Transfusion microbiology practice

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Foreword

When routine testing for hepatitis B surface antigen was introduced into transfusion services in the early 1970s, specialists in transfusion medicine became, through necessity, much better acquainted with their microbiological colleagues. With the advent of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) in the early 1980s and the routine availability of test procedures in 1985 for what was then called anti-HTLV III, the acquaintanceship ripened into a partnership. Today, a further decade down the road, with hepatitis C (but not yet G?), HIV 2 and in some places HTLV I and II now added to the list of potentially transmissible diseases for which testing is done, it appears that microbiology has become a subdiscipline of transfusion medicine, much as tissue typing did some 30 years ago. Perhaps, as this book's title would suggest, transfusion medicine represents a significant subset of microbiological practice.

The World Health Organization's Regional Office for the Eastern Mediterranean is to be commended for its foresight and initiative in sponsoring the production of this monograph, which, because of its comprehensiveness and relevance, will have worldwide impact.

The authors have done excellent work in bringing together the complete range of viral and nonviral transfusion-transmitted infections, the diagnostic and confirmatory tests available and the strategies underlying their use, and the appropriate handling of the data obtained.

An orthodox and conventional approach has been adopted which is intensely practical, and yet appreciative of the subtleties of testing and its interpretation. Where there are advantages and disadvantages in the employment of various types of screening test, these are dealt with in a rational and even-handed fashion and an excellent summary of this information is provided. The contemporary views of the importance of standard operating procedures (and who writes them) as part of a quality system are also well reflected.

In a succinct fashion, this technical manual brings together a wealth of relevant information about the microbiological aspects of modern transfusion practice. It will prove invaluable for medical, scientific and technical staff of

transfusion services, both large and small, and will also provide an unusual, if not unique, view of modern transfusion medicine as it impinges on the work of the microbiological laboratory staff.

Professor Robert Beal
President-Elect, International Society of Blood Transfusion
Former Director, Blood Department,
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Preface

The WHO Regional Office for the Eastern Mediterranean continues to support the development of blood transfusion services in the countries of the Region according to regional and country-specific plans. The activities aim at achieving the provision of safe blood and blood components, based on voluntary regular non-remunerated blood donation, without undue pressure or inducement. Ensuring blood safety is among the highest priorities of the Regional Office for the Eastern Mediterranean.

Many infective agents are potentially transmissible through the transfusion of blood and its derivatives. Although the relative importance of different agents may vary in different countries, and even within different areas of the same country, microbiology transfusion practice is a topic of global importance. Being aware of the importance of the subject to all regions of the world, we attempt to cover in this publication a wide range of infective agents, including those that may be of no special significance to countries of the Region. We have also tried to cover theoretical and practical aspects of modern transfusion microbiology.

This publication is intended for blood transfusion specialists, technologists and technicians, who must strive to ensure the provision of blood and blood derivatives free from contamination with infective agents.

The authors are grateful to Dr Hussein A. Gezairy, Regional Director for the Eastern Mediterranean, for his valuable and continuous support and to Dr M.H. Khayat, Deputy Regional Director for the Eastern Mediterranean for his continuous encouragement and advocacy. Our thanks are due to Professor Robert Beal, Australia, for reviewing the draft and for his constructive suggestions.

Abbreviations

ALT	alanine aminotransferase
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
GLP	Good Laboratory Practice
HAV	hepatitis A virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
HGV	hepatitis G virus
HIV	human immunodeficiency virus
Ig	immunoglobulin
IgG	immunoglobulin class G
IgM	immunoglobulin class M
OD	optical density
PCR	polymerase chain reaction
PTI	posttransfusion infection
PTNANBH	posttransfusion non-A, non-B hepatitis
QA	quality assurance
QC	quality control
QM	quality monitoring
RNA	ribonucleic acid
SOP	standard operating procedure

Chapter 1

Introduction

The provision of blood and blood products free from contamination by any infectious agents is globally the aim of all blood transfusion services and the staff working within them. However, in many countries there is great concern about the safety of the blood supply. Acute shortages of resources often result in the transfusion of unscreened or only partially screened blood. In other circumstances, even where screening is performed, the quality of the collection and laboratory screening procedures is often poor, and there is still a significant risk to recipients of blood transfusions.

In recent years the true significance of the potential for blood transfusion to be efficient vehicle for the transmission of infection has finally been acknowledged. Although the transmission of syphilis and hepatitis B virus (HBV) had been recognized for many years, it was the isolation and identification of human immunodeficiency virus (HIV) and the realization that transmission by blood transfusion was not only occurring, but transfusion was potentially a major route of infection, that rapidly pushed the issue of blood safety into prominence and made everyone aware of the need for, and importance of, microbiologically safe blood and blood products. The role of the transfusion microbiologist has thus grown rapidly with the need for more specialized staff who understand the nature of infectious agents and the problems in designing, implementing and maintaining effective and appropriate blood donation screening programmes.

The process of providing safe blood essentially begins with donor selection. Although the subject of donor selection is not covered in this manual, it is referred to because of its importance in the recruitment and retention of "safe" blood donors. The identification and deferral of donors considered to be "high-risk", by activity or association, is central to donor selection, which aims to minimize any risk of transmission of infectious agents by donations which may have been collected from donors in the window period of acute infection (infectious but not detected by routine screening tests).

Laboratory screening, however, remains at the centre of a safe blood supply. In most countries it is mandatory to screen donated blood for a minimum set of markers of infectious agents. The decision as to which infectious agents are screened for has to be based upon prevalence data for the particular donor population, together with any political, social or ethical considerations that may be relevant. Continuous monitoring of the prevalence of other agents that may be transmitted by transfusion is also necessary, to determine if and when screening should be introduced. To be effective, not only must screening tests be performed correctly, but they must have sufficient sensitivity to detect infections in otherwise apparently healthy, asymptomatic donors and sufficient specificity to ensure that the number of false positive reactions, and hence unnecessarily wasted donations, is minimized. For each infectious agent screened for, the most appropriate marker of infection must be identified and, wherever possible, either quantitatively or semiquantitatively, a minimum detectable level defined. All screening procedures must be carried out by trained staff following carefully the manufacturers' instructions, and applying the principles of Good Laboratory Practice (GLP) at all times.

This manual has been produced to try to provide a theoretical and practical text on key aspects of modern transfusion microbiology and the provision of a microbiologically safe blood supply. Thus, the theme throughout this manual is the provision of a safe blood supply through the appropriate and effective screening of donations. While individual countries may have their own specific problems and perspectives in providing a safe blood supply, the basic principles and procedures applied are common to all. Practical advice and guidance is provided on the design of screening programmes, the selection and performance of screening tests, maintaining quality and the confirmation of screening results. In addition, a section has also been included on transfusion bacteriology, environmental monitoring of work areas, donor arm cleansing and product sterility testing. It is hoped, therefore, that this manual will be of use to all staff working in transfusion services, providing an easy to use and straightforward reference text for both theoretical and practical aspects of the screening of donated blood for transmissible infectious agents.

Chapter 2

The transmission of infection by blood transfusion

2.1 Introduction

Since blood transfusion became a common and integral part of modern medicine, it has been realized that certain infectious agents can be passed from donor to recipient. Historically, when relatively few of the microorganisms identified today were known about, syphilis was thought to be the only "serious" disease that was transmitted. Ironically, even though syphilis is still screened for, it is currently the only one of the major transmissible diseases that can be treated successfully. However, while a number of infectious agents are transmitted by blood transfusion, many are not. Understanding why some agents are transmitted and others are not is essential for a full understanding of the subject of transfusion microbiology; screening policies and strategies can only be properly developed and implemented with this knowledge. In addition, prevalence data for infectious agents present in the local or national population are also required, to determine not only which transmissible agents are present in the donor population, but also which are already present in the patients likely to receive the blood. In some situations the prevalence of an infectious agent is so high in both the donor and patient populations that, even though the agent is transmissible, screening becomes inappropriate because very few donors do not carry the agent and very few patients are not already infected.

Furthermore, even agents that are clearly transmissible may not always actually be transmitted following the transfusion of an infected or potentially infected product; reasons for this also need to be understood. In the case of bacterial transmission, the bacteria themselves may not be quite so important as the toxins that they produce when multiplying in the blood pack or bottle. Often bacterial growth inside blood packs and bottles is self-limiting, but the toxins produced remain and may cause severe illness in recipients.

There are a number of particular characteristics of an infectious agent that make it potentially transmissible:

- asymptomatic infection
- presence in the bloodstream
- parenteral transmission
- survival during storage.

2.2 The infected blood donor

2.2.1 Asymptomatic infection

It is a fact that we are all reservoirs of a number of infectious agents. Most are relatively harmless, but some are not. Generally, in a healthy individual, there is a balance between the body's defences and the individual infectious agents, such that each is contained and does not result in an active infection. This balance is easily upset, however, enabling an agent to establish itself and give rise to a generalized systemic infection. Such an infection may be very mild and pass unnoticed, or asymptomatic infection, or moderate to severe and debilitating symptomatic infection. The precise course of infection clearly depends upon the particular agent and the individual infected, but whether an infectious agent gives rise to an asymptomatic or symptomatic infection, an individual will be infectious at some stage, although for a short period, during the course of the infection.

Clearly, a potential donor who has any recognizable clinical symptoms which could be due to an infection would not be bled. This assumes, however, that the donor selection and questioning procedures are adequate and that the donor would declare any symptoms appearing in the weeks before donation. Thus, with any infectious agent that always gives rise to clinical symptoms, infection is very unlikely to be transmitted by transfusion, because an infected donor would not be considered fit to donate. However, an asymptomatic infection in a donor may subsequently become symptomatic following donation. In this case it is essential to ensure that donors are also made aware of the importance of reporting bouts of illness within a certain time period following donation.

Unfortunately, with most infectious agents there is a clearly defined period following infection, the "window period", during which infection may not be detected, although the individual may be infectious. Following infection with any infectious agent there is an incubation phase during which the agent proliferates

and the infection establishes itself. The length of this period varies tremendously, depending upon the infectious agent, the individual and the particular marker used to detect the agent. Subsequently, the results of the immune response to the infection start to appear and, usually, antibodies against the agent become detectable; the window "closes" when this antibody becomes detectable.

2.2.2 Presence in the bloodstream and latency

An infectious agent must be present in the bloodstream of the donor at the time of donation in order to be transmitted. However, the agent must not just be present, but must be present in an infectious or potentially infectious form.

Infectious agents are carried in the bloodstream in a number of different ways, depending upon the particular agent and the stage of infection. As previously mentioned, most infectious agents are found in the bloodstream at some stage during the course of infection, although this may not represent an infectious stage. An infectious agent may be present in the blood stream free in the plasma (known as viraemia, bacteraemia or parasitaemia as appropriate), carried in the leukocytes (white blood cells) or carried in the erythrocytes (red blood cells).

Free in the plasma. Carriage free in the plasma is probably the simplest and most common form of carriage in the bloodstream. An agent can circulate in this way as a means of directly infecting other tissues or can be released from infected tissues into the bloodstream as part of the life cycle of the organism. Viruses, bacteria and protozoa can all be found carried free in the plasma. Agents which are transmitted by blood contact (see 2.2.3 on parenteral transmission) initially circulate from the point of entry to reach the tissues where primary infection will occur. Following the establishment of infection in the primary sites, some agents are then released back into the bloodstream to be carried to other tissues and/or render the individual infectious and able to facilitate transmission to another individual. An example of an agent which makes full use of the bloodstream is hepatitis B virus which, following entry into an individual, uses the bloodstream to reach the liver, following which new virus particles are then released back into the bloodstream for transmission to a new host.

