Diagnostic strategies for blood borne infections in Sweden
You are never too old to set another goal or to dream a new dream

C.S. Lewis
Diagnostic strategies for blood borne infections in Sweden
Abstract


In all kinds of transfusion of blood products and transplantation of tissue and organs the risk of spreading blood borne infections to the recipient is something to always have in mind. The main infections of interest are Hepatitis B, Hepatitis C, HIV-1/2, HTLV-1/2 and Syphilis.

These infections are spread mainly in three modes of transmission: by contaminated blood products or syringes, sexual transmission and mother-to-child transmission during pregnancy, delivery and breastfeeding. Screening assays for these infections are mainly based upon serological methods.

The aims of this thesis were to evaluate some of the screening assays and screening strategies for blood borne infections that are in use in Sweden, and to study the prevalence of one blood borne infection in Sweden. Our results show that the newest immunoassays for HIV have narrowed the diagnostic window, without being less specific.

We have also shown that a new automated Hepatitis C antigen assay has simplified the detection of viremia level in HCV-infected patients, at least in high viremic patients. The regulations stipulates antibody screening of HCV in blood donors, but a HCV core antigen assay could be a useful addition in the screening strategy, to prevent HCV transmission.

The new automated syphilis antibody assays used as first line screening assay in the reverse algorithm testing showed high sensitivity in our evaluation. Due to automation, they are preferred against the non-treponemal agglutination assays previously used. The specificity, though, differs between the automated assays, something to take into account when choosing an assay for blood donor screening.

Finally, a prevalence study on HTLV-1/2 infection in Sweden showed low prevalence, but the infection is 10 times more prevalent among IVF clients than among blood donors. Nevertheless, given the low prevalence and that the situation has not changed since last evaluation in the 1990’s, the current screening strategy of new donors only, seems appropriate.

Keywords: blood borne infections, screening assays, blood donors, HIV, HTVL-1/2, Hepatitis C, Syphilis.

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Sammanfattning

Vid screening av blodburna infektioner hos blodgivare och donatorer av vävnader och organ, är det mycket viktigt att de analyser som används har hög sensitivitet och specificitet. Främst är det hepatit B och C, HIV-1 och HIV-2, HTLV-1 och HTLV-2 och syfilis som är de infektioner som testas och kontrolleras hos blodgivare.

Dessa infektioner sprids huvudsakligen via tre vägar; smittade blodprodukter eller kanryler, sexuell överföring och mor till barn smitta under graviditet, förlossning och amning. Screening av dessa infektioner är huvudsakligen baserad på serologiska metoder. Dock är analyser för att upptäcka dessa infektioner bara en del av säkerhetsprogrammet. Nätbaserad enkät och intervju av givare ifråga om riskbeteende är minst lika viktig. Denna avhandling är dock inriktad på just testerna.

Syftet med denna avhandling var att utvärdera några av de screeninganalyser och screeningstrategier för blodburna infektioner som används i Sverige, samt att studera förekomsten av en blodburen infektion i Sverige.

Våra resultat visar att de nyaste fjärde generationens HIV-tester har minskat det diagnostiska fönstret, utan att vara mindre specifika. Dessa tester är också numera de som föreskrivs för blodgivare av Socialstyrelsen.

Vi har också visat att en ny automatiserad hepatit C antigen-analys kan förenkla kontroll av virusnivåer hos HCV-infekterade patienter, åtminstone hos högviremiska patienter. Denna test kan även vara ett complement till antikroppsscreeningen av blodgivare, som en extra säkerhet mot överföring av hepatit C-smitta.

De nya automatiserade syfilistesterna som används som primär screeningsanalys med den omvända testalgoritmen visade hög känslosighet i vår utvärdering. Beroende på efterfrågan på automatiserings, har dessa treponemala tester ersatt de tidigare så vanliga non-treponemala testerna för blodgivare. Specificiteten skiljer sig dock mellan de olika analyserna, något att ta hänsyn till när man väljer en analys för blodgivarscreening.

List of publications


III. Malm K, Duberg A, Sundqvist M, Fredlund H, Andersson S. Evaluation of a hepatitis C virus core antigen assay to monitor viral load in patients on antiviral therapy and in untreated patients. Manuscript


Other publications that is relevant for this thesis:


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**List of abbreviations**

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACU</td>
<td>Antenatal care unit</td>
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<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<td>Anti-HBc</td>
<td>Antibodies to Hepatitis B core antigen</td>
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<td>Anti-HBe</td>
<td>Antibodies to Hepatitis B e antigen</td>
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<td>Anti-HBs</td>
<td>Antibodies to Hepatitis B surface antigen</td>
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<td>ATLL</td>
<td>Adult T-cell leukaemia/lymphoma</td>
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<td>CD</td>
<td>Classification determinant (cell surface molecules)</td>
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<td>CDC</td>
<td>Centers of disease control</td>
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<td>CLIA</td>
<td>Chemiluminescent Immunoassay</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EBV</td>
<td>Epstein Barr Virus</td>
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<td>ECL</td>
<td>Electro-chemiluminescence</td>
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<td>EIA</td>
<td>Enzyme-like Immuno assay</td>
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<td>HAM</td>
<td>HTLV associated myelophaty</td>
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<td>HAV</td>
<td>Hepatitis A virus</td>
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<td>HBeAg</td>
<td>Hepatitis B e antigen</td>
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<td>HBsAg</td>
<td>Hepatitis B surface antigen</td>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>HDag</td>
<td>Delta antigen of Hepatitis D virus</td>
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<td>HDV</td>
<td>Hepatitis D virus</td>
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<td>HIV</td>
<td>Human Immunodeficiency virus</td>
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<td>HTLV</td>
<td>Human T-lymphotropic Virus</td>
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<td>ID-NAT</td>
<td>Individual NAT</td>
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<td>IDU</td>
<td>Intravenous drug user</td>
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<td>IVF</td>
<td>In vitro fertilization</td>
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<td>LIA</td>
<td>Line immunoassay</td>
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<td>MP-NAT</td>
<td>Minipool NAT</td>
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<td>NAT</td>
<td>Nucleic acid amplification test</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PHI</td>
<td>Primary HIV infection</td>
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<td>POC</td>
<td>Point of care</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>RIA</td>
<td>Radio immunoassay</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RPR</td>
<td>Rapid plasma regain</td>
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<td>STI</td>
<td>Sexual transmitted infections</td>
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<td>SVR</td>
<td>Sustained virological response</td>
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<td>TPHA</td>
<td>Treponema pallidum haemagglutination</td>
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<td>TPPA</td>
<td>Treponema pallidum particle agglutination</td>
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<td>TSP</td>
<td>Tropical spastic paraparesis</td>
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<td>VDRL</td>
<td>Veneral disease research laboratory</td>
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<td>WB</td>
<td>Western Blot</td>
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<td>WHO</td>
<td>World health organization</td>
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Introduction

Viral and bacterial infections are spread between humans in many different ways. Some infections are spread by direct contact with an infected person’s blood. These infections are often spread vertically between mother and child, and through sexual contact.

In an attempt to limit the transmission of these blood borne agents, screening programs of certain population groups, such as blood donors, are implemented in many countries, as well as awareness campaigns concerning sexual health, safe sex practices and needle exchange programs for intravenous drug users (IDU). Blood sampling and analysis of suspect infections are also an important tool for minimizing the risk for transmission, and in this context, safe, sensitive and rapid assays for diagnosis of these infections is of uttermost importance.

Blood borne viral infections

Hepatitis B

Hepatitis B is the first discovered human hepatitis virus. It was first detected as a protein that occurred in serum and plasma along with a history of multiple blood transfusions, for example in leukemia patients (1). The protein was called Australia antigen (AU-antigen), because it was first discovered in a serum sample of an indigenous Australian. Later the AU-antigen was associated with hepatitis B (2-3). This antigen is a glycosylated envelope protein of the mature Hepatitis B (HBV) virus, and was renamed Hepatitis B Surface Antigen (HBsAg). The virus is a double-stranded DNA-virus belonging to the hepadnaviridae family. The viral particle, also called the Dane particle, is 42 nm in diameter. The viral DNA genome, which is about 3200 bp, is surrounded by a protein core and a lipid envelope, where the HBsAg is located (4).

Acute infection of Hepatitis B may be asymptomatic, especially in neonates and small children. If symptomatic, they usually have non-specific features such as nausea, anorexia, headache and diarrhoea. Vague abdominal pain and splenomegaly/hepatomegaly may also occur. Jaundice can occur, but more often the infection is anicteric. In 95% of cases, the infection resolves, the virus is cleared and antibodies to the different viral antigens are formed in a certain sequence. The first antibodies appears when virus is still present. Those antibodies are directed against the core antigen, and are named anti-HBc (antibodies to Hepatitis B core antigen).
When the HBsAg has been cleared, the antibodies to the different viral antigens against this antigen, anti-HBs, will appear. A third antigen, the Hepatitis B e antigen (HBeAg) is associated with the nucleocapsid of the virus, and is used as an infectivity marker. Normally, when this antigen disappears, its antibody, anti-HBe, will appear. When both HBsAg and HBeAg are present, the viral load is very high. The natural course of Hepatitis B infection is shown in Figure 1. The antigen and antibody markers are used in diagnosis of the infection, where HBsAg is used as a primary screening marker for the infection.

![Figure 1. Clinical course of Hepatitis B infection. The antigen and antibody markers are used in diagnosis and monitoring of the infection. Reproduced by permission from Abbott Scandinavia, Solna, Sweden.](image)

Over 90% of infected individuals will heal completely and eliminate the virus. The infection can become chronic, which means an ongoing liver infection, which later can develop into liver cirrhosis and liver cancer. The younger the age of infection the higher risk of developing a chronic infection. Children under school-age (0-6 years) when infected are the most likely to develop chronic infection, where 95% are infected as neonates. This is why it is important to prevent mother to child transmission.

Approximately 30% of the world’s population shows serological markers of past or present HBV infection. The global prevalence varies with some highly endemic areas, including China, Southeast Asia, major parts of Africa parts of the Middle East and the Amazon basin in Latin America (5). In these areas the most common transmission route is perina-
tal, from infected mothers to neonates. The infection can be transmitted intrauterine, during labor and post-partum through breast-feeding or intimate contacts between mother and child, where transmission during labor is by far the most common route (6). In order to prevent mother to child transmission, vaccination programs and immunoprophylaxis routines have been implemented in many countries, resulting in a decrease in the infection in many parts of the world (7-8). Blood safety routines have almost eliminated the risk of infection in blood transfusion, at least in the developed countries. Vaccination programs addressed to intravenous drug users have also decreased the infection in this population group, otherwise common victims to this infection.

In Sweden the prevalence of Hepatitis B is relatively low. Less than 0.5% of the population are chronic carriers of the virus. About 1500 new cases of hepatitis B are reported each year. Of these, approximately 95% are chronic infections, mainly consisting of members of immigrant families, who contract the infection in their country of origin. The most common infection route for acute hepatitis B in Sweden is through heterosexual contact. Infection via drug injection is less prevalent than in the beginning of the 2000s (9). A study conducted in the Stockholm area among young people (15-22 years) attending youth clinics showed an overall prevalence of HBV markers of 1.8% (10). Among persons with origin in endemic regions, the prevalence of HBV-markers was 6.5%. Twenty-nine percent of the study group (n=464) had been vaccinated. To prevent mother-to-child transmission, all pregnant women in Sweden are, since February 2005, offered HBsAg-screening on their first visit to antenatal care. If HBsAg tests positive, screening for other HBV-markers is done, as well as counselling and follow-up (11). Children born to HBsAg-positive mothers are vaccinated after birth, and some regions in Sweden provide vaccination to all children.

