Detection of blood transmissible viral agents: implications for blood safety
Jongerius, J.M.

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Chapter 1

General introduction
Introduction

The safety of the blood supply has increased enormously over the last two decades. This is particularly due to the selection of voluntary nonremunerated blood donors, the continuous improvements and new approaches to laboratory testing of donated blood, and the use of specific viral inactivation methods for blood products. However, there still remains a residual risk of recipient infection from today's blood supply. The predominant source of this risk is attributable to blood collected from donors who are in the preseroconversion phase of infection, the so-called window period. Other sources of residual risk are emerging viral mutants, atypical seroconversions, immunosilent infections, newly identified blood borne viral agents and last but not least laboratory testing errors [1-3].

In The Netherlands as well as in many other countries, blood donations are routinely screened for hepatitis B virus (HBV), human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2), hepatitis C virus (HCV), human T-lymphotropic virus type I and II (HTLV-I and HTLV-II) and Treponema pallidum. Apart from the direct virus-detection test for HBV, the screening tests used are based on antibody detection. Despite the continuous improvement of such screening tests, the existence of a window period, remains a problem. To improve the safety of the blood supply, a new technology, nucleic acid amplification technology (NAT), was recently introduced in blood banking. Instead of screening for viral antibodies or proteins, using NAT, the presence of nucleic acid sequences, unique to a particular viral agent, can be determined in blood samples. Thus, (infectious) window period blood donations that otherwise escape detection may be identified. Routine HCV NAT screening of blood donations was implemented in July 1999 in The Netherlands. The HCV NAT minipool screening program was extended with HIV NAT in November 2000. The implementation of NAT for other blood borne viral agents is currently under discussion. Efforts to even further improve the safety of the blood supply will continue to be explored. However, the necessity and feasibility to achieve a "zero-risk" for blood transmissible viral agents is momentarily questioned,
especially in view of the high cost benefit of additional measures [4-6].

This thesis focuses on the limitations and new developments of laboratory testing for blood transmissible viral agents. In addition, the transmission of the recently identified GBV-C virus by blood was studied.

**Hepatitis B virus**

Hepatitis B virus (HBV) is an enveloped virus, 42 nm in diameter, containing a partially double stranded circular DNA genome. HBV belongs to the family of the *Hepadnaviridae*. At least six HBV genotypes, designated A-F, can be distinguished [7]. HBV is transmitted by parenteral routes, intimate contact and vertically from mother to child at birth. Hepatitis B surface antigen (HBsAg) is the major target for detecting acute or chronic infections with HBV. Antibodies to HBsAg confer protection after infection or vaccination. Antibodies to the hepatitis B core antigen (anti-HBc) become detectable 3-4 weeks after the appearance of HBsAg and usually persist for many years. Thus, anti-HBc antibodies are considered as a reliable marker of (past) HBV infection [8]. Currently, in The Netherlands, blood donor screening for HBV infections entirely relies on the detection of HBsAg.

Since the early nineteen seventies, diagnostic tests are available for HBV diagnosis. At present, HBV testing is performed using third generation immunoassays for the detection of HBsAg. Using these tests in blood donation screening does not completely eliminate the risk of HBV transmission by blood products. HBsAg tests may be negative in the window period of HBV infection, in the early convalescence phase (core window) of HBV infection, and during chronic HBV infection when very low levels of HBsAg are present [10-12]. Additionally, emerging HBsAg mutant forms of HBV, expressing the HBsAg “a” determinant with a changed amino acid composition, cause a threat to the safety of the blood supply [3,6,9].

The major antigenic domain of the HBsAg protein is the “a” determinant which is formed by a hydrophilic region located between amino acids 124 to 147. Antibodies to
the “a” determinant of HBsAg confer protection to HBV after infection or vaccination. Various HBsAg mutant forms, carrying one or more mutations in the HBV S (surface or envelope) gene compared to the wild type virus, have been reported. HBsAg mutant forms were initially observed in vaccinees, monoclonal antibody treated patients, as well as in patients without antibody to HBsAg. The most common HBsAg mutant form has a R (Arg) replacement of G (Gly) at position 145 of the envelope protein [13]. Other replacements include E (Glu) at position 141 and N (Asn) at position 126 [14]. Epitope changes of the ‘a’ determinant due to mutation(s) of the HBV S gene can result in false-negative results in the routinely used HBsAg screening assays [14-16].