Carried in leukocytes. Carriage in leukocytes, both lymphocytes and neutrophils, can occur in one of two ways: as free intact organisms in the cytoplasm, or as genetic material within the nucleus of the leukocyte. In some cases the same agent may be found in both forms, but at different stages of

infection. Although some leukocyte types will actively engulf individual infectious agents in their role as scavengers, agents which can be found in the leukocytes generally specifically infect these cells as part of their life cycle. Because leukocytes are nucleated and have a normal cellular cytoplasmic organization, once infected, the infection may persist for the life of the cell, and ultimately the life of the individual. A number of viruses can persist in an infected individual after the resolution of an acute infection, and after immunity has apparently built up. The virus can persist in a dormant form, even in the presence of specific antibodies against it, as viral nucleic acid integrated into the host cell's genome. However, it can subsequently reactivate following some future stimulus, and may result in another acute infection. This form of viral persistence is known as latency. Latency differs from chronic infection in that latent infections are completely inactive and the intact virus cannot be detected, while chronic infection is the result of persistent active infection, although often low-level, with continuing viral replication. Any infectious agent that may be present in leukocytes, in an active or latent form, may therefore be transmitted by transfusion.

Carried in erythrocytes. Some protozoan infections have a phase in which the organism is present in the erythrocytes. During this phase the organism usually matures into the next stage of its life cycle before being released from the erythrocyte, either while still circulating or in the liver or spleen. The organisms released may be able to infect other red cells as well as other tissues, so spreading the infection. The most common examples of such organisms are the protozoan *Plasmodium* spp., the causative agents of malaria.

2.2.3 Parenteral transmission

The route of transmission of an infectious agent clearly plays a part in determining whether transmission by transfusion is possible. Parenteral transmission is defined as the transmission of an infectious agent by any means except through the alimentary canal (gastrointestinal tract); thus parenteral routes include intravenous and intramuscular injections, direct contact between blood and other body fluids, any action or activity that leads to a break in the skin and direct blood to blood contact, and sexual contact. The transmission of any infection by blood transfusion can only be by the parenteral route, i.e. the intravenous or intramuscular infusion of blood or blood product, and generally

only infectious agents that are transmitted parenterally are transmitted by transfusion. Therefore viruses such as hepatitis B virus (HBV) and human immunodeficiency virus (HIV), most bacteria (bacteraemic donor), and protozoa such as *Plasmodium* spp. and *Trypanosoma* spp. are all transmissible, while viruses such as rhinoviruses and influenza viruses are not considered to be transmissible by this route. These types of virus are normally transmitted via aerosols through the mucous membranes of the mouth and buccal cavity, and although low-level viraemia may occur, blood-to-blood contact has not been reported to transmit infection.

There are, however, exceptions to these generalizations. For example, hepatitis A virus (HAV) has been reported to be transmitted by transfusion. Although HAV is an enterovirus, transmitted by the faeco-oral route and therefore not normally considered to be transmissible by transfusion, cases of transmission through large pool plasma products have been reported. It is presumed that a number of donors were infected with HAV and were viraemic at the time of donation. HAV is a nonenveloped virus and, like a number of other viruses, is not easily inactivated during the production processes; infectious virions present in the raw plasma may survive, resulting in an infectious final product.

2.2.4 Survival during storage

Blood and blood products may be stored in a number of different physical states, (e.g. whole blood, plasma, high concentration protein solution, lyophilized material) and at a number of different temperatures (−40 °C to 25 °C). Any agent present in the donated blood must be able to survive at least some of these storage conditions and the conditions during processing to be able to infect recipients of the products. Generally, viruses are best suited to this, and certain viruses, if present in the original donation, may be found in virtually all products prepared. This is especially true of the nonenveloped viruses referred to earlier. Bacteria can persist, but often they multiply briefly during storage and then die quickly in the generally nonoptimal conditions, although, as stated previously, they can leave toxins in the product which can cause severe illness. *Treponema pallidum*, the causative agent of syphilis, is one of the more unstable organisms and will usually survive for no longer than 72 hours at 4 °C.

2.3 The infected recipient

There remains then the question of why the apparent transmission of a transmissible agent may not give rise to infection in some patients; although blood transfusion is a particularly effective route of transmission, transmission and subsequent infection do not always occur, or are not always seen to occur. Reasons for this include the particular agent, the size of the infectious dose given, the particular blood product given, the condition of the patient and the patient's immune status, and the degree of posttransfusion patient monitoring performed.

a) Particular infectious agent

Although a donor may be infected with a particular agent, he may not actually be infectious, i.e. at the time of donation the infectious agent itself was not present in the bloodstream. Some donors infected with hepatitis C virus (HCV) possess circulating antibodies but the virus cannot be detected, and studies have shown that transmission of HCV in such instances is unlikely.

b) Size of the infectious dose given

The number of infectious particles needed to ensure infection varies with each infectious agent and in many cases is not known. In theory at least, the presence of just one infectious particle could transmit infection, but in practice it is likely that there is a minimum critical number. The volume of blood transfused also partly determines the size of infectious dose given. If, for example, a unit of blood contains just one infectious dose and only 100 ml of blood is actually transfused, there is a good chance that the infectious agent would not be transfused.

c) Blood product transfused

Not all agents will be present in all products made from an infected donation. Some viruses like HBV and HIV, which can be found as free virions in the plasma, may be present in nearly all products made, including large pool products. Other agents may only be associated with particular products because they are normally present only in certain components of the blood. For example, agents found in leukocytes, such as cytomegalovirus or toxoplasma, will rarely cause problems in plasma products or leukodepleted products; while agents found in erythrocytes, such as malaria, will rarely cause problems in plasma or platelet products.

d) Condition and immune status of the patient

The rate of blood loss and replacement and the immune status of the patient can influence the likelihood of infection. Patients receiving blood are often in generally poor health and cannot easily fight off any infection transmitted; however this is not always the case. Patients with massive trauma and rapid blood loss during surgery may be given many units of blood before being stabilized. A lot of this blood flows in and out of the body very quickly and it is thought that in such cases, depending upon the amount of infectious agent present in the donated blood and the volume of blood transfused, sufficient infectious particles would be washed straight through the patient so that infection would not occur. Immunosuppressed patients may be far more susceptible to certain infectious agents, especially viruses like cytomegalovirus and many bacteria, than nonsuppressed patients, and serious infections may develop from even very low levels of infectious agent. Conversely, patients already prescribed high doses of antibiotics or antiprotozoan drugs may not become infected after receiving blood infected with certain bacteria or protozoa.

e) Patient monitoring

In many countries, including most industrialized countries, the monitoring of patients for posttransfusion infection is not optimal. There are many reasons for this, but the net result is that in almost all countries the incidence of posttransfusion infection (PTI) is underreported. The effectiveness of any blood donation screening programme can be assessed accurately only by effective feedback from hospitals, i.e. by notifying the transfusion service of all suspected cases of PTI.

There are, of course, many reasons why PTI is underreported, often relating to the posttransfusion care and monitoring of patients. In a "healthy" recipient of blood, for example a previously healthy trauma victim, even if transmission does occur, an asymptomatic infection may follow which may never be diagnosed. A significant proportion of natural HBV infections are asymptomatic, the only indication being the subsequent, usually chance, finding of the antibody against HBsAg (anti-HBs), or other marker(s) of HBV infection, in individuals with no known history of hepatitis. The same can apply to other agents; unless symptoms appear or there is active monitoring of asymptomatic recipients over a period of time it is unlikely that many infections will be identified.

As can be seen, there are many reasons individual recipients may either respond differently to an infectious agent transmitted by transfusion, or may not become infected following the transfusion. However, the provision of safe blood is vital, because although the recipients themselves may not become clinically ill following infection, they will be infectious at some stage and may then act as the foci of new infections, either in the family environment or with casual partners and other contacts.

2.4 Donor and patient populations

The final issue in this chapter concerns the question of which infectious agents are actually present both in the donor population and in the patient population. Prevalence data for transmissible infectious agents are needed for any donor population, be it local or national, and for the hospital patient population, in order to ensure that all donation screening performed is both effective and appropriate. These data need to take into account any geographical or other variations, such as mixed ethnic groups in the population, and also need to be reviewed regularly to monitor changes in prevalence which could affect the blood screening strategies adopted. For each infectious agent screened for, there exist two important prevalence levels: a lower level at which, once reached, screening becomes necessary, and a higher level at which, once reached, screening may no longer be appropriate and may be stopped. To some extent these levels are simply theoretical figures, but they do serve to rationalize the process of selecting those infectious agents which may pose a real threat to the blood supply, not just a perceived threat based upon their infectivity and that does not take the actual prevalence into account. Thus, as a particular infectious agent appears in a population and starts to spread, the prevalence early on may be so low as to make screening inappropriate. However, as the prevalence increases the number of donors that could be infected starts to increase and screening becomes necessary. If the prevalence continues to increase, eventually the number of noninfected donors becomes very small and the supply of blood becomes seriously compromised. At this stage it may be appropriate, therefore, to cease screening for the agent. Such a decision is unlikely to compromise the safety of the blood supply because at the same time the increasing prevalence means that the number of naturally infected patients also increases, and possibly at a faster rate. Thus the significance and risk of any PTI may actually decrease because so many patients would already be infected before any transfusion took place.

Chapter 3
Transmissible viral infections

3.1 Introduction

The transmission of viral agents is one of the major problems facing transfusion medicine. Over the years the effectiveness of transfusion as a route of transmission for a number of viral agents has been demonstrated time and time again. Even with quality driven screening programmes, the nature of viral agents, and their incubation periods and infection patterns, means that there is still the problem of potential transmission through donations collected during the window period of infection, when the donor may be infectious but without any detectable markers of infection.

This chapter will look at the basic virology and epidemiology of transmissible viral infectious agents of most concern—hepatitis B and C viruses, human immunodeficiency viruses, human T cell leukaemia viruses and human cytomegalovirus. Mention will also be made of two other viruses that may be transmitted but that have much less clinical significance to any recipient and for which screening is rarely or only selectively carried out—hepatitis A virus and parvovirus B19.

3.2 Hepatitis B virus

3.2.1 General

Hepatitis B virus (HBV) is a DNA virus, a member of the hepadnavirus (*Hepadnaviridae*) family. The infectious particle, the Dane particle, is 42 nm in diameter and comprises the DNA genome encapsulated in core protein, which is then covered by an envelope of surface proteins. The hepadnaviruses are characterized by the production of a vast excess of the surface proteins, commonly known as hepatitis B surface antigen (HBsAg), as spherical and filamental forms which are released into the bloodstream with the infectious Dane particles. The Dane particles can reach concentrations as high as 10^8/ml–10^{10}/ml while the HBsAg particles typically reach levels of 10^{13}/ml. The virus is transmitted

parenterally, including by intimate contact, and has an incubation period of about 4–8 weeks. The prevalence of HBV varies worldwide from as low as 0.01% to 0.1% in developed countries with good public health to 20%, and even higher, in many developing countries. In the countries with a high prevalence, infection is commonly transmitted vertically from mother to infant at birth and during the early years, and horizontally during childhood through close contact. Parenteral routes commonly include: blood transfusion, notably in countries where blood is not screened; intravenous drug use; nonsterile surgical instruments, including tattooing and acupuncture instruments; and patient to staff transmission in hospital and other health care settings.

Infection may follow one of two courses: either acute infection with the subsequent clearance of the virus and development of immunity, or chronic infection with persistence of viral replication for extended periods, even through the lifetime of the individual. However, chronic infection may spontaneously resolve and the individual may then develop immunity; alternatively a stable chronic infection may reactivate with a resulting further acute episode. Although HBV infection can lead to severe disease (cirrhosis, hepatocellular carcinoma and liver failure; asymptomatic infections are very common) with most individuals infection resolves and immunity develops without any symptoms whatsoever. Figure 3.1 indicates the possible outcomes of infection with HBV.

