be present in blood immediately before a person gets sick, so that an individual with infection but no symptoms possibly could transmit SARS through a blood donation. To help determine whether or not an individual might be infected with SARS, a blood collection facility will ask a potential donor orally or in writing about any travel to a SARS-affected country or a history of SARS or possible exposure to SARS. Enzyme-linked immunoassays for detection of specific IgG and IgM antibodies and RT-PCR for detection of SARS coronavirus–specific RNA in SARS patients has been developed. Rapid, sensitive, and specific identification of SARS and other novel coronaviruses by molecular methods will be very important in the future.

Discovery of Unrecognized and Uncharacterized Viral Agents

Based on past history, it is not just a hypothetical risk that many people have been infected with unrecognized viruses, for example, many patients with symptoms of non A–E, G, and TTV post-transfusion hepatitis. It is still possible that unexplained cases of post-transfusion hepatitis may be caused by a new, undiscovered pathogen. In recent years, numerous new infectious agents found worldwide have been identified through time-consuming procedures. By the time a new virus, such as HCV, HIV, and SARS, is found, many people are infected and there could be a large number of fatalities. There is an urgent need to develop methods for rapid identification and characterization of previously unknown pathogenic viruses. The most recent technologies for detecting and identifying previously unrecognized pathogens are expression library screening, representational difference analysis, and broad-range polymerase chain reaction. But they are all time-consuming approaches. The new unrecognized and uncharacterized viral agents can be rapidly identified by some of the new molecular approaches, for example, subtraction hybridization (Hu and Hirshfield, 2005) and DNA microarray.

Conclusion

Ensuring the safety and efficacy of blood and blood products is a critical regulatory challenge. The high safety level of the blood supply is the result of continued improvements in blood donor screening and testing. It will be achieved by introducing more updated nucleic acid tests to the field of blood safety. Nucleic acid testing is a method of testing blood that is more sensitive and specific than conventional tests that require the presence of antibodies to trigger a positive test result. Also, NAT works by detecting the low levels of viral genetic material present when an infection occurs but before the body develops an immune response to a virus. This improved sensitivity should enable us to significantly decrease the infection window period, allowing for earlier detection of the infection and diminishing the

chances for transmission of the agent via transfusion. We are not only to protect the blood supply from known pathogens but also the emergence of new and unrecognized and uncharacterized infectious agents. The NAT methods are more sensitive and specific compared with non-NAT. In the future, NAT technology, such as PCR, may allow routine screening of donors for all the known and unknown pathogens of concern to blood safety.

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References

Biggerstaff, B.J., & Peterson, L.R. (2003). Estimated risk of transmission of the West Nile Virus through blood transfusion in the US, 2002. *Transfusion, 43,* 1007–1017.

Blut, A. (2000). TT Virus. *Infusion Therapy Transfusion Medicine, 27*, 114–116.

CDC. (1997). Immunization of health-care workers: Recommendations of the advisory committee on immunization practices (ACIP) and the hospital infection control practices advisory committee (HICPAC). *Morbid Mortal Weekly Rep, 46*, 1–42.

Chamberland, M.E. (2001). Emerging infectious disease issues in blood safety. *Emer Infect Dis, 7*, 552-553.

Cohen J. (1999). The scientific challenge of hepatitis C. *Science, 285*, 26–30.

Cossart, Y. (2000). TTV-a virus searching for a disease. *J Clini Virol, 17*, 1–3.

Das, K., Kar, P., Gupta, R., & Das, B. (2004). Role of transfusion-transmitted virus in acute viral hepatitis and fulminant hepatic failure of unknown etiology. *J Gastroenterol Hepato, 19,* 406–412.

Ginocchio, C.C., Kemper, M., Stellrecht, K.A., & Witt, D.J. (2003). Multicenter evaluation of the performance characteristics of the nucliSens HIV-1 QT assay used for quantitation of human immunodeficiency virus type 1 RNA. *J Clin Microbiol, 41*, 164–173.

Green, P.L., & Chen, I.S.Y. (2001). Human T-cell leukemia virus types 1 and 2. Section two: specific virus families, *Fields Virology*, Fourth ed. Lippincott Williams & Wilkins, Philadelphia, 1, (pp. 1941–1969).

Guichon, A., Chiparelli, H., Martinez, A., Rodriguez, C., Trento, A., Russi, J.C., & Carballal, G. (2004). Evaluation of a new NASBA assay for the qualitative detection of hepatitis C virus based on the NucliSens Basic Kit reagents. *J Clin Virol, 29,* 84–91.

Hersing, D.H. (1993). In vitro nucleic acid amplification techniques. In *Diagnostic Molecular Microbiology Principles and Applications.* Mayo Foundation, Rochester, MN, ASM Press, pp. 51–87.

Hu, Y., and Hirshfield, I. (2005). Rapid approach to identify an unrecognized viral agent. *J Virol Methods, 127*, 80–86.

Hu, Y., Shahidi, A., Park, S., Guilfoyle, D., & Hirshfield, I. (2003). Detection of extrahepatic HCV replication by a novel highly sensitive single tube nested-PCR. *Am J Clin Pathol, 119*, 95–100.