**Hepatitis D (Delta-virus)**

In 1977, Rizetto and colleagues found a new antigen-antibody system in Hepatitis B carriers, distinct from the other three systems in HBV-infected individuals (surface, core and “e” systems). This new antigen was called δ-antigen, and its corresponding antibody was consequently called anti-δ (12). Some years later, this antigen turned out to be the hallmark of a new virus, an incomplete small RNA-virus, which required HBV for its infection (13). The virus was called hepatitis D virus or deltavirus. It consists of a single-stranded, circular RNA and an antigen (the delta-antigen or
HDAg) enveloped by a lipoprotein coat which consists of HBsAg from Hepatitis B-virus (14). Thus, this virus is dependent on HBV-infection, which means that only individuals with previous HBV-infection can be infected with Hepatitis D virus (HDV). The infection of HDV can occur in two different patterns, co-infection and superinfection. In co-infection both HDV and HBV are transmitted to an uninfected individual simultaneously. Superinfection is when an individual is already chronically infected with HBV and also becomes infected with HDV infection. Co-infection is often self-limited, but can in some cases give a more severe progression, including fulminant hepatitis. When the patient recovers from HBV, the HDV-infection will also be cleared, but about 20% of coinfection leads to chronic infection and cirrhosis progression. Superinfection in patients with chronic HBV should be suspected when a stable chronic HBV infection suddenly worsens. HDV is associated with fulminant liver disease, cirrhosis progression and increased risk of hepatocellular carcinoma (15).

Eight genotypes have been identified so far, where genotype 1 is distributed globally, whilst genotype 2-8 do have more local distribution (16). Genotypes 2 and 4 are typically found in Japan and Taiwan, type 3 in the Amazon region and types 5-8 have African origin. Genotype 3 is known to give a severe form of the disease and occurs in outbreaks (17).

HDV infection is diagnosed by detection of antibodies to HDAg and HDV-RNA in serum or plasma. The initial step in screening for HDV infection is by antibody detection in HBsAg-positive patients. HDV antigen can also be measured with EIA-assays, but antigen is just transiently detectable in serum, and the marker is not a routine test. HDV-RNA assays are used to detect virus and to follow up treatment (18).

More than 15 million people are infected with HDV worldwide. The infection is endemic in Central Africa, South America, Asia and the Mediterranean Basin (18). Higher prevalence occurs in countries with a population of low socioeconomic status. But recent studies indicate an increasing prevalence in countries in Western Europe and the United States (18-21). Here the infection occurs primarily among individuals exposed to blood, such as intravenous drug users, and immigrants from endemic areas. Actions to prevent HBV-infection will, as HBV-infection is a presumption for HDV-infection, automatically also prevent HDV-infections.

In Sweden some 30 cases are reported every year, where the majority are infected before immigration to Sweden.
Hepatitis C
The discovery of HBsAg and its association with transfusion-transmitted hepatitis paved the way for screening tests for blood donors in order to prevent distribution of hepatitis infection from blood donations. In 1973, the Hepatitis A virus (HAV) was identified by Feinstone and colleagues, but this virus has another transmission route, as it is spread by the fecal-oral route (22). Still, there were cases of transfusion-transmitted hepatitis although the donations were tested for HBsAg before transfusion. As a matter of fact, the majority of these transfusion-transmitted cases proved to be unrelated to Hepatitis B, as 75% of all transfusion-associated hepatitis cases were not due to HBV, nor to HAV. In 1975, Feinstone and colleagues published a study on 22 patients with post-transfusion hepatitis, with no serologic evidence for hepatitis B. After studying several viral antibodies, such as antibodies to HAV, Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) the authors conclude that these hepatitis cases were probably caused by a then unknown viral agent (23). These cases of hepatitis were called Hepatitis Non-A, non-B, as the causng agent was unknown. In 1989 Choo et al (24) managed to clone the viruses’ genome; it was renamed to Hepatitis C and found to be the major etiological agent of transfusion-transmitted hepatitis, and also a major agent of chronic hepatitis infection worldwide (25).

![Figure 2. Hepatitis C genome organisation. One open reading frame encodes a polyprotein of 3010 amino acids. This protein is cut by viral and cell enzymes to active proteins. Source: Graham Colm, Wikipedia.](image-url)
Hepatitis C virus belongs to the Flaviviridae, and is a single-stranded RNA-virus, where the genome is surrounded by a 50 nm envelope. The genome encodes a polyprotein, which is cleaved into 3 structural proteins (core, E1 and E2) and 7 nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (26). The structure of the virus genome is described in Figure 2.

Many of the immunoassays for antibody screening are based on these proteins. In 1991, Choo et al managed to determine the complete HCV genome (27), and then several HCV isolates from different part of the world were sequenced. This led to the identification of several types with up to 33% dissimilarity over the viral genome. A genotype classification system for HCV-strains was established in 1994, which divided the strains into 7 genotypes (designated with Arabic numerals) and several subtypes (designated with lower case letters). The HCV genotypes are used as an epidemiologic tool, but can also affect some serologic assays, with indeterminate results as a consequence. It has been proposed that disease outcome, e.g. rate of evolution into chronicity after acute infection, and progression into liver disease, may also differ depending on genotype, although this is not clear and has not been proven (28).

The incubation period of Hepatitis C can vary between 14 days to several months. Usually, patients have mild or no symptoms. Consequently, it is generally unusual for infected subjects to notice the infection at all during this stage. Most of the infected patients learn about their infection during some kind of screening program. This is why data on the natural course of Hepatitis C infection is limited. Approximately 25-35% develop symptoms like malaise, weakness, anorexia and sometimes jaundice. Cases of fulminant liver disease have been reported, but are very rare (29). Up to 80% of the infected persons develop chronic infection. Of these, about 20% will develop cirrhosis within 10-20 years after the onset of infection. Among individuals with cirrhosis, the risk of developing Hepatocellular carcinoma (HCC) is about 1-4% per year (30). Niderau and colleagues shows in a study that cirrhosis patients have a 20% higher risk to catch HCC than non-cirrhotic patients (31). The course of chronic HCV-infection and risk factors for severe disease are shown in Figure 3.

Chronic HCV infection can be treated with interferon and anti-viral drugs. The duration of therapy and the outcome is highly dependent on genotype, where genotype 1 requires a longer duration of the therapy than genotype 2 and 3. However, new anti-viral drugs have recently been launched to the market, which are highly effective and will reduce the
therapy duration especially for genotype 1 (32). Moreover, with these new drugs, the interferon can be excluded, which is a considerable improvement, as the interferon has distressing side effects.

Diagnosis of HCV infection is based on antibody screening, antigen and nucleic acid detection. Antibody assays are usually based upon EIA-technique. A primary screening assay is followed up with a second, extended antibody assay to confirm antibody presence. Assays to detect HCV Core antigen or HCV-RNA are used to distinguish between cleared infection and ongoing infection. These assays are also used for therapy monitoring. Antibodies to HCV will remain whether the infection is cleared or not (33).

A recent study by Gower and colleagues estimates the global prevalence of antibodies to HCV at 1.6%, corresponding to 115 million infections worldwide. Of these 80 million individuals are assumed to be viremic, of which the majority are adults (34). The distribution of different genotypes varies from one geographic area to another. In Europe and USA, genotype 1, 2 and 3 are the most common, while genotype 4 exists predominantly in the Middle East and northern Africa (35). The distribution of genotypes worldwide is shown in Figure 4.
In Sweden approximately 2000 new cases of hepatitis C are reported each year. A majority of these are asymptomatic carriers and most of them have been infected in Sweden. The predominant route of transmission of hepatitis C is intravenous drug use (IDU). In 2012, 1'981 new cases were reported, and 46% of these stated IDU as the transmission route. The most common genotypes in Sweden are 1 (45%) and 3 (30%) proceeded by type 2 (20 %) (36). The risk of contracting HCV through blood transfusion in Sweden today is very small (37). In order to prevent transmission, several needle exchange programs have been tested, and are promoted by organizations such as the WHO. In Sweden, one such program has been initiated, in the southern region of Skåne (38). However, the role of these programs is disputed. Studies from other countries show that HCV continues to spread among IDU’s despite such efforts (39-40). HCV infection is easily spread, not only through syringes but also via paraphernalia used and shared by IDU’s (41).

### Human Immunodeficiency Virus (HIV)

The first cases of a new, previously unknown, disease emerged in the United States in the beginning of the 1980’s. The syndrome was characterized by a degradation of the immune system, which led to opportunistic infections such as severe pneumonia, epithelial tumors, disseminated fungal and mycobacterial infections. The syndrome was called AIDS (Aquired Immunodeficiency Syndrome). It was spread primarily among homosexual men and blood product recipients, indicating that the causal agent was,
like the hepatitis B infection, a blood borne infection (42). In 1983-84 it was discovered that this syndrome was caused by a retrovirus, first called Human T-lymphotropic Virus Type 3 (HTLV-III), then renamed Human immunodeficiency virus (HIV) (43-44).

Figure 5. HIV life cycle. Reproduced with permission from Mol Neurobiol. 46 (3): 614-638. Copyright © 2014 Copyright Clearance Center, Inc

Retroviruses are RNA-viruses which, when they have infected their host cells, utilize the enzyme reverse transcriptase, to transcribe RNA into DNA, which is then integrated into the host cell’s genome. Retroviruses are divided into seven subfamilies, of which the HIV belongs to the Lentivirus family. Lentiviruses are enveloped and the genetic information consists of two copies of single-stranded RNA. The virus, like all retroviruses, has three major structural genes, gag, coding for capsid proteins, env, coding for surface and transmembrane proteins and pol, coding for the viral enzyme systems. HIV has, in addition to these three main genes, supplementary genes that encodes for regulatory proteins and virulence factors (45). The gag-coded capsid protein p24 is an important marker in diagnosis of HIV. Shortly after discovering the HIV, another AIDS-causing virus was identified, which by sequence comparison differed by more than 55% from the previous virus. Thus, the two viruses were called HIV-1 and HIV-2. HIV-2 is predominantly found in West Africa (46). Research has found that these viruses have their origin in primates. Cross-species transmission of simian retroviruses is the origin of HIV, where HIV-1 origi-
nates from common chimpanzees and HIV-2 from Sooty mangabey macaques (47-49).

The lifecycle of HIV (and other retroviruses) is described in Figure 5. The virus infects CD4+ T lymphocytes, macrophages, brain microglia and dendritic cells, when the surface protein gp120 binds to cell receptors. The viral nucleic acid is released in the host cells cytoplasm, reverse transcriptase will help the synthesis of cDNA from the viral RNA. The DNA is integrated into the host cell’s DNA, to form a provirus, which can remain in a latent stage for a long period of time, to later become active, and start production of viral RNA. New viral particles are formed and then released from the host cell by budding, ready to infect new host cells (50).

After a short incubation period (1-3 weeks), a primary HIV infection occurs which in some infected individuals give rise to symptoms resembling mononucleosis, including fever, sore throat, fatigue and sometimes skin rash. This condition is called primary HIV infection (PHI). Some who are infected do not experience any symptoms at all during this stage of infection (51). The infection is chronic, and if it goes on untreated, the CD4+ lymphocytes will decline, and at a certain point, opportunistic infections will occur, and eventually AIDS will be manifested, see Figure 6. Many years can pass between first infection and occurrence of AIDS-related symptoms. During this latency, the infected individual is usually

![Figure 6. Time course of HIV infection. CD4+ cell count vis-à-vis HIV RNA copies during the natural course of infection. Based on Figure 1 in Pantaleo, G et al. (February 1993). "New concepts in the immunopathogenesis of human immunodeficiency virus infection". New England Journal of Medicine 328 (5): 327-335. Reproduced with permission, Copyright Massachusetts Medical Society.](image-url)
asymptomatic. CDC classifies the infection into three stages based on the CD4+ T-lymphocyte count (52).