3.2.2 HBV serology

Following infection the virus migrates to the liver and enters the hepatocytes where the viral DNA integrates with the host cell genome. The viral DNA then uses the cell's machinery to produce new viral DNA and a number of HBV proteins, including an excess of HBsAg, resulting in the sequential appearance in the bloodstream of Dane particles and uninfectious HBsAg particles; and then the other specific antigen and antibody markers of HBV infection are produced. Precisely which markers are present and for how long depends upon whether infection is acute and resolves or progresses to chronicity.

HBeAg is a nonstructural protein whose specific function is not clear. It appears soon after HBsAg is first detectable and is generally considered to be a marker of viral replication and hence infectivity; while HBeAg is detectable, HBV DNA is also detectable. HBeAg, however, declines before HBsAg is cleared. Seroconversion to anti-HBe is a first indication that the infection may be acute

Fulminant   Recovery and immunity
approx. 1%

Symptomatic   Asymptomatic
25%–30%   70%–75%

Acute infection

95%

Exposure to HBV

5%

Chronic infection

Cirrhosis   "Healthy carrier"
15%–25%   75%–85%

possible

Hepatocellular carcinoma
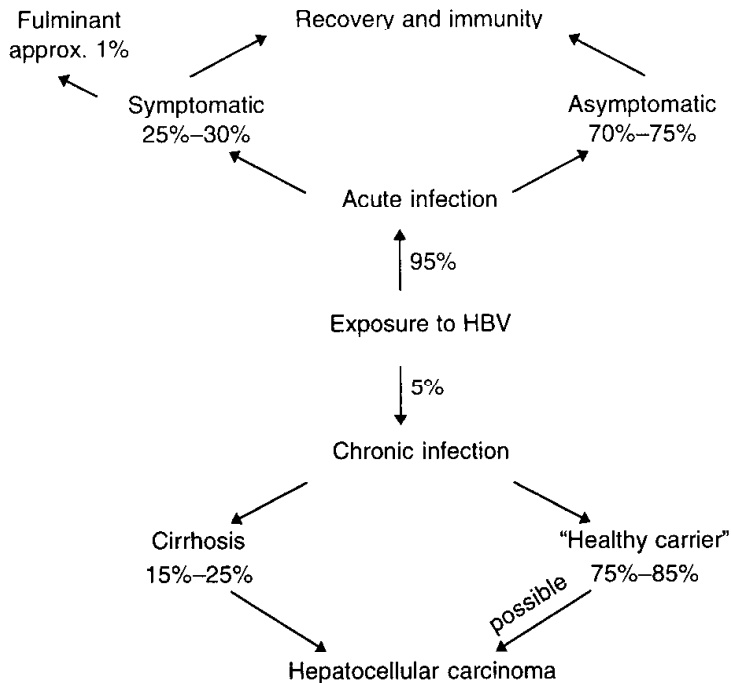
FIGURE 3.1 **Outcomes of infection with HBV**

and resolve naturally, and generally there is a short time gap between the disappearance of HBeAg and the appearance of anti-HBe.

HBcAg is the protein that makes up the core of the Dane particle. It is not found in the bloodstream although it can be found free in hepatocytes. Anti-HBc, however, is a particularly useful marker of infection which appears after HBsAg and HBeAg but may persist for the lifetime of an individual. IgM anti-HBc appears first, with IgG anti-HBc gradually increasing in titre and persisting after the IgM titre has declined. IgM anti-HBc is commonly used to identify acute infections.

Anti-HBs is a protective antibody which usually appears shortly after the disappearance of HBsAg although there are some individuals in whom there is an extended delay before the appearance of anti-HBs. Titres may decline over extended periods but recovery from acute infection does confer lifetime protection.
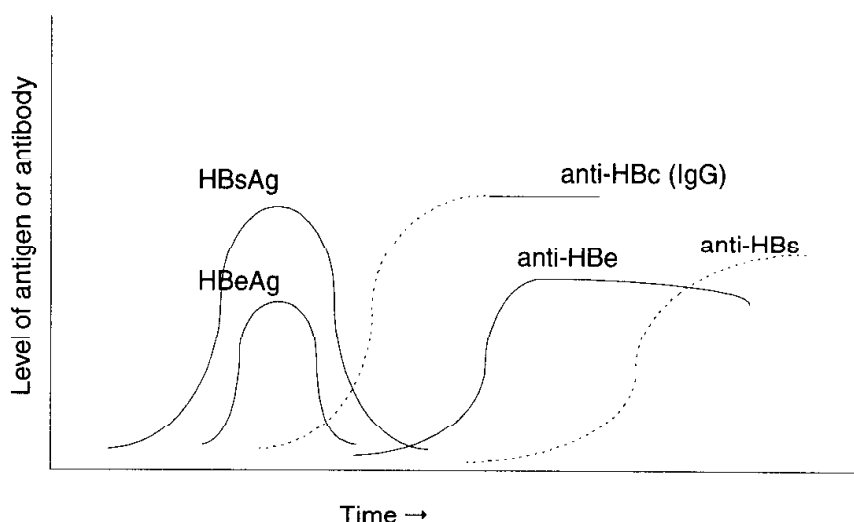
FIGURE 3.2 **Serological profile of acute HBV infection**

Acute infection (see Figure 3.2). The first detectable marker is HBsAg, which increases in titre quite rapidly and then starts to decline, normally being detectable for 2–12 weeks. Early on in acute infection, HBsAg may be the only marker detectable, and, in the absence of Dane particles, the individual may be initially uninfectious for a short period. HBeAg subsequently appears, but at a level below that of HBsAg, and then declines quite quickly, disappearing before HBsAg disappears. Anti-HBc (IgM + IgG) appears around the time that HBsAg levels peak, the IgM level declining gradually over a period of 2–16 weeks from first appearance, while the IgG level persists for many years, often for the lifetime of the individual. Anti-HBe appears as HBeAg disappears and may persist for many years although levels do slowly decline. Anti-HBs finally appears some time after HBsAg levels have declined and almost always marks full immunity, persisting for the lifetime of the individual. Occasional cases have been reported where anti-HBs has been detected in the presence of HBsAg.

Chronic infection (see Figure 3.3). Following their appearance, HBsAg and HBeAg persist, followed subsequently by IgG anti-HBc. While HBeAg persists, an infected individual has a chronic active HBV infection. In some individuals seroconversion to anti-HBe may occur; this could be at any time, with the consequent cessation of active HBV infection. Chronic infections may resolve after a period of time with cessation of liver disease and subsequent development of full immunity.
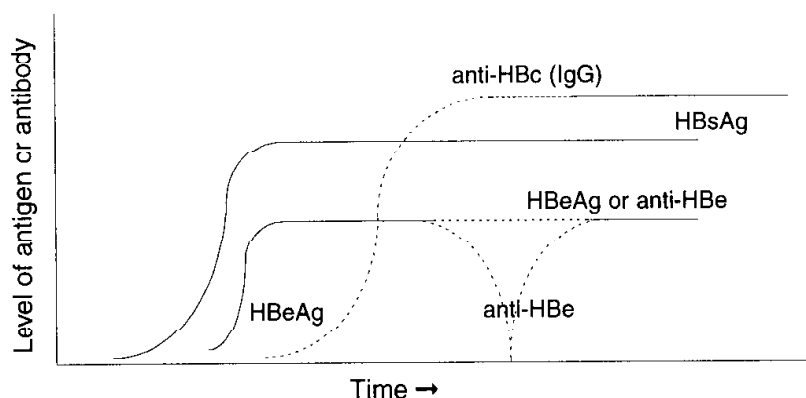
FIGURE 3.3 **Serological profile of chronic HBV infection**

3.2.3 Hepatitis B virus DNA

Using molecular techniques HBV DNA can be detected in serum during acute and chronic infections. In acute infections DNA can be detected in the bloodstream soon after HBsAg can be detected, but may not persist for long. Many studies have looked at the link between HBV DNA and HBeAg/anti-HBe and have found that 75% to 85% of HBeAg positive individuals are HBV DNA positive, while only 10% to 20% of anti-HBe positive individuals are HBV DNA positive. The detection of HBV DNA may have more value when performed in conjunction with anti-HBc screening to detect the HBV tail-end carriers (see 3.2.5).

3.2.4 Screening of blood donations for HBV infection

Detection of HBV infection in donated blood is best achieved by screening for HBsAg; this is universally accepted. HBsAg is the first marker to appear in the bloodstream, and persists throughout the period of infectivity. The other markers of HBV infection are of use in confirming infection (see Chapter 10) and determining the type and stage of infection, but apart from anti-HBc (see 3.2.5) have no value in routine blood screening. Detection of HBV DNA may be beneficial in assessing the individual's status, but again has limited value to blood screening: the techniques are not yet suitable for mass screening, and DNA levels

can fall to below detectable levels while infectivity may remain. The sensitivity and specificity of HBsAg assays are generally good with sensitivities increasing all the time, being currently around 0.1 iu/ml.

3.2.5 Anti-HBc screening

For many years the subject of anti-HBc screening of donations has been discussed. Before specific anti-HCV screening became possible, anti-HBc screening was considered by some to be a possible way of reducing the number of cases of posttransfusion non-A, non-B hepatitis (PTNANBH), at that time seen in large numbers. The identification of the hepatitis C virus (HCV) and the advent of anti-HCV screening have now demonstrated that this particular strategy had no value in the prevention of PTNANBH.

However, others consider that the value of anti-HBc screening is to identify that small number of donors who are either resolving an acute infection or clearing a chronic infection, are apparently HBsAg-negative on screening, but who may still have low-level viraemia and be infectious. These people have been called "tail-end carriers". In such individuals anti-HBc IgM and/or IgG may indeed be the only detectable circulating markers of infection, thus only detectable by anti-HBc screening. This situation could explain cases of reported posttransfusion HBV apparently resulting from transfusion of donations screened as HBsAg-negative; unfortunately in these situations it is often not easy to demonstrate that the patient had no other sources of infection. A major problem with anti-HBc screening is to then identify those donors who are truly anti-HBc only or who are naturally immune following infection earlier in life. Many anti-HBc screening tests have relatively poor specificity and there are also major problems confirming initial anti-HBc reactivity. Even if anti-HBs is also subsequently detected, it is generally agreed that those donors with low-level anti-HBs (usually less than 100 million iu/ml) cannot be considered to be sufficiently immune to be used.

While there is no doubt that there may be a small number of individuals who may be infectious but only have detectable circulating anti-HBc, it is unclear how many blood donors would fall into this group. However, the higher the prevalence of HBV in the population, the less significant an "anti-HBc only" becomes in transfusion practice. Currently, a number of countries screen all donations for anti-HBc while others do not. At present, however, there are insufficient conclusive data available to assess the true value of anti-HBc screening.

3.2.6 HBV mutants

There are currently two groups of HBV mutants identified; the pre-core mutants and the surface antigen mutants. The pre-core mutants have a normal HBsAg expression but missing or altered HBeAg expression. Currently, these mutants are not thought to present any threat to the blood supply because HBsAg expression is normal and blood screening would therefore not be compromised. However, the HBsAg mutants, originally termed vaccine escape mutants, are of concern to blood screening as HBsAg expression is altered such that some assays may fail to detect some HBsAg mutant forms. This is most likely where the assays use monoclonal antibodies, either in the solid phase or in the conjugate, as these antibodies are totally specific for the "a" determinant of HBsAg, which is the usual missing or altered protein of the HBsAg mutants. Assays using polyclonal antibodies are less likely to fail to detect HBsAg mutants as their reactivity is broader and usually encompasses other epitopes on the HBsAg molecules. Studies so far have found that most HBsAg assays from reputable international manufacturers do detect the majority of the HBsAg mutants available for study.