Schreiber et al. estimated the residual risk of transmitting HBV infection by the transfusion of screened donor blood in the USA to be 1 in ~ 63,000 [9]. The estimated length of the HBV window period used for calculating this risk was 59 days [17]. This risk was estimated by calculating the incidence of infection in repeat blood donors with the length of the window period as fraction of a year. As shown in Table 1, the incidence of HBV infection among repeat donors in The Netherlands during the period of 1995 through 2000 was 1.3 per 100,000 donors. Using Schreiber’s method, the residual risk of transfusion transmitted HBV infection in The Netherlands, for 1995 through 2000, is 1 in ~ 475,000 screened blood donations.

To further improve the safety of the blood supply, the implementation of HBV NAT testing in minipools is discussed. The currently used HBsAg screening tests are highly sensitive and HBV DNA levels are relatively low during the pre-HBsAg-positive infectious window period with an average of less than 1,000 copies per mL (range: 1 to 2,400 copies per mL; genome doubling time: 4 days) [6]. Busch et al. reported that implementation of HBV NAT reduces the HBV window period with approximately 6-15 days [1]. Based on this window period reduction estimate, the residual risk may decline in the Netherlands from 1 in ~ 475,000 to 1 in ~ 530,000 to 635,000 screened blood donations. Thus, the protected yield of implementing HBV NAT is small. For this reason, HBV is a less feasible target for NAT in minipools compared to HCV and HIV [6].
Table 1. Viral infections per 100,000 donors in The Netherlands [1]

<table>
<thead>
<tr>
<th>Year</th>
<th>HBV infection</th>
<th>HIV infection</th>
<th>HCV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>New *</td>
<td>Repeat †</td>
<td>New</td>
</tr>
<tr>
<td>1995</td>
<td>61</td>
<td>2.7</td>
<td>0</td>
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<td>1996</td>
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<tr>
<td>1998</td>
<td>63</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>1999</td>
<td>86</td>
<td>0.7</td>
<td>2.3</td>
</tr>
<tr>
<td>2000</td>
<td>44</td>
<td>1.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Mean</td>
<td>59</td>
<td>1.3</td>
<td>2.0</td>
</tr>
</tbody>
</table>

/ Sanquin jaarverslag 2000.
* first time blood donors.
† repeat blood donors.

Note: incidence among repeat blood donors has been used for calculating residual risks.

Human immunodeficiency virus

Human immunodeficiency virus (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS), is an enveloped single stranded RNA virus belonging to the lentivirus subfamily of the Retroviridae. It was originally named lymphadenopathy-associated virus (LAV) or human T-cell lymphotropic virus type-III (HTLV-III). The virus, which was first identified in 1983 is now designated HIV-1 [18,19]. A closely related strain, HIV-2, which is endemic in West Africa, has been identified in 1986 [20]. HIV-1 can be divided in three genetically distinct groups, group M (major), group O (Outlier) and the recently discovered group N [21-24]. In group M, the genotypes A through K can be distinguished [23]. HIV is predominantly transmitted by sexual intercourse, sharing of contaminated injecting equipment, and vertically to infants from HIV infected mothers. In the early stages of the HIV epidemic the receipt of HIV contaminated blood components was also an important route of spread. The first
reports on transfusion-associated AIDS were published in late 1982 and early 1983 [25-27]. HIV has been transmitted through receipt of whole blood, cellular components, plasma and clotting factors [28,29].

After the introduction of HIV-antibody testing in 1985, the incidence of transfusion transmitted HIV infection declined dramatically. The first commercially available tests employed HIV-1 lysate proteins for the detection of HIV-antibodies. Over time, HIV-antibody tests have been improved continuously to increase both sensitivity and specificity. At present, HIV antibody testing of blood donations is mainly performed using third generation “double antigen” immunoassays. These assays employ both recombinant HIV antigens and synthetic HIV peptides, enabling simultaneous and earlier IgM and IgG detection of HIV-1 and HIV-2 antibodies. In some countries blood donations are additionally tested for the presence of the HIV p24 antigen, which is detectable approximately 1 week before the appearance of HIV antibodies [30,31]. Currently, fourth generation tests are available which combine detection of HIV p24 antigen and HIV antibodies.