With anti-retroviral treatment, the chronic infection can be controlled and the AIDS stage can be avoided. New drugs and combinations are continuously developed, to prevent AIDS progression in the infected (53). The drugs are divided into several groups due to their function. There are drugs that prevent viral introduction in the host cell, drugs that inhibit the action to enzymes reverse transcriptase, integrase and protease. Combinations of drugs are used for treatment in order to prevent the virus from developing resistance to the drugs (54). Infection with HIV-2 gives more seldom symptoms of PHI, and is characterized by a lower viral load and a longer asymptomatic phase. The AIDS disease caused by HIV-2 is similar to that of HIV-1 (55-56).

Diagnosis of HIV infection is based upon serological and molecular methods. Modern immunoassays based on EIA-technique detect both antibodies (to both HIV-1 and HIV-2) and HIV-antigen in serum samples. Reactive samples are confirmed with both extended serological antibody assays (based upon the immunoblot-technique) and nucleic acid assays, to detect viral RNA. Several recombinant antigens and synthetic peptides are used as antigens in these assays. The core protein p24 is used as a screening marker for viral presence in serum samples.

According to WHO statistics, 35 million people lived with HIV worldwide in 2013. Of these, 3.2 million were children <15 years. In the same year, 1.3 million died from AIDS. The vast majority of HIV-cases were found in Sub-Saharan Africa (27.4 million cases, 71% of all cases) (57). In Sweden, about 6400 individuals lived with HIV in 2013. This corresponds to a prevalence of 0.06%. In 2013, 461 new cases were reported, where the majority (76%) had been infected in another country other than Sweden. The number of new cases has been between 350-500 each year during the last decade, see Figure 7 (58).
Soon after the discovery of the HIV, assays to detect antibodies were developed. Since 1985, in Sweden, all blood donations have been screened for antibodies, and since 2010, both antigen and antibodies have been detected by the assays. In many countries, nucleic acid testing is implemented. This has prevented HIV-infection by blood transfusion. No vaccine has been developed to date, but anti-viral drug treatment has limited the number of AIDS cases, at least in the countries with high socio-economic statuses. However, between 2005 and 2013, the Middle East and North Africa experienced a significant increase in mortality from AIDS (66%). Eastern Europe and central Asia also experienced an increase of deaths from AIDS during the same period, but in a more moderate sense (5%) (57). It is of utmost importance that HIV-infected persons can be diagnosed and treated, both in order to prevent AIDS development in infected individuals but also to prevent the further spread of the infection.

Human T-lymphotropic Virus (HTLV)
The first human retroviruses to be discovered was Human T-lymphotropic virus type 1 and 2 (HTLV-1/2). It was Gallo and colleagues who first isolated HTLV-1, and identified its association with blood malignancy (59). Independently, a research team in Japan found a retrovirus in patients with T-cell leukaemia. Shortly thereafter, a second human retrovirus was discovered, HTLV-2 (60-62). HTLV-1/2 belongs to the Deltaretrovirus genus, and recently other types of HTLV have been found (HTLV-3 and
HTLV-4) in the south of Cameroon in Western Africa (63). Like HIV, the viral RNA is single-stranded, and converted into DNA in the human host cell. The infection is lifelong. In contrast to HIV, HTLV exists predominantly as a cell-associated provirus, and can, as such, replicate passively during cell mitosis, and in that way infect new cells. The HTLV Tax regulatory protein is responsible for this proliferation of HTLV-infected cells. But HTLV can also be spread actively by new virions produced in infected cells which are transmitted to non-infected cells, similar to HIV (but to a lower extent). As cell-free HTLV is assumed to be poorly infectious, it is thought that the primary spread is by cell-to-cell fusion (64). Because of this, plasma viral load is not as high as for HIV and considered a poor prognostic marker and, thus, not used in clinical follow up routines. However, in a recent study a real-time PCR method for detecting viral RNA in plasma samples from HTLV-1 infected patients was applied. In this study Cabral and colleagues detected free HTLV-1 RNA in plasma, suggesting that viral replication occurs in plasma, indicating transmission pathways other than described above (65). Like other retroviruses, the genome consists of gag, pol and env genes, encoding for structural proteins and enzymes, but also two regulatory genes, tax and rex, for transactivator and regulator of expression.

HTLV-infection is usually asymptomatic. Most infected individuals live with the virus for their entire life, without showing any symptoms. Nonetheless there are two major diseases that HTLV-1 is associated with. These two diseases are Adult T-cell leukaemia/lymphoma (ATLL) and HTLV-associated myelopathy/Tropical Spastic Paraparesis (HAM/TSP). The risk of becoming affected with an associated disease depends on several factors, such as age when infected, geographical area and route of infection (66). Among HTLV-1-carriers, the lifetime risk of developing HAM/TSP is 0.3-4%, while the risk of getting ATLL is calculated to be 1-5%. HTLV-1 is also associated with a number of autoimmune symptoms, such as arthropathy, uveitis and polymyositis, and if including these syndromes, the overall lifetime risk of getting a HTLV-depending disease is close to 10% among infected individuals (67). As for HTLV-2, its association with disease is less clear. Compared to HTLV-1, the pro-viral load is lower. According to a study by Murphy et al, it is about five times lower for HTLV-2 than HTLV-1 (68). There is an association with neurological syndromes similar to HAM/TSP (69) and also other neurological disorders, like peripheral neuropathy and spinocerebellar syndrome (70).
ATLL is a malignancy of CD4+ lymphocytes, infected with HTLV-1 pro-virus. Most HTLV-1 carriers that develop this disease have been infected early in life, often by breast-feeding. The mean age at diagnosis of ATLL is 40-60 years old, varying depending on geographical location.

The association between HTLV-1 and myelopathy was established both in Japan and the Caribbean in the mid 1980’s. This disease was named thereafter HTLV-1-associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP) (71-72). The syndrome is caused by an inflammation of the white and grey matter of the spinal cord. Patients who face these symptoms do often have higher proviral load than asymptomatic HTLV-carriers (73). In most cases a symmetrical paraparesis gradually affects the lower limbs, slowly progressing and with no remission. Bladder disorders are also common, which leads to repeated urinary tract infections (74).

HTLV-1 and HTLV-2 infections are diagnosed primarily with serological assays to detect antibodies. Like other antibody detection tests, they are based upon EIA-technique for screening, and immunoblot assays for confirmation of antibodies. There are a few automated immunoassay platforms that provide HTLV-1/2 antibody assays (75).

It has been estimated that 10-20 million people worldwide are infected with HTLV-1. However, this estimation is based on studies with selected populations where HTLV-1 infection is overrepresented. On the other hand, data which are based on blood donors may underestimate the prevalence. The infection rate strongly varies across different geographical regions and in different populations (76). The highest prevalence is found in southern Japan, South America, the Caribbean and some parts of Africa. HTLV-2 has higher prevalence rates in some parts of South America and the Caribbean, but is also found to be prevalent among intravenous drug users in the big cities of North America and Europe. Prevalence studies have been conducted among IDU for instance in Sweden, Spain and in the US (77-79).

The prevalence in Sweden is in general low, apart from IDU’s in Stockholm where prevalence rates of 2-3 % have been reported (77). Blood donors and persons seeking care for infertility are groups that are mandatorily screened, and are also the groups where new cases are incidentally discovered. The number of new cases per year among new blood donors in Sweden are shown in Figure 8.
To prevent the spread of HTLV-infection, screening of blood donors, breast milk donors and patients at infertility clinics is either mandatory or recommended. Women with confirmed infection are advised to limit or exclude breast feeding of their children (80).

Other viral infections

There are some other viruses that can play a role in blood transfusion or organ transplantation. Cytomegalovirus (CMV), for example, can cause problems in immunocompromised patients. This virus exists in a majority of the adult population (40-100%), and transfusion of blood products to seronegative, immunocompromised recipients with seropositive products can be at potential risk of developing severe CMV-infection (81). Leucocyte depletion of blood products can prevent these events, as well as CMV-screening and the use of only CMV-negative blood to susceptible recipients (82).

West Nile virus (WNV) is a mosquito-borne infection that commonly infects birds, but can occasionally infect other species, like horses and humans. The infection has its origin in Uganda, but in the late 1990:s it was introduced to the North American subcontinent. The virus is a flavivirus, and infected subjects have a viremic period of 1-11 days, where the virus can be spread by blood transfusion. In 2002, at least 6 cases of transfusion-transmitted WNV-infection were confirmed (83). To prevent
this, nucleic acid testing of WNV in blood donors was implemented in the USA. In several European countries, blood donors that have visited the USA, are banned from donating for 1-2 months after returning (84).

There have also been cases described of transmission of other viral infections via blood donations, e.g. Chikungunya Virus, Dengue Virus and Hepatitis E (85-87).

**Blood Borne Bacterial infections**

**Syphilis**

Syphilis, or Lues, has been known to humanity for hundreds of years. Treponemal infection is believed to have its origin in eastern Africa, then having spread via Asia to the American continent. At the end of the thirteenth century, the disease came to Europe via sailors who had been to the “New World” (88). The primary transmission route for syphilis is by sexual contact, but it is also known that at some stage of the infection, it can be spread in blood products and through blood contact. Today, most of the blood products are refrigerated, and since treponema bacteria are sensitive to refrigeration, infection from transfusion-transmitted syphilis has almost disappeared (89-90). Nevertheless, many countries continue to test all their blood and blood component donations for antibodies to syphilis. The screening also serves as a marker for sexual risk behavior, an undesirable characteristic of blood donors.

Syphilis is caused by the spirochete *Treponema pallidum* subspecies *pallidum*. The disease is classified into several stages (91-92). During the primary stage of the disease, which appears about three weeks after infection, a lesion will arise at the site of the bacterial introduction. This lesion is a painless chancre, which contains proliferating spirochetes, which easily are spread via intimate physical contact. Within 3-8 weeks, the chancre is healed, but the bacterium has now spread systemically via the bloodstream. Within 3 months, the secondary stage symptoms will arise. This stage includes various symptoms, where a disseminated, maculopapular rash is the most common. Other symptoms that can appear during this stage are malaise, weight loss, muscle ache, lymphadenopathy, alopecia, meningitis, ocular inflammation and inflammation of mucosal tissues in the oral cavity and genitals. These symptoms resolve spontaneously, but if the patient is not treated, the secondary symptoms can reoccur during the first year of infection. If not treated, the infection evolves to the latent stadium, when the disease progresses into a chronic infection. The first
year of this asymptomatic stage is called early latent syphilis. The following years of latent infection is called late latent syphilis. In the latent stadium, the bacterium may seed the bloodstream intermittently. Sexual transmission in this latent stadium is rare, but infection of the fetus in pregnant women may occur, as the bacterium may exist in the bloodstream. The tertiary stage of the infection occurs in about 30% of infected individuals that do not receive treatment. Symptoms of this stage appear 10 to 20 years after initial infection. There are three forms of late syphilis; benign or gummatous, cardiovascular and neurosyphilis. Lesions in the central nervous system (e.g. syphilitic meningitis, visual changes, facial weakness) may also occur earlier in the infection, during the secondary stage. Late neurosyphilis includes general paresis and demyelination of nerves in the dorsal columns (91). See Figure 9.

Congenital syphilis, i.e. syphilis spread to the fetus during pregnancy, can occur at any stage of syphilis infection, and during any stage of pregnancy. However, the later in pregnancy and the earlier stage of infection, the greater risk of infection of the fetus (93). The symptoms can vary, are often very severe with multiple organ engagement and stillbirths are not uncommon. Congenital syphilis is very rare today in developed countries with proper antenatal screening programs. If syphilis infection in pregnant women is diagnosed and treated early in the pregnancy, the risk of congenital infection is considerably reduced (94).