3.3 Hepatitis C virus

3.3.1 General

Infections with the hepatitis C virus (HCV) have been recognized for many years, but the virus has only been identified and characterized in the last 10 years. The virus is an enveloped RNA virus that has been classified as a separate genus within the flavivirus (*Flaviviridae*) family. This has been deduced from the structure of the RNA and specific sequences within it. Although much is now known about the virus, the virus itself has still not been isolated as an intact virion; HCV RNA can be detected in the serum of infected individuals, but so far complete HCV virions have not been detected. The current knowledge about HCV and the diagnostic assays that are currently available have all arisen from the isolation of the complete viral genome from human plasma and the *in vitro* expression of the genome and subsequent characterization of the proteins expressed.

The virus is transmitted parenterally, including through intimate contact, although there has been some debate about the risk of transmission through sexual contact; the routes of infection are essentially the same as for HBV. The prevalence of HCV varies worldwide from as low as 0.1% in some industrialized

countries to as high as 40% in some developing countries. Following infection there is an incubation period from about 3 weeks to 3 months prior to the appearance of HCV RNA, followed by anti-HCV between 1 week and 4 weeks later. Infection with HCV can follow one of two courses: either acute infection followed by resolution of infection or chronic persistent infection. In about 50% of cases the infection is acute and resolves, usually within a year. Whether true immunity follows the resolution of acute infection is not yet clear; although HCV RNA is no longer detectable, the anti-HCV remaining may not protect the individual from subsequent reinfection.

The genome of the virus has been found to have certain areas with marked variation in the nucleic acid sequence. This variation has been identified as giving rise to a number of distinct virus subtypes; currently six major subtypes have been well characterized, and at least another two are being investigated. The subtypes are all geographically distributed and, importantly, do show some differences in the course and severity of infection and in the response to interferon therapy For example, subtype 2 is more resistant to current interferon therapy.

Since the identification of the hepatitis B virus and the introduction of HBsAg screening, residual cases of posttransfusion hepatitis have continued to be seen. These cases were not due to HBV or HAV, and no other viral agent could be identified, although it seemed very likely that a virus was involved. These cases were thus termed posttransfusion non-A, non-B hepatitis (PTNANBH), and for about 15 years many workers tried hard, and unsuccessfully, to identify the specific cause of such cases. This was finally achieved, however, when the HCV genome was isolated and *in vitro* diagnostic screening tests were produced. There is no doubt that the identification of HCV and the introduction of anti-HCV screening of blood donations has been a significant step in the prevention of cases of PTNANBH and thus has increased the safety of the blood supply.

3.3.2 HCV serology

The humoral immune response to HCV appears to be relatively weak; the antibodies are not present at high titres and the reactivity against the currently identified individual epitopes is very variable. Our knowledge and understanding of the serology of HCV is based solely upon the reactivity of infected individuals against the specific and defined antigens used in current screening tests. This has limited, to a certain extent, the development of our understanding, because only a

limited number of HCV specific antigens are currently available for use. These are not naturally occurring but are produced artificially, either as recombinant antigens or, less frequently, as peptide antigens. Thus, the antibody profile of the virus is restricted to the profile seen with the specific antigens available. These comprise antigens from the nonstructural (NS) regions NS3 (c33), NS4 (c100), NS5 and from the structural core region. In addition antigens from the envelope regions (E1 and E2) are being investigated as potentially useful antigens for the detection of HCV infection.

Although the precise antibody and nucleic acid profiles marking acute and chronic infections are not fully understood and known, current data seem to indicate that during acute infection antibodies against the NS3 (c33) and/or core regions of the genome are usually the first to be produced following seroconversion, and these then persist late in an acute resolved infection. Detection of antibodies to these proteins is generally more predictive of true infection than antibodies against the NS4 and NS5 regions, which generally appear later and disappear earlier (see Figure 3.4). Although usually antibodies to core, NS3, NS4 and NS5 are detected in truly infected individuals, in a significant number of infected individuals only antibodies to core, NS3 and NS4 are detected, and in a number of other truly infected individuals only antibodies to core and NS3 are detected. In a chronic infection it is likely that HCV RNA persists during the period of chronicity and in the presence of antibodies, which themselves also persist at reasonable titres for the period of chronicity (see Figure 3.5).
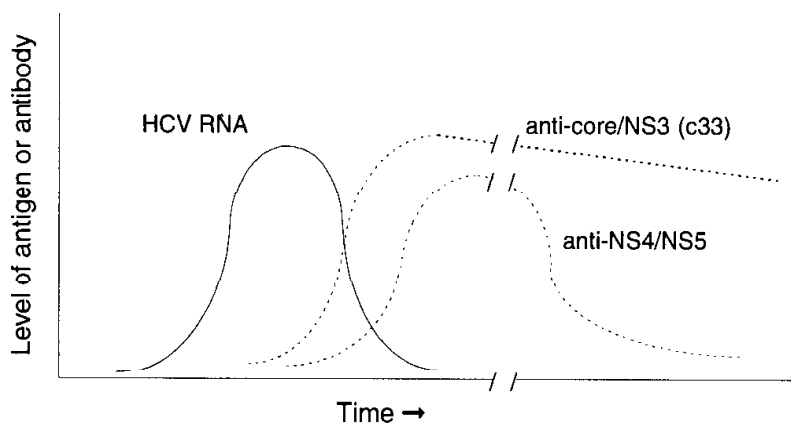
FIGURE 3.4 **Serological profile of acute HCV infection**
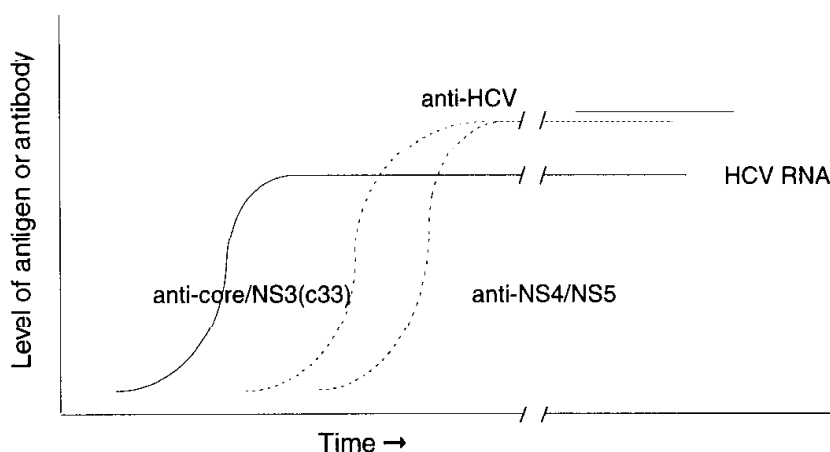
FIGURE 3.5 **Serological profile of chronic HCV infection**

3.3.3 Screening blood donations

Currently blood donations are screened only for anti-HCV. Screening for HCV RNA is, at present, not practical, and probably not appropriate in most cases. A major problem that faced transfusion services and laboratories at the start of anti-HCV screening was the generally poor specificity of anti-HCV screening assays. Even now, anti-HCV assays are significantly less specific than assays for other infectious agents, currently giving repeatedly reactive rates from 0.15% to 0.6%, compared with 0.01% to 0.1% for most anti-HIV assays. A second problem was, and still is, confirmation of the screening results (see Chapter 10). Again, because of the way in which anti-HCV screening has developed, the "confirmatory assays" currently available commonly incorporate, although presented in a different format, the same basic antigens as used in the screening assays; depending upon which assays are actually used, the antigens may thus be more or less similar between screening assay and the so-called "confirmatory assay". True confirmatory assays are not yet available.

Donors with indeterminate HCV serology are another major problem associated with anti-HCV screening. A number of individuals are found to be positive on screening but, when then tested by a "confirmatory assay", for example recombinant immunoblot assay (RIBA), are neither clearly positive nor

negative. They show some apparent anti-HCV reactivity, but insufficient to be sure of being able to distinguish true HCV infection from nonspecific reactivity. This is demonstrated by, for example, reactivity against core antigen only or against NS5 antigen only, when using HCV RIBA or Western blot. Depending upon the donor population and the prevalence of HCV, up to 30% of repeatedly screen reactive donors may be classified as HCV indeterminate. The problem with HCV indeterminate results is that, within the large number of nonspecific false positive results, some may actually indicate true HCV infection; either recent seroconversion when the antibody response has not yet developed fully, or, at the other extreme, resolved previous acute or chronic infection. Although, because they give positive screening results, such donations are unlikely to be transfused, there is a concern that any donors who may be or may have been truly infected with HCV should be referred for appropriate clinical intervention. Thus a number of studies are currently being performed to try to identify, as reliably and quickly as possible, donors with uncertain HCV serology but who may be truly infected.

3.3.4 Detection of HCV nucleic acids

Since the introduction of anti-HCV screening, a number of confirmed cases of transmission of HCV by confirmed anti-HCV-negative donations have been reported. These cases have been caused by donations collected from viraemic but anti-HCV-negative donors, most probably in the acute phase of infection, who cannot routinely be identified. Such window period individuals remain a problem for transfusion services, especially when many cases of HCV infection are asymptomatic and risk factors may not be readily identifiable. However, at present, methods to detect viral nucleic acids, such as the polymerase chain reaction (PCR), are not available in a format that is suitable for routine screening in a normal, nonspecialized laboratory. Indeed, even if suitable techniques were available, the cost of performing them might be prohibitive, especially when anti-HCV screening would also need to be performed. The extent of the problem of transmission of HCV by window period donors is also largely unknown. The cases reported may just represent the tip of the iceberg, being identified by chance rather than because of clinical concerns and subsequent investigation. While it is accepted that there is a potential problem, without sufficient data to confirm the full extent of any problem, additional screening cannot currently be justified.

3.4 Human immunodeficiency viruses

3.4.1 General

The human immunodeficiency virus (HIV 1 and 2) is a retrovirus and is the causative agent of AIDS. Although originally the virus was classified as human T-cell leukaemia virus HTLV III, it was subsequently reclassified as a lentivirus when it was realized that HIV is not oncogenic. The first reports of the isolation and identification of HIV appeared in 1984, although it is now realized that many cases of HIV infection and AIDS occurred previous to this date.

HIV is transmitted by the parenteral route, mainly through sexual contact, mother-to-infant transmission and, less commonly, by blood or intravenous drug use. Unprotected sexual contact is a major route of transmission in many countries, causing a high infection rate in young adults in those countries. This in turn adds to the problem of transmission from mother to infant *in utero*, during birth and in the perinatal period; transmission by breast milk is an additional route of childhood infection. Transmission by blood transfusion is a particularly effective means of transmission but one which is largely preventable with appropriate donor selection and donation screening.

Infection with HIV leads initially to a mild glandular fever-like illness 2–3 weeks after infection. In addition to the clinical symptoms, the CD4+ T cell count is lowered for a time. Most HIV infected individuals recover from this initial infection within 2–3 weeks, seroconvert and then remain asymptomatic for 5–10 years. However, during this period, individuals actually appear to undergo a persistent chronic infection which causes a gradual decline in CD4+ T cell numbers. When these numbers fall below a certain level the individual becomes susceptible to a number of infections and the symptoms marking the start of full-blown AIDS appear.

The prevalence of HIV varies from country to country and even within individual countries. In some countries the prevalence, because of firm public health measures and other interventions, is very low, 1 to 2 per million population, and is likely to remain so. However, in most countries the prevalence is gradually increasing; in some cases the rate of infection is high and in others it is low. Globally, WHO estimates in 1993 were that over 13 million young adults were infected with HIV and at least 2 million had progressed to AIDS, most of whom were either dead or dying. Currently, it is estimated that there are at least 5000 infections per day and by the year 2000, 30–40 million individuals will be

infected, with 1 million dying per year. The most serious problems appear to be in Africa where heterosexual transmission and mother to infant transmission account for a large proportion of infections. However, the number of cases in Asia is increasing rapidly, notably in south-east Asia and India.