The main source of residual risk for HIV infection in blood banking is attributable to donor blood collected during the window period of HIV infection. The incidence of such events is calculated 1 in ~493,000 in the USA [9]. The incidence of HIV infection among repeat donors in The Netherlands during the period 1995 through 2000 was 0.6 per 100,000 donors (Table 1). Using Schreiber’s method combined with a preseroconversion window period estimate of 22 days, the residual HIV risk for The Netherlands is 1 in ~2,780,000 screened blood donations [8,32]. To further diminish the risk of virus transmission via blood donated during the early window period of HIV infection various national blood transfusion organizations presently use, or are in the process of implementing, routine HIV NAT testing in so-called minipools varying from 24 to 96 donations. The Dutch blood supply foundation, Sanquin, has implemented HIV NAT minipool screening as of November 2000. HIV NAT testing narrows the HIV window period with approximately 10-15 days [1]. Thus, theoretically, the residual HIV risk attributed to window period donations in The Netherlands has declined from 1 in ~
2,780,000 to 1 in ~ 5,000,000 to 8,335,000 screened donations following NAT-testing.

Another important residual risk source is the emergence of HIV variants which may not be detected by commercially available antibody screening assays [2,3]. The isolation and characterization of the Cameroonian variant HIV strains ANT-70 and MVP-5180, resulted in modifications of HIV antibody tests to correct deficiencies in sensitivity [21,22,33]. Modified tests should always be evaluated, even to ensure the sensitivity in detecting the more common HIV strains [34]. The sensitivity problems (false-negative HIV tests) with a third-generation HIV antibody detection test (IMx HIV-1/HIV-2 Plus, Abbott) which occurred in 1996 in Europe further stress the necessity for continuous monitoring of routinely used screening tests [35,36].

**Hepatitis C virus**

Hepatitis C virus (HCV) is an enveloped single-stranded RNA virus [37]. HCV, the major causative agent of post-transfusion hepatitis non-A, non-B (PTH-NANB), was discovered in 1989. HCV has been classified within a third independent genus of the *Flaviviridae* family, which also includes flavivirus and pestivirus genera [38]. Different stains of HCV demonstrate a remarkable degree of nucleotide sequence diversity. Currently, at least 6 major genotypes can be distinguished [39]. HCV is predominantly transmitted by infected blood. The risk of sexual transmission of HCV is absent or very low and vertical transmission from an infected mother to child is infrequent [40-42].

After the cloning of a specific part of the HCV genome in 1989, the rapid development and introduction in 1990 of first generation anti-HCV screening tests for application in routine donation screening led to the prevention of PTH-NANB in ~63% of cases [43,44]. The first commercially available screening tests employed only the C100 recombinant antigen which is derived from the non-structural NS4 region of the HCV genome [38]. Second generation anti-HCV tests were introduced during 1991 and employed both structural (C22/core) and non-structural (NS3/NS4, C100 and C200) derived recombinant or synthetic antigens. Second generation HCV screening tests, which
showed a largely increased sensitivity in detecting anti-HCV antibodies, reduced the window period by several weeks [45,46]. The sensitivity of third generation anti-HCV tests, employing an additional recombinant antigen derived from the NS5 region of the HCV polyprotein, which were introduced in 1993, was slightly improved (with regard to anti-NS3 detection) compared to second generation tests. In contrast, the addition of the NS5 antigen had adverse effects on the specificity of the third generation tests [47,48].

Despite the continuous improvement of test sensitivity, the risk of HCV transmission due to window period donations has remained an issue. It may take as long as an average of 70 days after HCV infection before anti-HCV antibodies reach detectable levels [48]. The residual risk of transfusion-transmitted HCV infection in the USA was calculated to be 1 in ~103,000 screened blood donations [9]. The incidence of HCV infection among repeat donors in The Netherlands during 1995 through 2000 was 0.3 per 100,000 donors (Table 1). Accordingly, for The Netherlands, the residual HCV risk calculated by Schreiber’s method combined with a preseroconversion window period estimate of 70 days is 1 in ~1,725,000 screened blood donations [9,48]. HCV NAT screening in minipools (maximum 48 donations), a method which reduces the HCV window period with approximately 41-60 days [1], was implemented in The Netherlands in July 1999 [49]. Due to HCV NAT testing, the residual risk attributed to donations collected during the window period of HCV infection has theoretically declined from 1 in ~1,725,000 (when only screening of anti-HCV antibodies was used) to 1 in ~4,165,000 to 12,500,000 screened blood donations. Thus, the application of HCV NAT testing virtually eliminates the risk of HCV transmission by blood products. During the first two years of routine HCV NAT minipool screening of up to 48 donations, no HCV RNA-positive, anti-HCV-negative blood donations were found among ~1,800,000 blood donations collected in The Netherlands.