Figure 9: The natural course of untreated syphilis in immunocompetent individuals. Reproduced with permission from J Clin Invest. 2011;121(12):4584–4592. Copyright © 2014 Copyright Clearance Center, Inc
As the bacterium has not been cultured in vitro on artificial media, definitive diagnosis of infection is made with dark field microscopy (today a rare method), fluorescent microscopy or PCR methods in the primary stage of infection. Modern molecular techniques can also be used, to detect bacterial nucleic acid in samples from the chancre. However, most commonly syphilis is diagnosed using antibody detection, which can be used at all stages of the disease. Two kinds of antibodies are used in the diagnosis. The earliest assays to detect syphilis used so called non-treponemal antibodies. These antibodies are a due to the tissue damage that the infection causes. Cardiolipin is one such antigen that can be used as antigen to detect syphilis-associated antibodies. Individuals with positive non-treponemal antibodies may be infected with syphilis, but other conditions can also give rise to these kinds of antibodies e.g. autoimmune syndromes. A positive non-treponemal assay therefore needs to be followed up with an assay that detects treponemal antibodies, directed against the bacterium’s own antigens. Historically, the non-treponemal antibody assays have been used as screening assays (blood donor screening for example) and the treponemal antibodies as confirmation assays. The non-treponemal assays have been inexpensive and easy to use, but in recent years, due to automation of laboratory analysis, the treponemal assays are often used as screening assays instead, followed by the non-treponemal (95-96). This new screening algorithm, the so-called reversed algorithm, is adapted in many high resource settings, where there is an opportunity to use automated immunoassays to facilitate the screening process. In Sweden, the regulations for blood donor screening requires a treponemal assay as the first screening method, thus the use of reverse algorithm is the predominant testing algorithm for syphilis in Swedish laboratories.

According to the World Health Organization, the incidence of syphilis, is estimated to be 12 million new cases each year. The disease has a higher prevalence in developing countries, where 3-15% of women at childbearing age may have syphilis. Due to the fact that approximately 30% of pregnant women with syphilis will give birth to a stillborn child, and 30% will have children with congenital syphilis, it is critical that there is early detection and treatment to prevent both mother to child transmission and sexual transmission (97). In Sweden 200-300 new cases are reported each year. It is more common among men, and especially among men who have sex with men. In 2013, out of 275 reported cases, 29% had primary stage infection, 14% had secondary syphilis and 24% early latent infection. The 32 remaining percentage had an unclear stage of infection (98).
Syphilis infection is treated with antibiotics, using Penicillin G as the first drug of choice. This can be used at all stages of infection, and also on pregnant women to prevent congenital syphilis (99-100).

To prevent transmission of syphilis, it is important that screening programs for certain groups are implemented. Blood donors, pregnant women and STI clinic attendants are key groups to offer screening to. Early detection and treatment are crucial to prevent long term complication, congenital and sexual transmission. In many countries, screening programs for pregnant women are offered.

**Other bacterial infections**

As clinically healthy individuals normally do not carry bacteria in their bloodstream, the only bacterial infection to take into account (except from syphilis) is contamination of blood products during blood donations. Blood units can be contaminated during collection and processing. In general, individuals with bacteremia are symptomatic and thus excluded from blood donations. But sometimes, a low grade infection, or recovery from bacterial illness, can be asymptomatic and thus bacterial infections in the blood stream or transient bacteremia can cause spread of bacterial infections to blood product recipients. There are several examples of this. *Yersinia enterocolitica* is a bacterium that can multiply at a temperature of 4 °C and thus proliferate in stored red blood cell bags. Transmission can result in septicemia and endotoxin-mediated shock. In the United Kingdom, at least six cases, four of them fatal, have occurred since 1988 (101). Transient bacteremia can occur after dental treatment or even just tooth brushing. There have been cases of transmission of *Staphylococcus aureus* contamination due to donor undergoing dental repair some hours before donating blood (102).

In 1999, Sweden and Denmark experienced an outbreak of septicemia with *Serratia marcescens* among patients receiving red blood cells and platelets. The outbreak was due to contaminated blood collection bags. In this case, bags had been contaminated during the bag production, but bags can also become defect during the preparation of blood product process. On the whole, with the current screening programs of blood donors, bacterial infection from contaminated blood components is a far bigger risk than becoming infected with a blood borne virus. The current risk of receiving bacterially contaminated platelet concentrates may be 1000 times higher than the risk of transfusion-transmitted HIV, HCV, HBV and HTLV infection (103).
Other blood borne infections

Protozoan infections

There are some protozoan infections that are of interest in relation to blood transfusion. Once they were thought to be exotic in non-endemic developed countries but due to increased travel they have become more common globally.

Malaria, caused by the genus *Plasmodium*, is mainly transmitted by mosquitoes. The parasites invade the bloodstream after a mosquito bite, and after less than 60 minutes, they infect liver cells where they undergo nuclear division. After 5 to 31 days, the cells rupture and the parasites are released into the bloodstream. It then infects the red blood cells, eventually giving rise to the symptoms that consist of recurrent fever, headache, shivering, joint pain and vomiting (104). Thus malaria can be transmitted by blood transfusion, organ transplantation or sharing of needles in intravenous drug use. The problem of malaria in blood products is naturally more relevant in endemic areas, but with increased travel habits and migration, awareness of the risk of transmission is important. Many countries in low prevalent areas have overcome this problem by using specific donor-selection criteria (see below).

Other protozoan infections of importance attributed to blood transmission are Babesia, the etiologic agent that causes babesiosis, *Toxoplasma gondii*, Leishmania and *Trypanosoma cruzi*. The latter, giving rise to Chagas disease, is mainly a problem on the American continent. The protozoa are mostly transmitted by hematophagous bugs, but can also be transmitted by blood donation, organ transplant and from mother to child. The disease occurs in two stages; acute and chronic. The acute phase persists about 6-8 weeks, and is often asymptomatic. If not treated, the disease enters the chronic stage, and many carriers will be asymptomatic throughout their lives, and are potential transmitters and at risk for developing symptoms. These symptoms consist of severe gastro-intestinal disease and/or cardiac problems such as arrhythmias, heart block and heart failure (105). Most of the infected individuals live in Latin America, where it is estimated that 8 million people have Chagas disease (106). Due to the levels of travel and migration, most U.S blood donor centers screen their donors for *Trypanosoma cruzi*. Other countries, outside the American continent, often use exclusion criteria to prevent transmission of this agent by blood transfusion (107).
Blood-borne infection screening and diagnosis

Screening programs

Blood donors
WHO recommends mandatory screening of these four agents for blood donations; Hepatitis B, Hepatitis C, Human Immunodeficiency Virus and Syphilis (108). In addition to these, screening routines for other blood-borne infections can be implemented according to prevalence and incidence in the actual country. Every country should have a national strategy for blood safety when dealing with blood borne infections. When establishing such a program, it is essential to take into account criteria such as prevalence and incidence of infections, infrastructure of the blood transfusion service, screening costs and available resources. When it has been decided which infections to screen for, a screening algorithm should be established, to ensure consistency in screening tests, decision concerned with release and withdrawal of blood components. An algorithm is used to answer questions about screening, confirm assays, identify criteria for release of blood components, regulate how to deal with false reactive donor samples and set out protocol for taking care of confirmed positive donors. Each transfusion-transmissible infection requires its own algorithm.

Aside from screening programs, other donor selection criteria or extended screening of selected groups is used to prevent transmission of blood borne agents. To address the problem of malaria, screening strategies where donors from selected high-prevalent regions are tested for malaria can be used, as well as limited or permanent deferral of donors from endemic countries (109). Deferral routines should rely on four specific criteria from donor questionnaires, they are geographical location (travel or lived in endemic area), length of time in such area, length of time since the last visit to such an area and history of previous malaria (110). Similar routines are adopted concerning other agents of low prevalence in different regions. Selection questionnaires can also include questions about drug use, sexual behavior, piercing and tattoos. Most European countries omit men who have sex with men and sex workers as blood donors (111). These two risk behaviors often lead to permanent deferral while other sexual risk behavior, such as having more than one partner in a period of a few months, can result in a temporary deferral, which is also the fact with risk events such as piercing, tattoos and other skin lacerations. Deferral period of 6 or 12 moths are used (112-113).
In 2011 a survey among the members of the European Council was conducted, where data were collected concerning screening of transfusion-transmitted infection (114). Among the 47 member states, 32 answered the survey (70%).

All 32 reporting countries screened all blood donations for Hepatitis B (HBsAg), Hepatitis C (anti-HCV) and HIV (anti-HIV-1/2). 91% also screened each donation for Syphilis. 16 of the 32 countries used a combination assay in screening HIV, simultaneously detecting both antigen and antibodies to HIV, see Table 1.

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</tr>
</tbody>
</table>

Table 1: Blood donor screening in Europe. In addition, some countries also screened for Trypanosoma Cruzi, West Nile Virus, CMV and Parvo B19 virus in selected groups. “Some” in the table also means screening in selected groups. Adapted from Council of Europe, The Collection, Testing and Use of Blood and Blood Components in Europe
Nucleic acid tests (NAT) for HBV, HCV and HIV on every donation were performed in 56%, 57% and 58% of the participating countries respectively. Some of these countries tested for nucleic acid individually, some in minipools, consisting of 6 to 96 units in each pool. Italy, Greece and the United Kingdom also performed NAT-tests for West Nile Virus on every donation, Finland and Luxembourg did NAT-tests for Hepatitis A virus and Parvo B19. Seven countries reported HTLV-1/2 antibody screening on every donation, 3 did HTLV-screening on first time donors whilst 19 countries did not screen at all. In Spain, some centers screened all donations, while some centers did selective screening.

Incidence of blood borne infections among repeat blood donors, and prevalence in first blood donors were also reported in the survey. Latvia and Portugal reported the highest incidence of HIV infection among repeat donors, Greece reported the highest incidence of HBV and Lithuania of HCV. Looking at prevalence among first time donors, Moldova had the highest prevalence of HIV, Bulgaria and Romania the highest HBV and Lithuania the highest HCV prevalence.

In the United States, Centers for Disease control (CDC), have set up rules for blood donor screening. They include screening with both antibody and NAT tests for HIV and HCV, HBsAg and anti-HBc for HBV and antibody tests for HTLV-1/2 and syphilis. A NAT-assay for detection of West Nile Virus is also mandatory as there has been transmission of this virus from blood donations in the US (83-84).

In Sweden, the National Board of Health and Welfare stipulates the rules for donor screening for every blood donor centre in the country. They have also set an algorithm for every agent. Since 2010, HBsAg, anti-HCV, HIV antigen and antibody assay and Syphilis screening with a treponema-specific assay is mandatory. A reactive screening test should be followed by a retest in duplicate, and when repeatedly reactive, the blood component will be discarded, and the assay followed up with confirmatory testing. Additionally, for first time donors, anti-HTLV-1/2 and anti-HBc are also mandatory (115). The screening algorithm includes retest in duplicate for reactive samples, and a follow up with confirmatory assays on repeatedly reactive samples. Any blood donation with a repeatedly reactive marker is discarded, even if the reactivity is non-specific, i.e. confirmed not be caused by an active infection.
Tissue and organ donors
Donors of cells, tissue and organs can be divided into two main groups; living donors and deceased donors, the latter donating whole organs posthumously. In Sweden different screening programs have been implemented depending on which group the donor belongs to. Living donors, who provide for example tissue such as bone tissue or whole organs, such as kidneys, are obliged to be tested for the same agents as a new blood donor, except for HTLV-1/2, which is only mandatory for persons originating from or who have been living in endemic areas, or when their sexual partner or parents are from such an area. If the tissue or cells are to be stored for more than 180 days, a new sample is taken, before the tissue or cells can be used for transplantation. This repeated sampling can be avoided if a NAT assay is performed for HIV, HCV and HBV on the first occasion of sampling, in addition to the mandatory serological assays (116). For deceased donors, just one sampling occasion is possible, thus the demand for repeated testing does not apply to these cases. The Swedish directive is a direct implementation of the European Commission directive regarding certain technical requirements for the donation, procurement and testing of human tissues and cells (117). Living donors are in some way comparable to new blood donors, except for the fact that they are asked to donate shortly before a planned surgery, and thus not selected routinely, such as persons that seeks to become blood donors. This might reflect a slightly increased risk of transmissible infections than first time blood donors. Though very rare, there are cases described where infections have been transmitted through allogenic grafts and tissue. Eleven cases of transmission via bone allografts are listed by Pruss et al (118), where HIV was transmitted in 5 cases, HCV in 5 cases and HBV in 1 case. In 1984, Hoft et al reported HBV transmission through cornea transplantation (119). The viral burden in the cornea is very low, or even absent, in HIV or HCV-infected donors, and consequently, transmission risk is very low. A study from the United States Eye Bank association did not find any cases of HIV, HCV or HBV during 12 years (>400 000 transplanted corneas), where the donors had been screened with serologic assays (120). However, it has been described that HCV genome can be detected in the cornea of HCV-seropositive donors (121).