3.4.2 HIV serology

Seroconversion occurs usually 1–3 months following infection with HIV. Prior to seroconversion the virus can be detected in the bloodstream, and viral RNA and viral DNA can be detected in the lymphocytes. In addition, 1–2 weeks before seroconversion, HIV p24 antigen can be detected. This antigen is viral core antigen and is one of the most abundant proteins produced by infected cells. As the antibody levels rise the level of p24 antigen declines as the antigen is bound by the antibody (see Figure 3.6). Although not detectable by normal methods, antigen production does persist during the majority of the period until AIDS develops; the antigen is bound and effectively neutralized by circulating antibody. The antibody response itself has a number of components; specific antibodies are produced against the core proteins (p24, p17, p55) and the envelope proteins (gp41, gp120, gp160), and the pattern and nature of such antibodies are of value in identifying truly infected individuals.
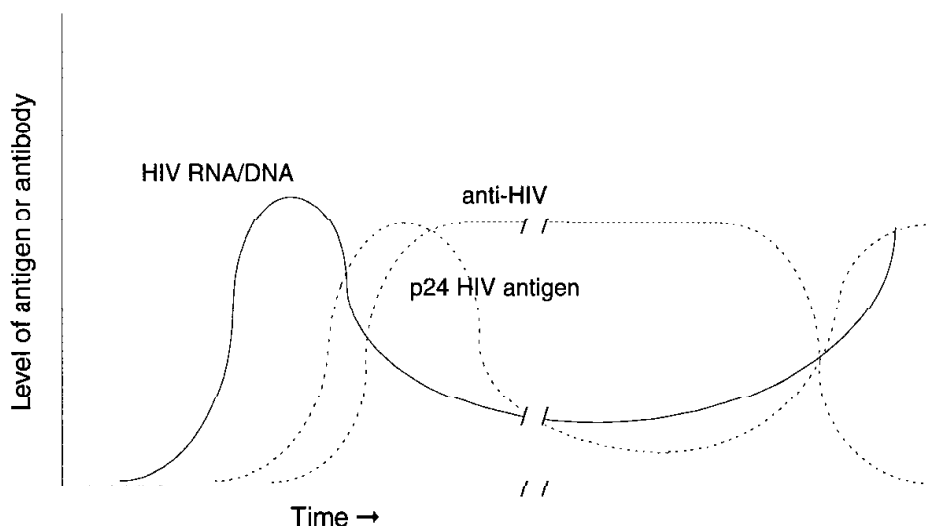
FIGURE 3.6 **Serological profile of HIV infection**

3.4.3 Screening blood donations

The vast majority of blood donations are screened for HIV infection using anti-HIV assays. In some countries, however, additional testing is performed using HIV p24 antigen assays (see 3.4.4). In the vast majority of cases anti-HIV screening will identify HIV-infected blood donations and prevent cases of posttransfusion HIV infection. Although only anti-HIV 1 screening was introduced at first, the emergence of HIV 2 meant that the anti-HIV assays had to incorporate specific HIV 2 antigens.

3.4.4 HIV p24 antigen screening

Late in 1995 the Food and Drug Administration of the United States of America decreed that HIV p24 antigen testing should start in the USA in addition to anti-HIV 1+2 testing. In a similar way to screening for HCV nucleic acid, the intention is to decrease the window period in an acute infection during which a potentially infectious but anti-HIV 1+2-negative donation could be made. However, in the case of HIV the situation is far more complicated as, although testing closes the window period by a few days, there still remains the risk of transmission from donors who are viraemic but p24 antigen and anti-HIV-negative. Screening for HIV DNA in addition would reduce the window period by perhaps one or two days more, while screening for pro-viral RNA would reduce it by about another week. Thus, screening for HIV RNA would actually be far more beneficial than screening for p24 antigen, although, like HCV RNA screening, this is currently not practical. It has been calculated that, in the USA, the introduction of p24 antigen screening may prevent up to 10 cases of transmission per year, but at a cost of about US$100 million. Whether p24 antigen screening will have any significant effect on the safety of the blood supply in the USA is, as yet, uncertain, but will be watched with interest.

3.4.5 HIV 1 subtype O

In May 1994 reports were published of a new subtype of HIV 1, subtype O. Although there are many subtypes of HIV 1, this one was of particular concern, as some of the then current screening tests did not detect all examples of the new subtype. The subtype was first identified in one country in west Africa, but has now been identified, although in very small numbers, in a number of other countries. The identification of a number of examples of HIV 1 subtype O enabled

a panel of samples to be put together and commercial assays to be tested against the panel. A small number of tests detected only a few of the samples while most tests detected 60% to 90% of samples. Interestingly, most of the assays detected subtype O through cross-reactivity with HIV 1. However, most commercial assays used for blood screening have now been modified to be able to detect all examples of subtype O; while cross-reactivity is still used by some assays, the majority of modifications have included specific subtype O antigens. Although a potential problem for transfusion services, cases of subtype O are very rare and most assays will be able to detect most examples.

3.5 Human T cell leukaemia viruses

3.5.1 General

The human T cell leukaemia virus I (HTLV I) was the first human retrovirus identified. The virus is an oncogenic virus causing adult T cell leukaemia and lymphoma (ATLL or ATL) and tropical spastic paraparesis (TSP), also known as HTLV I associated myelopathy (HAM). A second virus, HTLV II, has also been identified in specific groups of individuals, for example intravenous drug users, although no significant disease process has yet been associated with this virus. Most infections with HTLV I are asymptomatic and remain so. However, there is a small risk that disease may develop any time up to 40 years after infection. ATLL can present as an acute leukaemia of CD4+ lymphocytes, most often between the ages of 30 years and 50 years. Death usually occurs within a year of the onset of symptoms. TSP is a progressive disease involving the degeneration of neurons in the spinal cord, leading to gradual paralysis of the lower limbs.

It is thought that the virus is virtually always cell associated, being present in CD4+ lymphocytes, and is transmitted in these cells parenterally via blood or semen, or from mother to infant via breast milk. The transmission by breast milk is a major route of infection in some areas where HTLV I is endemic, for example some areas of the islands of south-west Japan. Studies on the transmission of HTLV I indicate that the virus is not normally transmitted *in utero* but is transmitted in early life through breast milk equally to both male and female children. However, later in life sexual transmission is almost exclusively from male to female. Blood transfusion is another potentially significant route of infection. Early studies demonstrated the efficiency of transfusion transmission, but found that cell free products, such as plasma, did not transmit infection.

The virus is distributed across the whole world, but is concentrated in specific areas and countries, mainly in the tropics. Even within countries, only certain areas may have high prevalences of HTLV. This seems to indicate that in isolated or relatively self-contained communities, the combination of horizontal and vertical transmission can maintain levels of infection from generation to generation. The countries and regions with the highest prevalences of HTLV include the Caribbean, southern Japan, equatorial Africa, Papua New Guinea and parts of south America. Prevalence figures range from 5% to 30% and also increase with age in many populations.

3.5.2 HTLV serology

Following infection with HTLV I there is an incubation period from 30 days to 90 days before seroconversion. Prior to seroconversion viral RNA can be detected in lymphocytes. At seroconversion antibody to HTLV appears and this is the major target for the diagnosis of HTLV infection. After seroconversion the antibody generally persists for life, even if clinical disease subsequently develops later in life. The serological responses to HTLV I and HTLV II are very similar and specialized tests are needed to be able to discriminate reliably between HTLV I and HTLV II. The virus itself cannot be grown easily from infected cells and needs a number of growth factors and cell stimulators to be able to demonstrate viability.

3.5.3 Blood screening

The potential significance of blood transfusion as a route of transmission has meant that in a number of endemic countries screening of donations has been carried out for some time. In some nonendemic industrialized countries with mixed populations screening has also been introduced. In many other countries there is debate over the need and value of screening donations.

Blood donations are screened for an indication of current or previous HTLV I/II infection by screening for antibodies against the viruses—anti-HTLV I + II. There are now a number of reasonable anti-HTLV screening tests commercially available which have good sensitivity and specificity. In addition, confirmatory assays are now available.

3.6 Human cytomegalovirus

3.6.1 General

Human cytomegalovirus (HCMV) is a member of the herpesvirus family, a group of DNA viruses that are widespread in the general population and characterized by their ability to give rise to persistent latent infections. The virus is the largest of the herpesviruses and has the largest genome of any known virus. HCMV is globally the most common virus to be transmitted *in utero*, affecting up to 2.5% of all live births, and causing serious disease in symptomatic individuals. Globally between 40% and 90% of adults are infected with HCMV, the prevalence usually increasing with age from late childhood onwards; in endemic areas the prevalence can reach 90% in young adults. It has been recognized for more than 25 years as a potentially serious complication of blood transfusion, but only in certain patient groups. Infection in healthy immunocompetent individuals is usually asymptomatic, any symptoms resembling a mild glandular fever–like illness, which is essentially clinically insignificant unless occurring in a pregnant woman.

The virus is transmitted by the parenteral route and during acute infection may be present in most body fluids. During acute infection the virus infects circulating leukocytes, and eventually may persist latently in those cells. In addition, latent infection has been demonstrated in the endothelial cells lining the walls of blood vessels. "Latency" is the property of some viruses to persist in an individual following acute infection, not as intact virus but as viral nucleic acid only. This viral nucleic acid integrates into the host cell's DNA and therefore replicates with the host cell. At any stage the nucleic acid can become active and the infection reactivate, resulting in an acute episode.

As acute infection resolves, specific circulating antibody to CMV appears. However, because of latency this anti-HCMV is not highly protective and serves to identify potentially infectious donors. Following acute infection, it is quite possible that later on an individual may undergo another acute infection, either due to reactivation of latent virus or reinfection from an external source. This is not thought to be a serious problem in immunocompetent individuals but can be serious in immunocompromised individuals. Many such patients, for example those with leukaemia and transplant recipients, may face serious illness from HCMV. In previously infected donors, it is the latent virus in the transfused leukocytes that is thought to reactivate and give rise to posttransfusion HCMV

infection. Leukodepletion of blood products has been shown to prevent HCMV transmission, even from previously infected donors.

3.6.2 Screening for HCMV

HCMV-infected blood donors are identified by screening for anti-HCMV. Screening assays are generally enzyme immunoassays (EIAs), although a latex agglutination assay is commercially available and particularly suitable for a small number of samples and in situations where resources are limited. Unfortunately, as discussed above, the presence of anti-HCMV does not mark immunity, but rather indicates previous infection and thus a donor with latent and potentially infectious virus. Therefore, only donations from donors who have not been infected with HCMV are suitable for transfusion to immunocompromised patients.

Most currently available assays are total antibody assays that detect IgM, IgG and IgA anti-HCMV. It is unclear whether the ability to detect other Ig classes apart from IgG is important; recent acutely infected individuals may have high levels of IgM, although in many cases detectable IgG is also present, albeit at a low level.

Transmission by blood transfusion is only really of significance in immunocompromised recipients, including neonates, and such recipients should receive blood that has been screened for HCMV. However, there is no clinical justification for the selection of HCMV-screened blood for immunocompetent patients. Anti-HCMV screening of donations is therefore generally selective: only a small number of donations need to be screened for HCMV, and the anti-HCMV-negative donations identified are then used selectively only for those patients who need anti-HCMV-negative blood.

3.7 Parvovirus B19

The parvoviruses are the smallest human viruses, 20–25 nm in diameter, nonenveloped and with a linear single-stranded DNA genome. Because they have such a small genome, 4.5–5.5 kbp, they are only able to replicate in dividing cells. Human parvovirus B19 is an erythrovirus that is of minimal clinical significance in healthy individuals, but leads to acute infection which may cause of erythema infectiosum (slap cheek syndrome) in children, abortion and hydrops in pregnant

women and aplastic crisis in patients with chronic haemolytic anaemia. In many countries there are regular annual, or even more frequent, outbreaks of parvovirus B19 infection in the general population.

The virus may be transmitted by a number of routes: most commonly by respiratory secretions and close contact, especially among children, from mother to child through the placenta and by blood transfusion. Commonly the virus infects red cell progenitor cells causing the disappearance of erythroblasts and reticulocytes and a drop in haemoglobin level.