Hollinger, F.B., & Jake, T. (2001). Hepatitis B virus. Section two: specific virus families. *Fields Virology.* Fourth ed. Lippincott Williams Wilkins. & Philadelphia, 87, (pp. 2971–3036).

Katsoulidou, A.S., Moschidis, Z.M., Gialeraki, R.E., Paraskevis, D.N., Sypsa, V.A., Lazanas, M.C., Tassopoulos, N.C., Psichogiou, M.A., Boletis, J.N., Karafoulidou, A.S., & Hatzakis, A.E. (2004). Clinical evaluation of an HIV-1 and HCV assay and demonstration of significant reduction of the HCV detection window before seroconversion. *Transfusion, 44*, 59–66.

Krajden, M., Comanor, L., Rifkin, O., Grigoriew, A., Minor, J.M., & Kapke, G.F. (1998). Assessment of hepatitis B virus DNA stability in serum by the Chiron quantiplex branched-DNA assay. *J Clin Microbiol, 36*, 382–386.

Lamballerie, XD. (1996). Serological and molecular biology screening techniques for HCV infection. *Nephrol Dialysis Transplant, 4*, 9–11.

Abbott Laboratories. 2003. LCx HIV RNA quantitative assay from Abbott Laboratories. Abbott Laboratories, Abbott Park, IL.

Major, M.E., Rehermann, B., &, Feinstone, S.M. (2001). Hepatitis C viruses. Section two: specific virus families. *Fields Virology*, Fourth ed. Lippincott Williams & Wilkins, Philadelphia, (pp.1127-1161).

Mornet, E. (2004). DNA, 50 years of the double helix: from the concept of molecular hybridization to microarrays. *Gynecol Obstetr Fertil, 3,* 895–899.

Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., & Mayumi, M. (1997). A novel DNA virus (TTV) associated with elevated transaminase levels in post-transfusion hepatitis of unknown etiology. *Biochem Biophys Res Commun, 241,* 92–97.

Okamoto, H. Nishizawa, T., & Ukita, M. (1999). A novel unenveloped DNA virus (TT virus) associated with acute and chronic non-A to G hepatitis. *Intervirology, 42*, 192–204.

Palmer, S., Wiegand, A.P., Maldarelli, F., Bazmi, H., Mican, J.M., Polis, M., Dewar, R.L., Planta, A., Liu, S., Metcalf, J.A., Mellors, J.W.,& Coffin, J.M. (2003). New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol, 41*, 4531–4536.

Pass, R.F. (2001). Cytomegalovirus. Section two: specific virus families. *Fields Virology*, Fourth ed. Lippincott Williams & Wilkins, Philadelphia, (pp. 2676–2705).

Hayden, R.T. (2004). In vitro nucleic acid amplification techniques. In: Persing, D.H., Tenover, F.C., Versalovic, J., Tang, Y.W., Unger, E.R., Relman, D.A., & White, T.J., eds., *Molecular Microbiology: Diagnostic Principles and Practice.* Washington, DC, ASM Press, pp. 43–69.

Peter, J.B., & Sevall, J.S. (2004). Molecular-based methods for quantifying HIV viral load**.** *AIDS Patient Care STDS, 18*, 75–79.

Revelle, M. (1995). Progress in blood supply safety. *FDA Consumer Magazine, 29*, 21–24.

Roche. 2003. Roche Amplicor HIV-1 Monitor UltraSensitive quantitative assay. Roche, Nutley, New Jersey.

Schena, M. (2002). Introduction to microarray analysis. *In Microarray Analysis.* John Wiley & Sons, Hoboken, NJ, pp. 1–26.

Starkey, W.G., Millar, R.M., Jenkins, M.E., Ireland, J.H., Muir, K.F., & Richards, R.H. (2004). Detection of piscine nodaviruses by real-time nucleic acid sequence based amplification (NASBA). *Dis Aquatic Organisms, 5,* 93–100.

Stramer, S.L., Glynn, S.A., Kleinman, S.H., Strong, D.M., Sally, C., Wright, D.J., Dodd R.Y., & Busch, M.P. 2004. Detection of HIV-1 and HCV infections among antibody-negative blood donors by nucleic acid-amplification testing. *N Engl J Med, 19*, 819–822.

Tang, Y.W., Procop, G.W., & Persing, D.H. (1997). Molecular diagnostics of infectious diseases. *Clin Chem, 43*, 2021–2038.

U.S. Food & Drug Administration. (2004). Testing requirements for communicable disease agents. *Federal Register, 21CFR 610:40*, 75–80.

Wang, Q.M., & Heinz, B.A. (2001). Recent advances in prevention and treatment of hepatitis C virus infections. *Progress Drug Res*, Spec, 79–110.

Yu, X., Susa, M., Knabbe, C., Schmid, R.D., & Bachmann, T.T. (2004). Development and validation of a diagnostic DNA microarray to detect quinoloneresistant *Escherichia coli* among clinical isolates. *J Clin Microbiol, 42*, 4083–4091.