Regarding deceased donors, samples should be drawn as soon as possible after death, and not later than after 24 hours. In general, commercial serologic assays are not adapted to cadaveric samples, which complicate the testing. However, studies have been done to address this, both for
serologic and NAT assays (122-124), which show that these assays are also reliable for cadaveric samples.

For donators of germ cells, testing for HTLV-1/2 is mandatory in Sweden, in addition to the tests that are demanded for other living tissue or organ donators, regardless of if the donator comes from an endemic area or not (116). This is also the routine for in vitro fertilization (IVF) clients, as described below. However, the requirement of HTLV-1/2-testing is not stated in the European Directives, only for individuals originating from endemic areas as described above (117).

Antenatal and infertility screening
Maternal health programs often include screening for infections that can jeopardize the pregnancy and transmit infectious agents to the fetus during pregnancy or to the newborn during delivery and breast feeding. In 2014, Piso et al summarized recommendations for maternal infection screening from international evidence-based guidelines (125). According to their summary, Hepatitis B and HIV screening were the most recommended agents for universal screening. Screening for syphilis was also recommended, but not to the same extent. Bernloehr et al (126) published a survey in 2005 on guidelines of antenatal screening among 25 member states in the European Union. Twenty out of the 25 countries did have existing national guidelines for antenatal care, including guidelines on infection screening. Eighteen countries recommend testing for syphilis, 17 recommend testing for Hepatitis B, 12 for HIV and 7 countries recommend testing for HCV. The principal reason for screening is the possibility of preventing transmission to the child, so focus should be on agents where some action can be undertaken, not on testing that primarily causes anxiety, where no preventing action exists.

Regarding syphilis, antenatal screening has been proven to prevent transmission of congenital syphilis. In a study performed in 1999 by Alexander et al, mothers who tested positive for syphilis at the antenatal care unit (ACU), and got treatment with penicillin G injections were compared to women who’s syphilis infection was discovered at delivery and who consequently did not get treatment during pregnancy (127). In 198 out of 204 cases of syphilis infection detected at the ACU, treatment was successful and prevented transmission to the child. Lack of prenatal screening leads to failure to identify infected mothers and thus unnecessarily increases the cases of congenital syphilis infection.
The risk of a HBsAg positive woman transmitting HBV-infection to the child during pregnancy and delivery is relatively high. If the mother is HBsAg and HBeAg positive, the risk is as high as 90%, if the mother is anti-HBe positive and HBeAg negative, the risk is 5-20% (128). If the HBsAg status is known at an early stage in the pregnancy, the transmission can easily be prevented by Hepatitis B vaccination, starting within 12 hours after birth. It has been shown that if the mother has a very high viral load and detectable HBeAg during the last trimester of the pregnancy, the risk of vaccination prophylaxis failure increases (129). Therefore, HBsAg positive pregnant women are followed up with tests for HBeAg and HBV-DNA viral load. Antiviral treatment with lamivudine can be used in cases of high viral load, as this together with vaccination after birth effectively reduces the transmission (130).

Prevention of mother-to-child transmission of HIV is a very important key in reducing HIV and HIV-related child mortality globally (131). Without intervention, the risk of transmission ranges between 20% and 45%. With intervention, the risk is as low as 1-5%. Transmission can occur in utero, perinatal and postnatal, and through breastfeeding. In Sweden, anti-retroviral prophylaxis was introduced in 1994, to HIV-positive pregnant women, and thus the incidence of mother-to-child transmission of HIV decreased from 25% to 8%, and decreased further to less than 1%, when introducing a combination anti-retroviral therapy and cesarean section. In Sweden around 40-50 children are born to known HIV-positive women and since 2006, 2 children have been born who have acquired the infection from their mother. In addition, two infected children were born to mothers who did not know of their HIV positivity (132). Since 1988, all pregnant women who visit antenatal care units in Sweden are offered HIV screening. Today, all pregnant women are offered tests for HBsAg, Syphilis and HIV.

<table>
<thead>
<tr>
<th></th>
<th>HBsAg</th>
<th>anti-HBc</th>
<th>Hepatitis C</th>
<th>HIV</th>
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<th>HTLV</th>
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<tr>
<td><strong>Breast Milk donors</strong></td>
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<tr>
<td><strong>Pregnant women</strong></td>
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</tbody>
</table>

*Table 2: Swedish screening programs. Black: Mandatory screening, Red: Offered screening, Green: Regional regulations*
For IVF-clients, it is the same regulation both for so called partner donation (donation of reproductive cells between a man and a woman who declare that they have an intimate physical relationship) and for germ cell donators, where the cells are to be donated to a third party. In case of positivity for any of the mandatory agents, an individual assessment is done, to decide if there is a risk of transmitting the infection to the child or not, before decision on IVF treatment can be made. There are for example methods to minimize HIV and HCV transmission through sperm wash before assisted reproduction (133). The Swedish screening programs are shown in Table 2.

Other screening programs
Other population groups that are subject to screening programs, mandatory or optional are donors of breast milk, IDU’s when participating in needle exchange programs and patients who are on hemodialysis or are to undergo some kind of surgery. In the case of breast milk donors, HTLV screening is particularly important, as breast feeding is the main transmission route for that infection (134). Mandatory HTLV-1/2 assays for breast milk donors are an easy way to avoid HTLV-infection in infants. In high prevalent settings, HTLV-1/2-screening should be included in the antenatal screening of all mothers.

![Figure 10. Mother-to-child transmission of HTLV-1 can easily be prevented by avoiding or shorten the time of breastfeeding. Reproduced with permission from Pediatr Infect Dis J. 2013 Feb;32(2):175-7. Copyright © 2013, (C) 2013 Lippincott Williams & Wilkins, Inc.](image-url)
Screening methods

Agglutination/Flocculation

Screening of blood borne infection is generally done either with serological assays, or with molecular assays, the so called NAT. The earliest serological assays performed were the flocculation test for non-treponemal syphilis antibodies. These are simple assays that are based on antigen-antibody interaction. Two bacteriologists, Herbert Edward Durham (1866-1945) and Max von Gruber (1853–1927), discovered specific agglutination in 1896 (135-136). Carbon/cholesterol particles are coated with the antigen, which in the case of non-treponemal assays, consist of cardiolipin/lecithin. These particles are suspended in a buffer to an appropriate dilution.

![Figure 11. Interpretation of RPR agglutination. The test can be performed both qualitatively and quantitatively. Reproduced by permission from BD Bioscience Europe, Stockholm, Sweden](image)

Antigen suspension and the patient sample are mixed together, either on a glass slide or a plastic coated card. The sample and reagent are then allowed to react for about 5-10 minutes on a rocker. When antibodies are present in the serum sample, they react with the antigen to form antigen-antibody complex. This appears as flocculation of the carbon particle, showing characteristic macroscopic clumping of the particles. These assays are still widely used globally, to detect syphilis antibodies both in blood donors and clinical samples. As they are non-treponemal antibodies, they need to be confirmed with treponema-specific antibodies in order to diagnose syphilis.
Agglutination assays are also used to determine treponema-specific antibodies. Animal blood cells or latex particles can be used as the solid phase in these assays (137-138). Avian erythrocytes are usually used in Treponema Pallidum Haemagglutination (TPHA) and latex particles in Treponema Pallidum Particle Agglutination (TPPA). The test procedure of agglutination assays can be performed on slides, in tubes and in microtiter plates. Sample (with or without the antibodies in question) and particle suspension are mixed and then allowed to react for a sufficient length of time (from minutes to hours according to assay and agent) and the result is interpreted as agglutination (clumping of the cells/particles) or not.

The advantage of agglutination and flocculation assays is that they are simple and quick to perform, but with a large number of samples, the logistics can be difficult to achieve. Another disadvantage is that the interpretation of the results needs to be carried out manually, which requires skilled personnel.

Enzyme Immunoassay (EIA)
A major step in the evolution of serological assays for antibody and infection screening was the development of the EIA-technique. Its predecessor was the radioimmunology assays (RIA). The assay principles for these methods are the same, but different detection agents are used. RIA-assays were first described in 1960 (139).

Antigen or antibodies (depending on what is to be detected) are coated on a solid phase, usually the bottom of a microtiter plate or on plastic beads. A sample is added to the well or tube, often diluted in an incubation buffer. After incubation at the appropriate temperature (often 37°C) the plate is washed in a buffer several times, to wash away unbound antibodies and other proteins. Specific antibodies or antigens will remain, as they are bound by the specific antigen-antibody reaction. After the washing step, a conjugate is added to the reaction well. A conjugate can consist of antibodies directed against the bound antibodies/antigens, conjugated with a radioactive isotope (RIA) or an enzyme (EIA). The plate will again be incubated for a predetermined time, to let the antibodies and antigens bind together. After this second incubation, a wash step follows, to remove unbound material.
To detect the sought after antibodies, a substrate is added to the wells (EIA), for which the conjugate enzyme is catalyst, and a colour change will take place, if there are the sought after antibodies/antigens present in the sample.

The colour change is read using a spectrophotometer, as an absorbance unit. A cut off absorbance, often established by controls run in the same assay round, will determine if the analyte is present or not. In the RIA-assays, the presence or absence of the analyte is determined in a gamma counter. Because radioactivity requires special precautions and can be a health threat, it is today almost entirely replaced by EIA in infection serology.

The EIA method was first described in 1971 (then under the name ELISA, Enzyme-Linked Immunosorbent assay) by the Swedish researchers Eva Engvall and Peter Perlman and by the Dutch researchers Anton Schuurs and Bauke van Weemen independently (140-141). This method and its further developments has been revolutionary in the field of serological assays. The first generation of EIA-assays used whole viral or bacterial lysate as antigen, Newer generations utilize recombinant antigens and synthetic peptides, which has led to more sensitive and specific assays. Newer generations of EIA’s do also have antigens as conjugates, enabling

Figure 12: Schematic description of EIA-assay technique. Adapted from Gan SD, Patel KR, J Invest Dermatol Copyright: Nature Publishing Group
both IgG and IgM antibodies to react. In the case of HIV-assays, for example, this has resulted in the earlier detection of antibodies and a narrower pre-seroconversion window. One advantage of EIA-assays compared to agglutination assays, is that the interpretation is done with a spectrophotometer, and consequently eliminates the manual aspect and therefore also a subjective interpretation. It is an easily performed step-by-step assay method, but requires quite a lot of manual work. However, automated EIA platforms, so called EIA-processors, are available on the market in order to reduce levels of manual handling.

Automated immunoanalyzers
The demand of rapid, automated assays for screening large quantities of samples in blood bank facilities and other large-scale settings have encouraged the development of automated immunoanalyzer platforms. These assays are essentially based on the EIA-technique. In contrast to EIA-processors, where the samples are processed in batches, these assay platforms are run with random access, which makes it easy to analyze single samples continuously. Instead of microtiter plates, the solid phase consists of microparticles in solution.