About one week after infection there is a high-level but short viraemia during which virus titre may reach as high as 10^{11} virus particles per millilitre. This viraemia can be detected by using molecular techniques such as hybridization or PCR. After this the IgM antibody level starts to rise and symptoms may appear. After 3 to 4 weeks the IgG level starts to rise, reaching a peak after about 6 weeks; in healthy individuals full immunity develops and persists for life.

The screening of individual blood donations is not normally performed, as most recipients of blood are either immune or would not suffer any significant disease if infected. However, in some countries plasma pools are screened for the presence of viral nucleic acid before processing. The virus is, however, a good model virus for monitoring the effectiveness of viral inactivation procedures in raw plasma and fractionated blood products as its nonenveloped nature makes it very resistant to most of the viral inactivation procedures used in the preparation of plasma products.

3.8 Hepatitis A virus

Hepatitis A virus (HAV) is an enterovirus, a member of the *Picornaviridae*, family that has been classified as a separate genus—*Hepatovirus*. The virus is a nonenveloped RNA virus and may persist in fractionated plasma products. The clinical symptoms of HAV resemble those of HBV although the onset is usually more abrupt but the preicteric stage is less prolonged. Globally, most infections occur in children and the severity of disease increases with age. About 0.1% of infections lead to death due to fulminant hepatitis. However, chronic infection does not develop, and those infected individuals who have nonlethal infections recover completely with no long-term sequelae. Most infections last for about one

month, although rarely some infections may relapse and last for as long as 6 months.

The virus is normally spread only by the faecal–oral route, but some cases of transmission by blood products have been reported, e.g. iv immunoglobulin preparations. The transmission of HAV is very dependent upon poor sanitation and standards of hygiene, and hence it has a very high prevalence (over 90%) in most developing countries, but a falling prevalence in industrialized countries. Large quantities of virus are shed in the faeces during the acute stage of infection.

Following infection, virus levels build up in the gastrointestinal tract and virus is excreted in the faeces. A viraemia may be present in a number of infected individuals, but this may be at a very low level and only detectable by the most sensitive techniques. After 2–3 weeks IgM antibody against HAV starts to appear, followed by IgG anti-HAV. By about 6 months the IgM has disappeared but the IgG titre has stabilized and gives immunity to HAV for life.

3.9 New hepatitis viruses

Recently there have been reports of new hepatitis viruses which may be present in a significant proportion of blood donors, and which may be responsible for those residual cases of posttransfusion hepatitis (PTH) that cannot be shown to be due to viral hepatitis of type A, B, C, D or E. Generally NANBH can be divided into three groups based upon mode of transmission: community-acquired, parenteral and enteric. Virtually all enteric cases are identified as being caused by HEV. Most cases of parenteral and community-acquired NANBH are now known to be due to HCV, but a small number are not, and are thought to be due to another as yet unidentified viral agent or agents.

Studies in the USA have identified putative viruses that may be responsible for such residual cases of NANBH. Essentially there have been two viruses/ groups of viruses identified: the GB viruses (subdivided into three types: GBV-A, GBV-B, GBV-C) and the new hepatitis virus: hepatitis G virus (HGV). These viruses have all been identified as almost certainly members of the *Flaviviridae*, transmitted parenterally, and as present in a significant number of asymptomatic and symptomatic individuals although not necessarily associated with any specific disease process. The GBV-C and HGV viruses identified have been found to have greater than 90% homology at the genomic level and to be virtually

identical in their structural organization, suggesting that there is a high probability that the two may be strains of the same virus.

Unfortunately, at present, tests for these viruses depend upon molecular rather than serological principles. The polymerase chain reaction (PCR) has been used extensively to identify the presence of virus in samples from infected individuals. Liver enzyme levels, notably ALT, have also proved to have some predictive value in marking acute infection with active hepatitis. Serological tests, however, have not proved to be straightforward, with problems encountered in identifying potential antigens that are capable of detecting specific antibody in infected individuals. In a similar way to the development of anti-HCV assays, structural and nonstructural proteins have been identified from the viral genomes and expressed to produce tentative diagnostic antigens. However, little is known currently about the serology of these viruses, and those assays that have been put together and used have given some contradictory results. Prevalence figures for GBV-C and HGV, derived from PCR results and serological studies, give figures varying from 2% in volunteer blood donors to as high as 30% in some west African populations.

The role and significance of GBV-A and GBV-B are harder to assess. Phylogenetically GBV-A and GBV-C are more closely related than GBV-B. Primate animal studies have shown that GBV-A does not cause hepatitis or establish an antibody response, while GBV-B does and a degree of protective immunity is established in those animals. Limited comparative prevalence studies have demonstrated the presence of antibodies against GBV-A and GBV-B in different patient and risk groups, but the prevalences have all been significantly less than that of GBV-C/HGV.

Chapter 4

Transmissible nonviral infectious agents

4.1 Introduction

The transfusion transmission of nonviral infectious agents is an uncommon but potentially serious and sometimes fatal complication of transfusion.

Transmissible nonviral infectious agents form a large and heterogeneous group comprising bacteria, rickettsiae and protozoa, all of which may be transmitted by transfusion, but with varying degrees of efficiency and with widely ranging clinical significance. Currently however, there have been no confirmed reports of the transmission of fungal infections by blood transfusion. In some countries there is a very significant problem with the transmission of bacterial or protozoan infections because of the high prevalence of some of these agents. Unfortunately, suitable screening tests are not available for many of them, and thus specific screening is not always possible. Alternative procedures have to be used to minimize any risks of transmission. These may include donor exclusion based upon clinical history and local prevalence data, and environmental monitoring to ensure the cleanliness of blood collection, processing, storage and transport systems. However, as with screening for viral infections, the best monitor of the effectiveness of any nonviral infectious disease screening procedures is the active monitoring of recipients for signs of posttransfusion infection. It is clear that such incidents, when not fatal, are greatly underreported and without such data it is hard to determine the effectiveness of current procedures and whether any improvements in the screening and selection procedures are required to increase further the safety of blood transfusion.

In the case of bacterial agents, there are two broad routes of contamination: intrinsic and extrinsic.

Intrinsic contamination is due to bacteria present in the donor's bloodstream at the time of donation (bacteraemic donor), and may include such organisms as *Treponema pallidum* and *Yersinia enterocolitica*.

Extrinsic contamination is due to bacteria that have entered the blood pack from the environment during collection, processing or other handling, storage or transport, and may include such organisms as *Pseudomonas* and *Staphylococcus* spp.

In either case, bacterial growth within the blood pack will give rise to endotoxins, which are generally the major cause of the posttransfusion sepsis seen after transfusion of such an infected donation. Although there are many bacteria that can grow in the blood bags under the storage conditions used, in some cases growth is restricted and the bacteria themselves do not survive past a few multiplication cycles; the toxins they produce however, can build to high levels even from relatively few bacteria.

The presence of bacteria in donated blood, especially exogenous bacteria, is increasing in importance as, although uncommon, posttransfusion bacterial sepsis has a high mortality rate. Numerous instances of infected red cell products have been reported, and the introduction of the storage of platelet concentrates at 20 °C to 24 °C for up to 5 days has provided an ideal environment for the growth of any contaminating bacteria present, whether endogenous or exogenous.

4.2 Bacteria

4.2.1 Endogenous bacteria

4.2.1.1 General

If a donor is bacteraemic at the time of donation, it is most likely to be due to a low-grade asymptomatic infection, often with only a short period of bacteraemia. Major potential endogenous contaminants of donated blood include *Treponema pallidum* (the causative agent of syphilis), *Brucella melitensis* (the causative agent of brucellosis) and *Yersinia enterocolitica*. The presence of antibody to *T. pallidum* or *B. melitensis* indicates a potentially infectious donation which may transmit infection; the organisms, however, do not normally multiply in the blood or products during storage. However, the presence of organisms such as *Y. enterocolitica* or other species such as *Salmonella* or *Campylobacter* can be more significant, as not only can the bacteria multiply to large numbers during storage, but they also produce significant quantities of bacterial toxins. Transfusion of products containing such organisms can therefore lead not only to sepsis but also to septic shock.

BOX 4.1 Nonviral infectious agents that may be transmitted by blood transfusion

Bacteria

Exogenous bacteria	*Staphylococcus epidermidis*
	Pseudomonas spp.
	Serratia marcescens
Endogenous bacteria	*Treponema pallidum*—syphilis
	Borrelia burgdorferi—Lyme disease
	Brucella melitensis—brucellosis
	Yersinia enterocolitica (and others)
Rickettsiae	*Rickettsia rickettsii*—Rocky Mountain spotted fever
	Coxiella burnetti—Q fever

Protozoa

	Plasmodium spp.—malaria
	Toxoplasma gondii—toxoplasmosis
	Trypanosoma spp.—Chagas disease, sleeping sickness
	Leishmania—leishmaniasis

4.2.1.2 Treponema pallidum—*syphilis*

The spirochete *Treponema pallidum* is the causative agent of syphilis. Spirochetes are a class of bacteria that have thin flexible helical walls and are extremely motile. Treponemes cannot be cultivated on artificial media although they can be cultivated in cell culture or in animals. They are very characteristic organisms and can be seen easily using dark-field microscopy; this allows quick clinical diagnosis if treponemes can be isolated from a suspected treponemal lesion.

Following initial infection the course of infection can follow several stages leading to primary, secondary and tertiary syphilis. During initial infection the treponeme passes, normally through the mucous membranes, into the lymphatic system. The regional lymph nodes become enlarged as the treponemes divide and eventually they are released into the bloodstream from which they spread around the body. Usually the primary site of infection is marked by a lesion known as a chancre, which is full of treponemes. Although the chancre may heal and disappear completely, the lymph nodes may still be swollen and continuing treponemal division may give rise to secondary syphilis, which is characterized by the appearance of many lesions similar to the original chancre. These lesions, however, may be found on the skin as well as mucous membranes and are highly

infectious; nonvenereal transmission may occur at this stage. Also at this stage, all parts of the body may be involved and give rise to a wide variety of clinical symptoms. After 1–2 months the lesions normally gradually disappear and the infection becomes latent again. Tertiary syphilis may then present any time from 5 to 40 years from the primary infection. At this stage there is usually involvement of the central nervous system with the potential for serious brain damage.

Because treponemes are released into the bloodstream as part of their life-cycle there is the potential for transmission by transfusion. They are particularly fragile but can be transmitted by transfusion if they are present in the donation. However because they are very heat sensitive, storage at 4 °C soon destroys the organism; it is generally held that any spirochetes present in the pack would be destroyed within 72 hours of storage at 4 °C. As spirochetes can be seen only for short periods during infection, identification of infected individuals relies upon serology. Blood is therefore screened either using nonspecific tests, or for the presence of specific antibody to *T. pallidum*, as evidence of current or previous infection with *T. pallidum*. All positive donations are normally considered to be infectious and not transfused. Although the vast majority of cases of syphilis antibody identified in blood donors are due to "old" infections that have been treated successfully and present no risk of transfusion transmission, cases of recent primary acute syphilis are occasionally identified. In some countries, where there is a low incidence of syphilis and where all blood is stored before use, the value of such screening is often questioned as cases of posttransfusion syphilis are rare and any that may occur can be successfully treated with no lasting sequelae. However, syphilis screening of donated blood, no matter what the prevalence, has been considered to have value as a "lifestyle" indicator, as individuals exposed to syphilis may also have been exposed to other sexually transmitted diseases (such as HIV) and therefore should not donate.