Recent studies have shown that the HCV core antigen is detectable throughout the viremic window period of HCV infection [50-52]. It was shown that the core protein becomes detectable, on average, within 1-2 days after the appearance of viral RNA [50-52]. Currently, a new diagnostic test is commercially available for the direct detection of
the HCV core antigen. This assay appears to be suitable for high-throughput screening of blood donations [53]. HCV core antigen testing may be a useful alternative for HCV NAT, especially in countries which lack routine application of NAT.

**GB virus type C**

Through the identification of HCV and the development of screening tests it became possible to show that HCV was responsible for the vast majority of PTH-NANB cases [44,45]. However, approximately 10 percent of PTH-NANB are unrelated to HCV infection, suggesting the existence of additional causative agents (non-A-E hepatitis) [54]. In 1995, Simons et al. identified a novel RNA virus, designated GB virus type C (GBV-C), in patients with clinical evidence of hepatitis of unknown etiology [55]. In addition Linnen et al. independently identified a similar RNA virus, designated hepatitis G virus (HGV), from plasma of a patient with chronic hepatitis [56]. Sequence analysis indicated that GBV-C and HGV are different isolates of the same virus. The homology between GBV-C and HGV at the nucleotide and amino acid level is approximately 86 and 95%, respectively [57]. Both viruses are classified in the family of *Flaviviridae* but are distinct from HCV. The homology at the nucleotide and amino acid level between GBV-C/HGV and HCV is approximately 25 and 29%, respectively [57,58]. The GBV-C/HGV genome of 9,392 nucleotides encodes for a single large polyprotein [56-59]. GBV-C/HGV is transmitted parenterally, sexually and perinatally [60-63]. In the further part of this section GBV-C/HGV will be referred to as GBV-C.

GBV-C detection predominantly relies on nucleic acid amplification by reverse-transcription polymerase chain reaction (RT-PCR) using specific primers from the 5' non-translated region, the NS5a or the NS3 region [57,59]. In contrast with HCV, the envelope E2 region of the genome, encoding for the E2 protein, is not hypervariable [58,64]. This finding may be of importance for the effectiveness of the humoral immune response against GBV-C [65]. In 1997, Tacke et al. reported the development of an immunoassay for anti-E2 antibody detection based on a recombinant derived E2 protein [65]. It was
concluded that a humoral immune response to E2 is associated with a loss of detectable GBV-C RNA. Thus, anti-E2 may be a useful marker to confer a past/resolved GBV-C infection. However, simultaneous GBV-C RNA-positivity and GBV-C anti-E2-positivity, representing early viral clearance, incomplete protection by circulating GBV-C anti-E2 antibodies or false-positive EIA results, was found in several individuals [65].

Three out of 79 (4%) patients enrolled in a prospective study on the incidence of transfusion-associated GBV-C infection were found to be infected with HCV before transfusion and the cause of acute hepatitis could not be determined. 63 (80%) had transfusion related HCV infections of which 6 (10%) were co-infected with GBV-C. Three (4%) recipients were found to be solely GBV-C RNA-positive after transfusion. The remaining 10 (13%) recipients had no serological or molecular viral marker for any of the established hepatitis viruses [59,60]. The 3 recipients infected with GBV-C only had mild hepatitis (no jaundice; mean peak alanine aminotransferase level: 198 U/L) and the combined HCV and GBV-C infections were no more severe than HCV infections alone. In this study no causal relationship between GBV-C and hepatitis could be established. Thus, GBV-C accounts for only a minority of non-A-E hepatitis cases. In addition, a study performed by the Sentinel Counties Viral Hepatitis Study Team revealed that GBV-C was not implicated as an etiologic agent of non-A-E hepatitis [66]. It was also found that GBV-C infection did not lead to chronic disease and did not affect the clinical course of acute disease among patients infected with hepatitis A, B or C [66]. From these and other studies it can be concluded that GBV-C infection is not unequivocally causally related to acute or chronic hepatitis and does not alter the natural history of other chronic liver diseases [59,60,66,67]. Thus, given the apparently low or absent pathogenicity of GBV-C the need of blood donation screening procedures to prevent GBV-C infection is highly questionable [68].
Aims of the studies described in this thesis

The aims of the studies described in this thesis are:

1) to investigate a number of currently used, improved and or new methods for the detection of blood transmissible viral agents (chapter 2, 3, 5, 6, 7).

2) to determine the prevalence of GBV-C in various groups of patients and blood donors (chapter 8).

3) to determine the incidence of post-transfusion GBV-C, HCV and HBV infection in patients who underwent cardiac surgery (chapter 9).

4) to investigate the efficacy of a new strategy to retrospectively investigate the infectivity of blood products in a case of post-transfusion HBV infection (chapter 4).
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