The detection of analyte in these platforms is often based on other measurements than colour change. A commonly used technology is the chemiluminescence (CLIA). Instead of an enzyme, the conjugate is labeled with acridinium, an ester that produces light in the presence of alkaline hydrogen peroxidase (142). Energy is released when the reaction takes place, and the light emission and its intensity is measured by optic in the instrument. Several screening platforms on the market utilize this technology (143-144). With a predefined calibration curve, a cut off value is established to interpret results as reactive or non-reactive. These assays can also be used in quantitative analyses. A different kind of CLIA is electrochemiluminescence (ECL), which utilizes ruthenium as light emitter. The antigen-antibody-microparticle complex is bound to an electrode, and after removal of non-bound material a voltage is applied which induces the CLIA-reaction (145). As these automated immunoassay platforms are often used in areas with high socio-economic standards, with often low prevalence of blood borne infection, high specificity is a key factor in screening of blood bank samples, to prevent unnecessary deferral of donated blood.
NAT-assays

The use of molecular assays has become increasingly common when screening blood donors for blood borne viruses. These assays are typically based on polymerase chain reaction (PCR) methods. This method utilizes the ability of the polymerase enzyme to copy and amplify specific sequences of single-stranded nucleic acid fragments. By using a reaction mix with target-specific primers and nucleotides, the polymerase synthesizes a new copy of the DNA-fragment in cycles, thus generating multiple copies of the requested sequence.

![Polymerase chain reaction - PCR](https://commons.wikimedia.org/wiki/File:Polymerase_chain_reaction.png)

**Figure 13. Schematic description of Polymerase chain reaction.**

*From: “Polymerase chain reaction” by Enzoklop - Own work. Licensed under CC BY-SA 3.0 via Wikimedia Commons*

This method was first described in the 1980’s (146). It involves three steps, the first being the denaturation step, where the double stranded DNA is separated into single-stranded DNA. The next step is the annealing, which refers to when oligonucleotide primers bind to its complementary part on the DNA. Thirdly, during the elongation step, the polymerase will synthesize a new DNA strand complementary to the DNA template with the nucleotides in the reaction mix. This procedure will then be repeated in cycles resulting in exponential amplification and thus generating a sufficient number of DNA copies, enough to detect in a detection system.

When using NAT-assays for blood donor screening, a multiplex assay for HIV and HCV detection is usually used, and for cost reasons, the donation samples are often pooled into so called “minipools”. The number of samples in each pool can vary between 6 and up to 96 (114). The sensi-
tivity of the assays decreases when pooling samples compared to individual testing (147). Minipool NAT for HBV, which may replace HBsAg testing is also implemented in some countries. Multiplex assays that detect all three agents have also been developed (148).

The new antigen and antigen/antibody combination (combo) assays for HIV and HCV are in fact almost as early to detect infection as NAT-testing in minipools. A combo assay for HIV is well established in HIV-screening (149), and efforts are being made to develop a similar assay for HCV. To date, no combo assay for HCV donor screening has been reliable enough to be released on the market (150). Such an assay adapted for an automated immunoanalyzer would be a welcome addition to the screening resources.

<table>
<thead>
<tr>
<th></th>
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<tr>
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<td>5,5</td>
<td>35</td>
</tr>
<tr>
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<td>(5-11)*</td>
<td>45</td>
</tr>
<tr>
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<td>NA</td>
</tr>
<tr>
<td>Antibody test</td>
<td>14</td>
<td>60</td>
<td>NA</td>
</tr>
</tbody>
</table>


NAT: Nucleic acid amplification test. NA: Not applicable, Ag/Ab Combo: Antigen/Antibody combination assay

Rapid point-of care assays

The market of “easy to perform” point of care (POC) assays is continuously growing. There are tests adapted for settings with lack of instrumentation and laboratory facilities, and there are also tests adapted for domestic use outside clinics and health care centers.
These kind of simple and quick assays are often based on immunochromatography techniques. The principle of this serological method is generally the same as in EIA-methods, except that the reaction is carried out on a chromatographic paper with capillary action. The antibody used in the assay to test for the sought after antigen is labeled with colloidal gold and attached to a sample pad. When the patient sample, together with a sample buffer, is dropped onto the pad, the antigen present in the sample will react with the gold-labeled antibodies. The complex will move along a chromatographic paper with the buffer, and reach a corresponding antibody immobilized on the paper. An immunocomplex will then be formed, that results in a colored line. The assay is often very fast, and a result may be read after 15-20 minutes (151). Although simple and easy to perform, the interpretation sometimes is not straightforward, as the resulting lines can be weak, and non-specific lines can occur. Trained and skilled personnel are required. Despite this, some of these assays are also marketed for private use.

For blood donor screening, most of these assays are not sufficient. However, a few POC assays, especially for HIV antibody detection, have high sensitivity and specificity and, thus, in health centers in regions with limited access to specialized laboratory facilities, they may have its natural place.

Confirmatory assays
Reactive results obtained from screening assays need to be confirmed, both with repeated testing with the same method, and with a second independent method. In the case of HIV, the usual routine also includes a second sampling, to ensure that the sample has not been mixed with the wrong identity, before diagnosis. After repeating the positive result in duplicate, and at least two of three results are reactive, a second, confirmatory assay is performed.

The Western blot (WB) method is the traditional method for confirming HIV antibodies. It was for a long time considered the gold standard to confirm HIV antibody positivity. WB is also used to confirm HTLV-1/2 antibodies. The method uses electrophoretic separated HIV antigens derived from lysate of virus grown in culture. The antigens are separated in a sodium dodecyl sulphate (SDS) polyacrylamide gel, and then then transferred ("blotted") to nitrocellulose paper (152). The paper is then cut into thin strips, and incubated with serum sample diluted in buffer. The assay procedure is then based on EIA-technique, where the anti-human IgG is
labeled with alkaline phosphatase in order to bind to the antigen-antibody complex on the strip after the first wash step. During the next step a chromogenic substrate produces a dark brown color, so if antibodies are present, dark bands will appear on the nitrocellulose strip. Depending on what antibodies are present in the sample, a band profile will appear on the strip (the combination and intensity of bands that are present). Assigned assessment criteria are used to interpret the result, and designate the sample as positive, negative or indeterminate.

Line immunoassay (LIA) is a version of WB used to confirm a reactive result for HIV, HTLV and HCV (153-154). The LIA assays use well-defined antigens derived from the agent whose antibodies are to be confirmed. These antigens are fixed on a nylon strip. For HIV and HTLV the antigens are type specific, so that the test can both confirm antibodies and discriminate between HIV-1 and HIV-2 and HTLV-1 and HTLV-2 respectively. The nylon strip with the antigens is incubated in a trough with an incubation buffer and the sample.

![Discriminatory bands Confirmation bands Control bands](image)

*Figure 14: HTLV LIA strip. Adapted from package insert INNO-LIA™ HTLV-I/II Score assay.*

HBsAg can be confirmed with a neutralization assay. Before the sample is incubated with the solid phase antibodies it is pre-treated with neutralizing antibodies (anti-HBs) which binds to the HBsAg in the sample, thus blocking the antigen from binding to the microparticles during the next step. The sample, which is pretreated with blocking specific antibodies is compared to a sample which has not been pre-treated. If the resulting signal or absorbance is reduced by at least 50% in the sample portion that was pretreated, compared to the untreated sample, it is considered confirmed positive with HBsAg.
For syphilis, a second EIA or immunoassay can be used to confirm the treponemal antibodies. In addition, non-treponemal antibodies confirms an ongoing infection. Treponemal syphilis antibodies can also be confirmed with a LIA assay.
Aims

The general aim of this project was:
To evaluate screening assays and diagnostics strategies which are used for blood borne infections in Sweden.

The specific aims were:
To evaluate fourth generation HIV screening assays commonly used in blood donor screening.

To evaluate assays and algorithms for syphilis screening and confirmation.

To study the prevalence of HTLV-1 and HTLV-2 infection in Sweden.

To evaluate a HCV Core antigen assay in comparison with a molecular HCV-RNA assay in the detection of viremia and in monitoring HCV therapy.
Material and methods

Samples
Serum samples for the evaluation of screening assays (paper I and IV) were collected from different sources. Previously confirmed positive samples from the routine screening were used for sensitivity evaluation, as well as samples that had shown non-specific reactivity previously, i.e. samples that were reactive in the screening assay but not confirmed as positive. The latter were included to challenge the specificity evaluation. In paper III samples previously analyzed with a molecular assay to detect viremia were used to be compared to a serological assay to detect viral presence.

Furthermore, commercial seroconversion panels (paper I) and other commercial and institutional performance panels (paper I and IV) were used to compare the assays performance in the seroconversion window and at different stages of the infection. In addition, samples from patients that were sampled early in the infection were also tested as seroconversion panels (paper I).

Sample panels from earlier studies were also used (paper IV). A cohort from prevalence studies in Guinea-Bissau were used to obtain an adequate number of syphilis positive sera to study sensitivity for syphilis assays.

Samples from routine diagnostics used for prevalence studies were made anonymous before screening. In population groups were the prevalence was assumed to be low, samples were pooled into pools of 10 samples before analysis (paper II). In paper II samples were collected from pregnant women, hepatitis C positive patients and IDU’s. Samples that were analyzed retrospectively had been stored at -20°C—70°C. For HCV viral load testing, the samples had been stored in -70°C

Samples from healthy blood donors were used for specificity evaluation in paper I and IV.

Assays
In paper I three automated immunoassays for HIV screening were evaluated. All three assays were 4th generation EIA-based assays, called combo assays, detecting both HIV-antibodies (both HIV-1 and HIV-2) and HIV p24-antigen. All three assays were constructed and manufactured by Abbott Diagnostics (Abbott Laboratories, Abbott Park, IL, USA). Two of the assays; Architect HIV Ag/Ab Combo and PRISM HIV Ag/Ab Combo utilize chemiluminescence technique (described above) whilst the third
assay, AxSYM HIV Ag/Ab Combo uses alkaline phosphatase to yield a fluorescent product that is measured by the instrument optics. These assays were compared to older third generation assays, both a traditionally microtiter format assay, Murex HIV-1.2.O, and two automated assays, AxSYM HIV1/2 gO and PRISM HIV O Plus ChLIA (all three assays were also from Abbott Laboratories). At the time of the evaluation Abbott had almost 2/3 of the screening market for blood borne infections in Sweden. The data available on clinical evaluations of the testing systems were very limited. This was an important reason for initiating the evaluations.

In paper II some of the samples and pools of samples were analyzed with the microtiter EIA assay Murex HTLV I/ II (Murex Biotech Ltd, Dartford, UK) and with the new automated assay from Abbott Laboratories, Architect rHTLV-I/II, the latter evaluated during the study period (75). Confirmation of HTLV-1/2 screening positive samples was performed with the INNO-LIA HTLV I/II Score (Fujirebio Europe, Gent, Belgium)

In paper III the nucleic acid amplification assay COBAS TaqMan HCV Test v 2.0 (Roche Molecular Systems Inc, Pleasanton, CA, USA) for quantitative analysis of HCV-RNA was compared to a serological HCV antigen assay; Architect HCV Ag assay from Abbott Laboratories.