Nonspecific screening tests. Syphilis serology began with the Wassermann reaction; this was based upon complement fixation following the mixing of test serum with fetal liver extract from fetuses that had died from congenital syphilis. However, subsequent work demonstrated that this was a nonspecific reaction and the same reaction could be achieved using normal beef heart extract. The active antigenic component of the Wassermann reaction was found to be the tissue substance called cardiolipin, present in varying amounts in all tissues. This particular reaction was developed further by a number of other investigators resulting in a number of modified tests including the Venereal Disease Research Laboratory (VDRL) and rapid plasma reagin (RPR) tests, both of which are still

used frequently in many countries. Both tests are rapid tests based upon mixing a suspension of cardiolipin, lecithin and cholesterol (VDRL antigen) with test serum and looking for clumping of the cardiolipin, the RPR being enhanced by the adsorption of the VDRL antigen onto carbon particles, which enables any clumping to be seen a lot easier. Although nonspecific cardiolipin tests may give rise to a high number of false-positive reactions, they are useful because in treated acute infections anticardiolipin titres fall and the tests become negative. Confirmatory testing algorithms still use cardiolipin tests to determine true serological status.

Specific screening tests. These screening tests detect specific antibody to *T. pallidum* and include a number of different assay formats. The most commonly used test, and the most suitable for the mass screening of blood donations, is the *Treponema pallidum* haemagglutination assay (TPHA), which is based upon the agglutination of nonhuman red cells coated with treponemal antigens. A number of enzyme immunoassays are now available, but in many countries the cost of these, compared with the significantly cheaper and generally just as effective TPHA, is prohibitive. Other tests in use, such as the *Treponema pallidum* immobilization test (TPI) or the fluorescent treponemal antibody-absorption (FTA-ABS), are primarily confirmatory tests and not suitable for mass screening.

4.2.1.3 Borrelia burgdorferi—*Lyme disease*

Like syphilis, Lyme disease is caused by a spirochete, *Borrelia burgdorferi*. The organism is carried by a number of insect vectors, mainly by ticks of the *Ixodes* genus, but also increasingly it has been found in other blood-feeding insects such as horseflies and mosquitos; it is likely that human transmission is possible via these routes. The disease was first identified near Lyme, Connecticut, in the USA, but is now the most common tick-transmitted infection in the USA. and is also known to be endemic in many other parts of the world. The disease is generally seasonal and marked by unique skin lesions, rash, fever and lymphadenopathy. This may progress to meningoencephalitis or myocarditis, and then arthritis. A high percentage of infected individuals develop chronic joint disorders.

Although no case of posttransfusion Lyme disease has yet been reported, the potential for transmission remains. This is especially so as *B. burgdorferi* can retain viability in blood stored for up to 6 weeks, in contrast to *T. pallidum* which, as mentioned previously, loses viability in blood after 48–72 hours at 4 °C.

Enzyme immunoassays (EIAs) are available which detect specific antibodies against the organism; however, as cases of spirochetaemia are generally symptomatic, careful donor selection should ensure that any potential risk of transmission is minimized.

4.2.1.4 Brucella melitensis—*brucellosis*

Brucellosis (undulant fever) is caused by the bacterium *Brucella melitensis* which is normally acquired from an infected animal source. Normally it is not transmitted from person to person but, because there is a period of bacteraemia, transfusion transmission may occur. The organism normally enters through the mucous membranes of the throat from where it migrates to the regional lymph nodes. Here it multiplies before being released into the bloodstream from where it enters and resides in the reticuloendothelial systems of different tissues. Infection is characterized by general malaise and an undulating fever. Chronic infection normally follows which may last for many years with bouts of sometimes quite serious illness. However, while the organism is prevalent in many parts of the world, brucellosis has only rarely been reported after transfusion; although this may actually reflect poor reporting of posttransfusion infections and the true incidence of transmission may be at a higher level. The active deferral of donors at risk of exposure to or previously infected with *Brucella* should minimize any risk of transmission.

4.2.1.5 Yersinia enterocolitica

Yersinia enterocolitica is a Gram-negative bacteria that may be present as an asymptomatic bacteraemia in donors at the time of donation. The extent and significance of such infections are hard to assess as effective mass screening procedures are currently not feasible, and only sporadic monitoring is performed.

However, *Y. enterocolitica* has been recognized as a potentially serious microbial agent present in donated blood. Initially it was thought that the organism persisted free in the blood, and because it was psycryophilic (able to grow at low temperatures), it was able to multiply in the blood pack at the low storage temperatures used, eventually reaching high enough numbers to cause posttransfusion sepsis in the recipient. Recent data suggest, however, that in an infected individual the bacteria are phagocytosed but survive intracellularly in the circulating leukocytes. During storage of the blood collected from such an individual, the natural breakdown of the leukocytes releases the bacteria, which

are then able to grow at 4 °C. This results in the build up of large numbers of bacteria and their toxins in the stored unit, with the potential for causing both posttransfusion sepsis and septic shock in the recipient. It is now generally accepted that the early removal of leukocytes from donated blood will reduce significantly any risk of sepsis due to the growth of *Y. enterocolitica* during storage. Interestingly, platelet concentrates (although containing some leukocytes) appear to be less effective in supporting the growth of *Y. enterocolitica*, therefore presenting less risk of transmission.

Note. A particular concern with the use of pre-storage leukodepletion was that the leukocytes present, particularly in platelet concentrates, might actually play a role in the prevention of growth of any contaminating bacteria that are free in the plasma. Leukodepletion might therefore increase the likelihood of posttransfusion septicaemia or septic shock in certain cases. However, studies using a number of exogenous and endogenous bacterial species could find no increased rate of contamination in leukodepleted, compared with nondepleted, platelet concentrates.

4.2.1.6 Other endogenous bacteria

Cases of posttransfusion sepsis caused by a number of other endogenous bacterial species are occasionally reported. Donors may have a transient bacteraemia, possibly as a result of a low-grade gastrointestinal infection, following dental procedures or an insect bite, which may subsequently result in posttransfusion sepsis due to organisms such as salmonellae, campylobacteria, streptococci and staphylococci.

4.2.2 Exogenous bacteria

4.2.2.1 General

A number of bacterial species such as *Pseudomonas*, *Serratia* and *Staphylococcus* may be introduced into the blood pack from the environment during or after donation, either at venepuncture or during subsequent processing, storage or transportation of the blood. These essentially environmental organisms are generally more likely to cause problems in products stored at higher temperatures, such as platelet concentrates, because they are able to multiply to high numbers producing high levels of bacterial toxins. There have been many cases of posttransfusion septicaemia involving single or multiple recipients infected by the same source, and involving a large range of bacterial species, both Gram-positive and Gram-negative. However, while a large number of bacterial species have been implicated, in most cases the precise source of the

contamination could not be determined. Platelet concentrates are generally considered to be the most common source of posttransfusion septicaemia although the ratio of cases of sepsis to death is higher than with cases of posttransfusion septicaemia following transfusion of red cell products.

Recently, an outbreak of posttransfusion septicaemia due to *Serratia marcescens* was reported. The outbreak affected transfusion centres in both Denmark and Sweden and was traced to blood bags contaminated on their outer surfaces during manufacturing or packaging. Six patients were affected, one of whom died, and 4000 units of blood and an unknown number of platelet concentrates had to be discarded. Studies on the growth of *S. marcescens* in artificially infected blood packs have since demonstrated that not only does the organism grow well at both 4 °C and 22 °C, but that natural protection mechanisms such as phagocytosis and complement-mediated killing were not effective in destroying it.

4.2.2.2 Detection of bacterially infected donors and donations

Unfortunately, those bacteria which contaminate rather than cause a specific recognizable disease are not at all easy to detect, either in donors or their donations. Because low-level bacteraemias may not give rise to any symptoms in the donor, it is virtually impossible to identify and defer such donors; donor pre-selection is therefore inappropriate. In some countries the deferral of donors with a history of unexplained diarrhoea or vomiting within a month prior to donation was tried with a view to reducing the risk of transmission of *Y. enterocolitica*. However, no real benefits were found and a number of donors were found to have been unnecessarily deferred.

Although screening donations would, in theory, identify infected donations, mass screening systems are not available that are capable of detecting the range of organisms potentially present, detecting them within a reasonable period of time and at the low levels at which they may be present and still cause problems. In addition, the cost of such screening is totally prohibitive. Thus, at present, the prevention of bacterial contamination cannot rely upon laboratory screening, and endogenous organisms are likely to remain a problem for some time to come. The problems caused by exogenous organisms, however, can, in most cases, be effectively resolved with the application of principles of cleanliness to collection, processing, storage and transportation of blood and products. Minimizing the

environmental bacterial load significantly decreases the likelihood of introduction of bacteria from external sources.

4.2.3 Rickettsiae

The rickettsiae are smaller than most other bacteria and grow only inside animal cells. With the exception of Q fever, all rickettsial diseases are transmitted between animals via a blood-feeding insect vector. Transmission of rickettsial infections by blood transfusion can occur, but cases are extremely rare; only cases of the transmission of Q fever and Rocky Mountain spotted fever have been reported. In both cases infectious organisms are shed into the bloodstream, and it is at this stage that the potential for transmission exists. However, most infections at this stage are symptomatic, and donor selection procedures should identify any potentially infectious donors. Laboratory screening tests are available but are very specialized and not ideally suited for the screening of blood donations.

4.3 Protozoa

4.3.1 General

Parasitic protozoan infections are a significant problem in many developing countries. Often a large proportion of the blood donor population is infected, and the prevention of transmission by transfusion is a problem. Conversely, protozoan infections in most industrialized countries are far fewer and are largely restricted to the potential risk of transmission of malaria by donations collected from donors who may have been infected while travelling in malarious areas on holiday or on business. In recent years, however, the risk in industrialialized countries has increased, in terms of both the number of donors potentially exposed to parasitic infections and the number of different organisms involved, as the number of individuals travelling abroad and variety of destinations have increased, and also as migrants from endemic areas move into nonendemic areas and donate blood.

4.3.2 *Plasmodium* spp.—malaria

Malaria is caused by the parasite species *Plasmodium*, four of which are recognized as the causative agents of human malaria: *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. It has long been recognized as a transmissible infection,

and cases of transmission, and sometimes death, occur still. Malaria is carried by the anopheles mosquito and is injected into humans through the bite of an infected feeding mosquito. There are two stages to the life cycle of the parasite, one in the mosquito host and one in the human host. The infective form (sporozoite) travels to the liver where it divides many times before being released back into the bloodstream (merozoite). This merozoite infects red cells in which another replication cycle then takes place, after which the red cell bursts, releasing more merozoites into the bloodstream to infect yet more red cells. After a number of such cycles some of the merozoites will form male and female forms (gametocytes) at which stage the human cycle finishes. The gametocytes are then ingested by a feeding anopheles mosquito whereupon the mosquito stage of the organism's life cycle begins again.

Donations collected at the stage when the red cells are infected could contain potentially infectious red cells, or even free merozoites, which could result in transmission of infection to the patient. Because of the great variation in prevalence of malaria in different countries, approaches to minimizing any risks of transmission vary. In nonendemic countries the risk of malaria comes from donors who have travelled in malarious areas. In this case the use of standardized country risk groups (i.e. listing countries and their associated malaria risk) and donor questioning is generally sufficient to identify and defer any "at-risk" donors. In low-prevalence endemic countries donor questioning may be effective if, for example, cases are usually symptomatic and therefore would not go unnoticed, or if malaria is restricted geographically. Failing this, however, many countries screen donations by preparing and looking at thick films. While this may identify a donor with a high parasitaemia, the technique is not sensitive enough to detect most infections and certainly is not suitable for mass screening. In highly endemic areas donor questioning and thick films may have some value, but whether there is any value to malaria screening at all has to be considered. If the prevalence is very high it is likely that most patients would already be infected, and it could therefore be argued that transfusing potentially infected blood would not have any serious sequelae. This is an ethical and moral dilemma, but in such a situation it is very likely that if all infected donors were deferred there would be no donors left and therefore no blood for transfusion.