In paper IV six treponemal and three non-treponemal assays were performed. The treponemal assays were one agglutination assay; SeroDia TP-PA (“TP-PA”, Fujirebio Diagnostic Inc, Malvern, USA), three EIA-assays in microtiter format; TrepSure Anti-treponema EIA Screen (“TrepSure”, Phoenix Bio-Tech, West Oakville, Ontario, Canada), Enzywell Treponema IgG (“EnzyWell”, Diesse Diagnostica, Siena, Italy) and Captia Syphilis-IgM capture (Trinity Biotech Plc, Wicklow, Ireland). The latter was a test for IgM antibodies only. Two automated immunoassays both based on chemiluminescence technique; Architect Syphilis TP (“Architect”, Abbott Diagnostics) and Liaison Treponema Screen (“Liaison”, DiaSorin, Saluggia, Italy) were included and a point-of-care, immunochromatographic assay, DPP Syphilis Screen & Confirm assay (“DPP SS&C”, ChemBio Diagnostic Systems Inc, Medford, USA). The latter also had a non-treponemal component, which was one of the non-treponemal assays, together with Macro-Vue RPR Card test (“RPR”, Becton Dickinson and Company, Franklin Lakes, USA) and Oxoid VDRL Carbon antigen test (“VDRL”, Oxoid Ltd, Basingstoke, Hampshire, United Kingdom). To confirm discordant results between the TP-PA and TrepSure assays, an immunoblot assay, Inno-LIA Syphilis score (Fujirebio) was used.
Data collection
In paper I was collected and compiled from routine HIV-screening with the third and fourth generation HIV-tests. Data from blood and plasma donors, both new and regular, pregnant women and clinical samples was included. Data from seroconversion cases was also obtained.

Paper II concerned several population groups, where the data from the infertility screening was collected via enquiry directed to the IVF-clinics in Sweden. Data from blood donor screening was obtained from the national blood donation services. Information on the IDU population group was provided from the baseline study on blood borne infections among injecting drug users (155).

Ethical considerations
For study I and IV, this was considered a technical evaluation since all used samples had been screened before for the infection (cleared by the Regional Ethical Board at Uppsala University, Sweden).

For study II and III, ethical approval had been obtained by the Regional Ethical board at Uppsala University, Sweden.
Results and discussion

The evaluation of the 4th generation HIV screening assays covered 243,577 samples tested with the older 3rd generation assays (including one microtiter EIA and two automated immunoassays) and 182,888 samples tested with the new, 4th generation assays (three automated immunoassays). The total specificity for the 4th generation comboassays were 99.91%, 99.95% and 99.97% respectively for AxSYM, Architect and PRISM (only blood donors were tested on PRISM), see Table 4.

<table>
<thead>
<tr>
<th>AxSYM HIV Ag/Ab Combo</th>
<th>n</th>
<th>Reactive (init/repeat)</th>
<th>Specificity (init/repeat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma donors</td>
<td>18 506</td>
<td>21/5</td>
<td>99.88%/99.97%</td>
</tr>
<tr>
<td>Blood donors</td>
<td>10 154</td>
<td>16/4</td>
<td>99.84%/99.96%</td>
</tr>
<tr>
<td>New donors</td>
<td>798</td>
<td>2/2</td>
<td>99.75%/99.75%</td>
</tr>
<tr>
<td>Clinical exc pregnant</td>
<td>4 935</td>
<td>39/29</td>
<td>99.55%/99.76%</td>
</tr>
<tr>
<td>Pregnant</td>
<td>1 740</td>
<td>12/10</td>
<td>99.31%/99.43%</td>
</tr>
<tr>
<td>Total</td>
<td>36 148</td>
<td>96/54</td>
<td>99.80%/99.91%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Architect HIV Ag/Ab Combo</th>
<th>n</th>
<th>Reactive (init/repeat)</th>
<th>Specificity (init/repeat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma donors</td>
<td>22 867</td>
<td>35/4</td>
<td>99.85%/99.98%</td>
</tr>
<tr>
<td>Blood donors</td>
<td>15 374</td>
<td>22/10</td>
<td>99.86%/99.94%</td>
</tr>
<tr>
<td>New donors</td>
<td>664</td>
<td>0/0</td>
<td>100%/100%</td>
</tr>
<tr>
<td>Clinical exc pregnant</td>
<td>6 386</td>
<td>38/29</td>
<td>99.69%/99.83%</td>
</tr>
<tr>
<td>Pregnant</td>
<td>2 561</td>
<td>4/2</td>
<td>99.84%/99.92%</td>
</tr>
<tr>
<td>Total</td>
<td>47 852</td>
<td>99/45</td>
<td>99.83%/99.95%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PRISM HIV Ag/Ab Combo</th>
<th>n</th>
<th>Reactive (init/repeat)</th>
<th>Specificity (init/repeat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood and plasma donors</td>
<td>64 453</td>
<td>NT/20</td>
<td>NT/99.97%</td>
</tr>
</tbody>
</table>

Table 4. Specificity for three 4th generation HIV screening assays. Both initial and repeated specificity is reported.
The AxSYM system failed to detect one HIV-2 positive sample (n=40), all other previously positive HIV-1 and HIV-2 samples tested (in total 174 samples, 122 HIV-1 and 52 HIV-2) were detected by the three assays. Twenty-seven samples from 13 patients with primary HIV infection (PHI) were positive when tested with the combo assays, but still negative with the antibody-only assays. The seroconversion panels included were also positive in an earlier bleed than the third generation assays.

Taken together, the new HIV combo assays had superior sensitivity and specificity when compared to the previous generation of HIV screening assays. The failure to detect one of the HIV-2 samples with one of the assays could be an indication that HIV-2 detection is not optimal, a fact that had been observed before (156). But it can also be due to the fact that the HIV-2 samples were not of optimal quality because of storage and transport conditions on the site of collection (Guinea-Bissau, West Africa). Even so, a slightly lower ability to detect HIV-2 antibodies than HIV-1 antibodies may be the cause.

As Sweden is counted as a low prevalent area for HIV, a serological combo assay, detecting both antigen and antibodies to HIV, is considered an adequate alternative to NAT testing of blood donors. It has been shown elsewhere that the diagnostic window differs by approximately one day compared to minipool NAT (157). With the sensitive antigen/antibody combo assays available, the serological testing seems sufficient to prevent HIV transmission in low prevalent areas, along with blood donor questionnaire and interview concerning risk behavior between donations. The specificity is crucial when testing blood donors, as a poor specificity means unnecessary donation deferral. Concerns that the combo assays may have less specificity than the older antibody-only assays can be dismissed, as our study shows an excellent specificity for the three assays evaluated.

Since 1st April 2010, the use of HIV combo assay is a requirement for blood donor screening in Sweden, as decided by the National Board of Health and Welfare (115).

**Paper II. Prevalence of HTLV-1/2 in Sweden.**

Prevalence studies among five different population groups showed that overall, the HTLV-1/2 prevalence in Sweden is low. Among the studied groups, the prevalence was highest among IDU:s and people having other blood borne infections. The IVF clients had a ten-fold higher prevalence than blood donors, see Table 5. The prevalence in the IDU group was
comparable to a previous study conducted in the same area (Stockholm) in the beginning of the 1990:s (77). The majority of the infected in this group had HTLV-2 (93%).

Data concerning new blood donors was collected from the national blood donation services comprising of the years from 1995 to 2007. Sixteen HTLV-1 and 2 HTLV-2 infected donors were detected; the majority of the infected originated from, or had a partner from endemic areas. The clearly lower prevalence in this group compared to IVF-clients may indicate that the selection procedure for blood donors is well functioning and appropriate.

![Table 5. Seroprevalence of HTLV-1/2 per studied group](image)

Nearly 12,000 pregnant women were tested, with no evidence of HTLV-infection. A previous study from seven European countries showed a prevalence among pregnant women of 0.044% (158). Due to the fact that the samples came from women from a non-urban area, a study in a more densely populated region, with a larger proportion of immigrants from endemic areas, may show different results. It is possible that the IVF-clients reflect the overall prevalence in the general population. On the other hand, IVF clients may be a group that is more exposed to sexually transmitted infections, for example chlamydia infection, which can cause infertility. This may be the cause of a higher infection prevalence in this group. If the prevalence of HTLV-1/2 among pregnant women is extrapolated according to the rate found in the 2003 European study, or 6-times higher than the rate for blood donors, and up to the IVF client rate of 2.3 per 10,000 individuals found in this study, it could mean that approxi-
mately 15 – 20 women with HTLV-1/2 infection are giving birth each year (about 90,000 pregnancies/year in Sweden). If the transmission rate is 30%, it equates to about 5 – 6 children each year becoming infected with undiagnosed HTLV-1 or HTLV-2.

Concerning the IDU group, a comparable prevalence, which occurred during the 1990’s, was observed. The HTLV-positive individuals were more often positive with HIV, HCV and HBV (ongoing or cleared infection), than their negative counterparts. They were also more likely to have had periods of homelessness, and significantly older than the individuals who tested negative. Compared to the previous survey, they were approximately 10 years older, which may suggest that the transmission is low among the younger IDU’s and that the HTLV-infection in this population group may disappear within a generation (159).

Paper III. Evaluation of a Hepatitis C core antigen assay.

A total of 732 plasma samples were collected from 121 individuals, that over a three year period had been sampled three times or more, were used to analyze HCV viremia with a HCV-RNA assay (COBAS TaqMan HCV Test v 2.0). One hundred of these patients were sampled before, during and after Interferon and anti-viral therapy.

In 432 samples, HCV-RNA was detected, with concentrations ranging from <25 IU/mL to 34,200,000 IU/mL. The remaining 300 plasma samples had no detectable HCV-RNA. In 348 of these RNA positive samples, reactivity to HCV core antigen was detected (>3,0 fmol/mL), thus, in 84 samples with viremia, no HCV core antigen could be detected (<3,0 fmol/mL). The antigen negative samples had HCV-RNA levels ranging from <25 IU/mL to 4540 IU/mL. Of 358 samples reactive to HCV core antigen, 10 had no detectable HCV-RNA. Taking all samples together this implies a sensitivity for the antigen assay of 80.6% and a specificity of 96.7%. A large difference in sensitivity was observed for patients on treatment (61.9%), compared to 95.1% for samples from untreated patients. The specificity was 96.8% and 100% respectively.
Table 6. Sensitivity and specificity for Architect HCV Core Ag assay

<table>
<thead>
<tr>
<th></th>
<th>Samples taken during therapy (Pos HCV-RNA)</th>
<th>Samples from untreated patients (POS HCV-RNA)</th>
<th>Total (Pos HCV-RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive HCVCoreAg</td>
<td>126 (117)</td>
<td>232 (231)</td>
<td>358 (348)</td>
</tr>
<tr>
<td>Negative HCVCoreAg</td>
<td>284 (72)</td>
<td>90 (12)</td>
<td>374 (84)</td>
</tr>
<tr>
<td>Total</td>
<td>410 (189)</td>
<td>322 (243)</td>
<td>732 (432)</td>
</tr>
<tr>
<td>Sensitivity (95% CI)</td>
<td>61.9% (55.0%-68.8%)</td>
<td>95.1% (92.4%-97.8%)</td>
<td>80.6% (76.9%-84.3%)</td>
</tr>
<tr>
<td>Specificity (95% CI)</td>
<td>95.9% (93.3%-98.5%)</td>
<td>98.7% (96.2%-100%)</td>
<td>96.7% (94.7%-98.7%)</td>
</tr>
</tbody>
</table>

This is probably a result of a larger proportion of samples of a low viremic level in the treated population (102 samples < 5000IU/mL versus 15 samples <5000IU/mL in untreated patients), but it may also reflect a difference in the dynamics of the viral markers in treated and untreated individuals. Different genotypes were compared and a good correlation between the two assays was shown for all tested genotypes (1a, 1b, 2b and 3a) and overall. The correlation coefficient ranged between 0.79-0.91.

Figure 15. Correlation between HCV-RNA and HCVCoreAg assays. r=0.79
Fifty-eight of the 100 patients on treatment with interferon and antiviral drugs had samples taken in both week 1 and 4 after therapy start. Nine of these had undetectable HCV core antigen in week 1 and 4, and all nine achieved sustained virological response (SVR = undetectable HCV RNA levels (<50 IU/mL), 24 weeks after completion of treatment). Eighty-five patients had samples taken both 4 and 12 weeks after therapy start, and among these 49 had undetectable HCV core antigen in both weeks 4 and 12, 35 of these achieved SVR. The results suggest that negative core antigen assay in week 1 and week 4 predicts virologic negativity in week 12 (Early virological response=HCV RNA detectable at week 4 but undetectable at week 12). It may also predict SVR, as all patients with antigen negative samples in week 1 achieved SVR. This study, like previous similar studies (160-161) suggests a usefulness of HCV core antigen tests early in the therapy schedule, to predict therapy outcome. As the assay in many respects is easier and quicker to perform and less costly than a HCV-RNA assay, it can also act as an alternative to detect ongoing viremia in antibody-positive samples, useful for example in needle stick injury events. HCV Core antigen assays could also be an alternative to NAT testing of blood donors, in addition to HCV antibody testing.
Paper IV. Evaluation of serological assays for diagnosis of syphilis.

The diagnosis of syphilis is most frequently dependent on antibody detection with serological assays. Many of the assays are old and screening has traditionally been performed with cardiolipin-based non-treponemal assays, which detect antibodies that are formed due to the tissue damage that is caused by the infection. These assays are cheap and therefore widely used in screening activity. In the last decade, treponemal-specific antibody assays adapted to automated immunoanalyzer platforms have been developed, leading to the use of these tests for primarily screening. As the diagnosis of syphilis is complex and difficult, including both types of assays for definitive diagnosis, an evaluation of tests used in the new screening algorithm, with treponemal assays as first line screening test is of great value.

Non-Treponemal assays

595 (82%) of the samples tested with the RPR Card test, VDRL assay and DPP Syphilis Screen & Confirm assay (non-treponemal component, DPP SS&C) (n=729) had concordant results in all three assays. Using the RPR test as a gold standard assay (217 positive samples), the overall sensitivity of the DPP SS&C assay and the VDRL test was 86.5% and 77.1%, respectively. The overall specificity was 91.5% and 93.2% for the DPP SS&C assay and the VDRL test, respectively.

The major advantage of the DPP SS&C assay is that the interpretation of the results is objective, using an automated reader. The flocculation assays, RPR and VDRL, are read manually, requiring skilled personnel. As this POC-assay provides a simultaneous test for treponemal antibodies, it is suitable for health care centers, where a direct and rapid test result is required (162). As the sensitivity performance is lower than the traditional flocculation assay, a negative result should be followed up with extended analysis, if infection is suspected.

The RPR assay is still the most sensitive, but has not been evaluated for cerebrospinal fluid samples (CSF), as is the case for the VDRL assay.

A sensitive assay with some kind of automated interpretation (non-subjective), for example an EIA-assy, would be a desirable supplement to the assays currently available.
Treponemal assays
Out of 619 samples 553 (89%) that were tested with all the six treponemal methods (Captia Syphilis-IgM capture excluded) had concordant results in all assays. The TP-PA assay was indicated as the “gold standard” and compared to this assay the other assays had sensitivities ranging between 93.2% (DPP SS&C) and 100% (TrepSure and Liaison) and specificities ranging between 87.6% (Architect) and 98.2% (Liaison), see Table 7. The low specificity for the Architect assay was mainly due to the samples from Guinea-Bissau, which might have been of suboptimal quality, e.g. due to the previous local storage conditions, which may have affected the specificity determinations.

<table>
<thead>
<tr>
<th>Treponemal assays</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrepSure</td>
<td>100%</td>
<td>97.9%</td>
</tr>
<tr>
<td></td>
<td>(99.2%-100%)</td>
<td>(95.5%-99.2%)</td>
</tr>
<tr>
<td>Enzywell</td>
<td>96.3%</td>
<td>94.9%</td>
</tr>
<tr>
<td></td>
<td>(94.0%-97.8%)</td>
<td>(91.6%-97.1%)</td>
</tr>
<tr>
<td>Architect</td>
<td>99.8%</td>
<td>87.6%</td>
</tr>
<tr>
<td></td>
<td>(98.7%-100%)</td>
<td>(82.8%-91.4%)</td>
</tr>
<tr>
<td>Liaison</td>
<td>100%</td>
<td>98.2%</td>
</tr>
<tr>
<td></td>
<td>(98.8%-100%)</td>
<td>(95.7%-99.4%)</td>
</tr>
<tr>
<td>DPP SS&amp;C, trep</td>
<td>93.2%</td>
<td>97.6%</td>
</tr>
<tr>
<td></td>
<td>(90.9%-95.5%)</td>
<td>(95.8%-99.4%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-Treponemal assays</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDRL</td>
<td>77.1%</td>
<td>93.2%</td>
</tr>
<tr>
<td></td>
<td>(67.2%-87.0%)</td>
<td>(91.0%-95.4%)</td>
</tr>
<tr>
<td>DPP SS&amp;C, non-trep</td>
<td>86.5%</td>
<td>91.5%</td>
</tr>
<tr>
<td></td>
<td>(81.9%-91.1%)</td>
<td>(89.1%-93.9%)</td>
</tr>
</tbody>
</table>

Table 7. Sensitivity and specificity for five treponemal and two non-treponemal assays. TP-PA and RPR used as Gold Standard

The TrepSure assay, which is a microtiter plate EIA-format, was together with the TP-PA assay the best performing assay in terms of sensitivity and specificity, followed by the automated assays Liaison and Architect. If using the reversed test algorithm for syphilis screening, an automated method would be the best method of choice for primary screening, followed by, if reactive, a second treponemal assay, where both TP-PA and
TrepSure would be excellent methods of choice. Even though TrepSure has slightly higher sensitivity, the TP-PA has advantages in practical handling, as it is easy to perform single sample analysis, and also gives the opportunity to quantify the antibodies.

The advantage of the dual POC-assay is that it provides the opportunity for rapid diagnosis and treatment of syphilis as well as counseling for patients during the same visit. This is especially important in the effort to eliminate mother-to-child transmission of syphilis and accordingly congenital syphilis, and together with a rapid POC HIV-assay it has an important role in antenatal care units (163).

The reverse algorithm of syphilis screening has been implemented in many European countries, as it provides a more sensitive first assay in early, primary infection. In Sweden the National Board of Health and Welfare requires a treponemal assay, as a screening assay for blood donations.

The disadvantage of this regimen is that in low-prevalent settings, non-specific reactions may lead to unnecessary deferral of donated blood units. As the treponemal antibodies seem to persist for a lifelong period, past cleared infections will lead to donor deferral, which is not the case if using a non-treponemal assay as the primary screening method (164). On the other hand, the non-treponemal assays can be reactive in conditions other than syphilis infection, e.g. some autoimmune syndromes. Accordingly, in a higher resource setting, where the reverse sequence algorithm is preferred for screening, an automated treponemal immunoassay for initial screening subsequently followed by the TrepSure test or TP-PA assay as a second treponemal assay, appear highly effective. A quantitative highly sensitive non-treponemal assay, e.g. the Macro-Vue RPR Card test, could then be used as a supplementary test to evaluate activity of the syphilis infection.

However, syphilis diagnosis is challenging, as both the treponemal and non-treponemal assays yields false-reactive results. Studies performed to compare the old algorithm (non-treponemal first) with the new, reverse algorithm (treponemal first) have mainly been done in low-prevalent populations, where the problem with false-reactive treponemal tests can be met with a second treponemal assay to confirm the antibody positivity. On the other hand, a study by Hunter at al in 2013 showed that isolated reactivity in an automated treponemal assay (not confirmed with a second treponemal assay) may represent true evidence of *T. pallidum* infection (165). However, the slightly higher sensitivity of the treponemal assays in early and late latent syphilis infection supports the use of the reverse algorithm (166).
Conclusions and future perspectives

In Sweden, contrary to many other European countries, serology screening is still a requirement for blood and tissue donor infection screening. Due to the low prevalence of blood borne infections, and a careful selection procedure of blood donors, the risk of early, undetectable infections is very low. This has led to the assessment that NAT-assays are not currently necessary. A study has estimated that the cost-effectiveness ratios are far beyond what is usually considered cost-effective if NAT-assays would be adapted in Swedish blood donor screening (157). With this in view, the immunoassays used for serological screening needs to keep up a high sensitivity and specificity. Since the study in paper I, several new, automated HIV combo assays have been made available. The sensitivity and specificity for these new tests need to be continuously monitored.

Regarding HCV screening, the current HCV antibody assays required for blood donor screening does have quite a wide diagnostic window. Looking at the last ten years (2005-2015) there have been two incidents in Sweden where blood recipients have been infected by blood components donated of a HCV-infected seroconversion window phase donor. The HCV Core antigen assay evaluated in paper III could be a suitable addition to the antibody assays. More studies on the assays effect on seroconversion samples might be useful. The HCV Core antigen assays would provide a great improvement of HCV detection without implementing NAT testing.

Syphilis infection has had an upward trend both in Sweden and other western European countries over the last ten years (98). The diagnosis of the syphilis infection is challenging and is still substantially based on serologic assays. The use of the reverse screening algorithm, with a treponemal assay as first line screening test, and the launching of several new automated immunoassays, requires updating the diagnosis criteria. Even if syphilis infection is increasing, Sweden is still considered as a low prevalent area.
Specificity monitoring should be done regularly especially considering the tests used for blood donor screening. Several of the assays that have been evaluated in paper IV showed excellent properties that may be used for confirmation of immunoassay-screened reactivity.

As for the non-treponemal assays, used for determining active infection, an objective assay with manual-independent reading would be an improvement to diagnosis. Maybe the immunochromatographic POC assays with an automated interpretation reader could fulfill this need. As these kinds of assays become more and more common, both in health care centers and for sale in drugstores for private use, the evaluation of their quality is constantly required. As new assays are launched on the market continuously, evaluation is important in order to verify the quality of those assays.

The prevalence of certain blood borne infections is unknown, as several of them have a mild or asymptomatic course. This may imply an unawareness of the infection. In the case of HCV, individuals that received a blood transfusion before 1991 (the year of implementation of HCV antibody testing of blood donors) and were transfused with contaminated blood products may have had an asymptomatic infection and therefore

Figure 16. Number of reported cases of syphilis in Sweden 2000-2013. Adapted with permission from the Public Health Agency of Sweden
never been tested. Efforts have been made in parts of Sweden to invite those individuals to be sampled and analyzed.

HTLV-1 and HTLV-2 are also infections, which are asymptomatic for many years after infection. As these viruses are quite rare in northern Europe (except for HTLV-2 in the major cities, among IDU’s) very little attention has been paid to them. The only groups that are screened on a regular basis for these infections are blood donors and IVF clients. Still, the prevalence in Sweden is not known. A national prevalence survey among pregnant women and their partners would be a suitable follow-up to the relatively small and local overview covered in paper II. As some of the Swedish regional councils are carrying out needle exchange programs, a HTLV-1/2 prevalence study among the participants might also be of importance to learn about the HTLV-1/2 prevalence nationwide in these population groups.

HTLV-1/2 screening is mandatory for new blood donors and IVF clients. Possibly a third group, breast milk donors, should be added to the mandatory regulation, as this is the main route of transmission of HTLV-1/2 infection.

We had also planned to study the prevalence of another blood borne infection, West Nile virus. However, due to limited funding, this had to be postponed. The travelling habits among the Swedish population have changed, and the risk of becoming infected with microorganisms that are a potential threat to the blood supply may increase. A survey among blood donors to detect antibodies of such infections could provide information about possible future challenges in the blood safety.
Acknowledgements

When I started this journey for more than 8 years ago, I could not imagine how much work it would entail, and at the same time, how much pleasure. Having the opportunity to work on this thesis, in this interesting field has been a great privilege. However, this would not have been possible without the tremendous help and support of many people. I would like to express my sincerest gratitude to everybody who has contributed to this thesis, and in particular to:

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