Although malarial antibody tests are now available, their sensitivity and specificity are not optimal and therefore such tests not totally reliable. In addition, they would not be able to identify very recently infected parasitaemic individuals in whom specific antibody had not yet appeared. The cost of these tests is also

high, and at present it would appear that their main value in donor screening would be in nonendemic countries, to screen donors who had travelled to endemic countries and been deferred, and so return them to active status as soon as possible.

4.3.3 Trypanosoma cruzi—Chagas disease

Chagas disease is caused by the parasite *Trypanosoma cruzi*. The disease is confined mainly to the American subcontinent, where it is endemic in Latin America and increasing its presence in the southern states of the USA as migrant workers from Latin America move north. This spread in migrant workers may also affect other industrialized countries as these migrant workers travel to these countries. However, it is likely that this spread is self-limiting; infected individuals may present with the disease in other countries but the living conditions are usually not the same and the insect carriers (reduviid bugs) are not present, so preventing the full life cycle of the organism from establishing.

The parasite is carried in the gut of members of the family of reduviid bugs (triatomids) and is excreted in the faeces. These bugs live in the cracks in the walls and ceilings of buildings and at night feed on human blood. The bug normally defecates while feeding, and the open feeding site acts as the site of entry for the parasite. The entry site, generally on the face, swells to produce the typical chagoma of the disease. However, while this route is generally the major route of infection, blood transfusion is now considered to be the second most important route of transmission in endemic areas. When ingested by the reduviid bug the organisms enter the midgut where they replicate to form epimastigotes, which are then converted into trypomastigotes before being excreted in the faeces. Once in the human bloodstream, the trypomastigotes develop into amastigotes which infect the regional lymph nodes and may eventually infect virtually every organ of the body. The organism multiplies in the cells of the infected organs and when these cells burst trypomastigotes are released into the bloodstream. However, these cannot multiply in the human. They need to be ingested by a feeding bug, enter its gut and then start the whole cycle again. Although the liver and spleen are usually infected, the most characteristically infected organ is the heart. The amastigote forms replicating in the heart appear to induce an inflammatory response which causes enlargement of the heart. Central nervous system (CNS) involvement is common, often leading to meningoencephalitis.

Identification of infected donors can be problematical as more than 20% of infected individuals may be asymptomatic. Donor selection can identify symptomatic individuals, but that is of limited value in endemic areas. In nonendemic areas, in a similar manner to malaria screening, donors who either come from or have visited endemic areas can be deferred to minimize any risks of transmission. There are now a number of screening tests available that detect specific antibody. In recent years these assays have improved greatly in both specificity and sensitivity. However, there is no real standardization of *T. cruzi* serology and currently no reliable confirmatory tests available. Lastly, there is an effective way of inactivating organisms in the actual blood donations. For at least 40 years some Latin American countries, mainly in hyperendemic areas, have used gentian violet at a final concentration of 125 mg/500 ml added to the primary blood pack as an apparently totally effective way of inactivating any organisms present in the donation. After 24 hours at 4 °C in the presence of gentian violet *T. cruzi* is killed. Although effective in killing *T. cruzi* the use of gentian violet is not without some problems. It is unsuitable when products are required within 24 hours of collection, and some recipients of donations containing gentian violet have ended up with purple staining of the skin and mucosa; rouleaux formation and microagglutination have also been reported.

Although still a significant problem in Latin America, a number of countries in the Eastern Mediterranean Region with more developed transfusion services have managed to control *T. cruzi* and minimize any risks of transmission without compromising the availability of blood and blood products.

4.3.4 *Leishmania* spp.—leishmaniasis

Leishmaniasis is an infection of the reticuloendothelial system but it exists in three main forms: cutaneous, mucocutaneous and visceral (kala-azar). Cutaneous leishmaniasis results in skin lesions, subcutaneous leishmaniasis results in ulcers on the oral and nasal mucosa, and visceral leishmaniasis results in infection of the reticuloendothelial system in the liver and spleen. It is thought that the basic differences between the three types of infection result from the differing ability of the parasites to invade the body. The disease is found in a broad band extending around the globe across central and south America, central Africa, the Middle East, Pakistan, China and south-east Asia. Although a number of leishmania species exist, morphologically they are almost identical; differences are apparent only when molecular techniques are used to examine their DNA. The parasites

are transmitted through the bite of infected sandflies of the genus *Phlebotomus*, with each parasite species being restricted to a particular *Phlebotomus* species. The reservoirs for organism vary in different regions but invariably include rodents and other small wild mammals, although in urban areas dogs and even humans can serve as reservoirs.

The parasite is carried in the insect's gut where it develops into the motile promastigote which migrates to the pharynx from where it can be injected into a new host. In the human host these injected promastigotes invade the reticuloendothelial system where they develop into the amastigote forms which are eventually released into the bloodstream, and which may be ingested by a biting sandfly, so completing the cycle.

Identification of infected individuals is generally based upon clinical diagnosis, and, although more straightforward in cases of cutaneous and subcutaneous leishmaniasis, is complicated by the fact that a large percentage of cases are asymptomatic. Specific reliable diagnostic tests are not available widely, and not suitable for blood screening. Although potentially a threat to the blood supply in endemic areas, parasitaemia is generally transient and at a low level, consequently there is a low risk of transmission. This is supported by the lack of reports of transfusion transmission even in endemic areas.

4.3.5 *Toxoplasma gondii*—toxoplasmosis

Toxoplasma gondii is globally one of the most widespread vertebrate protozoan parasites; in some countries up to 95% of adults may have been infected with the parasite. Following resolution of acute infection, circulating antibodies appear but the organism persists latently in the circulating leukocytes. Reactivation of this latent infection has been reported, notably in immunocompromised individuals. The acute infection in healthy individuals is generally asymptomatic and not associated with any morbidity; however, in immunocompromised individuals infection is far more severe with the possibility of central nervous system involvement, myocarditis and pneumonia. Congenital infection can give rise to serious complications involving the liver and the central nervous system, and even abortion or stillbirth.

Members of the cat family are the hosts of *T. gondii*, and mice are thought to act as intermediate hosts helping to maintain the life cycle of the organism. It replicates in intestinal cells of cats resulting in the excretion of infectious oocysts,

containing sporozoites, in the faeces. After ingestion by another animal, including by humans, these oocysts release the sporozoites which then infect and multiply in a wide variety of other cell types including the reticuloendothelial system, leukocytes, and eventually the central nervous system.

Transmission of the organism to humans is thought to be mainly through eating raw or undercooked meat or contact with the faeces of infected animals, notably domestic animals and especially pets. Transfusion transmission has occasionally been documented in immunosuppressed individuals, with sometimes fatal consequences. To minimize any risk of transfusion transmission, susceptible patients who are seronegative and immunosuppressed could be provided with blood screened for the presence of antibody to *T. gondii* or with blood leukodepleted, ideally by filtration, prior to transfusion.

4.3.6 *Babesia microti* and *B. divergens*—babesiosis

Babesiosis is due to infection with the tick-borne protozoan parasite *Babesia* (*B. microti* in north America and *B. divergens* in Europe) which infects red cells and causes a sometimes serious malaria-like illness. Details on the global spread of babesiosis are not known but at present it appears that it is restricted to parts of the USA and Europe. The organism can survive in red cells for at least one month under normal blood bank storage conditions and, like plasmodium, can be transmitted by transfusion of blood from an infected asymptomatic individual. There have been only a few cases of transfusion-transmitted babesiosis reported and no deaths have so far occurred, although it is likely that a number of cases have gone unreported following asymptomatic infection in the recipient. Laboratory screening is not possible at present, and donor selection procedures have to be relied upon to minimize any risks of transmission.

4.4 Creutzfeldt–Jakob disease

Although thought to be caused by either a slow virus or a prion, the precise nature of the agent causing Creutzfeldt–Jakob disease (CJD) is still unclear. It is now generally considered that there is a possibility that it may be transmitted by blood donations from individuals who have received either pituitary growth hormone or human gonadotropin preparations. Current data suggest that the agent may be present in blood although, as yet, no clear confirmed cases of transfusion

transmission have been documented. The risk, if any, of transmission of CJD by blood transfusion remains to be established and might in any case vary between different countries depending upon the type of treatment given, the source of the preparations used, the exclusion policy implemented and the date of implementation.

Chapter 5
Screening tests

5.1 Introduction

There are many laboratory screening tests now available for the detection of specific markers of infectious diseases. Although most of these tests are suitable for blood screening, some are not, being specifically designed for clinical diagnosis, although this is mainly due to assay format or presentation rather than any performance aspects. Also, some assays are specifically designed to work only on certain automated systems, systems which may work well but are wholly inappropriate in transfusion laboratories. In general, most modern assays produced by well known international diagnostics companies perform well and offer the reliability and reproducibility required for blood screening.

At present there are three main formats of assay usually used for blood screening, although these are based upon just two assay principles, and there are many variants of these main formats. The two principles are that of agglutination and immunoassay; both essentially serological techniques based upon specific antigen–antibody reactions and the subsequent detection of those reactions. The main formats of the assays are single use rapid devices, particle agglutination assays and enzyme immunoassays, all of which are used in blood screening globally. Other types of assay are occasionally used, but tend to be for more specialized investigations, such as confirmatory or reference work. However the suitability of the individual assay formats in any particular situation needs to be assessed and determined locally according to needs and available resources, a subject that is covered in Chapter 6.

5.2 Particle agglutination assays

Particle agglutination assays are based upon the premise that antibody or antigen coated particles are agglutinated in the presence of the complementary soluble antigen or antibody. The tests are usually performed in microwells and the results are read, usually by eye or sometimes by automated pattern reading, simply by looking for agglutination in the bottom of the microwell.

The particles used in the standard particle agglutination assays are either nonhuman red cells (normally avian or ovine) or gelatine or latex particles, and are coated with either antigen or antibody depending upon the particular infectious agent. These assays are performed by first diluting the samples; normally serum is the preferred sample type although plasma may be suitable for some tests. The dilution of the sample is an important step in the assay as high serum concentrations can cause false-positive results. In addition, the diluents used in particle agglutination assays are often quite complex solutions containing blocking agents, detergents and high molecular weight hydrophilic compounds to minimize false-positive reactions and to enhance and aid true agglutination. A specified volume of diluted sample is then transferred into a microwell and the appropriate volume of particles added and mixed well. The microwell is a plain uncoated microwell which serves as the reaction vessel. The bottom of the microwell is U-shaped, helping to form the correct settle patterns of the particles. The reaction is incubated at room temperature on a vibration-free surface and the results read after 45 to 90 minutes, depending upon the test requirements. During the incubation period the particles will gradually fall out of suspension to form a pattern on the bottom of the microwell, and if the sample contains the particular marker being screened for, agglutination of the particles will occur. This can be seen as a mat of agglutinated particles covering the bottom of the well, while a negative, nonagglutinated well, can be seen as a single button of free particles in the middle of the bottom of the well (Figure 5.1).

The results can be read by simply scoring positive or negative on the basis of the presence of agglutination. If suitable automated readers are available, the agglutination patterns can be read automatically by the reader scanning set points across the bottom of the well and converting the values obtained into optical density (OD) values which can then be processed in the same way as EIA OD

Positive   Negative

FIGURE 5.1 **Typical particle agglutination assay settle patterns**

values. Included in each test kit are a set of controls that have to be run with each microplate. The results of these control samples are used to ensure that the test run has performed satisfactorily and that the agglutination patterns are as expected.

Particle agglutination assays are usually the cheapest assays available and from a technical point of view are equally suitable for small or large numbers of samples. One potential problem with particle agglutination assays is that samples need to be diluted, and sometimes to quite high dilutions. These dilutions need to be performed in a separate microplate to the agglutination reactions and therefore the diluted samples need to be transferred from the dilution plate to the assay plate.

5.3 Enzyme immunoassays

5.3.1 General

The enzyme immunoassays (EIAs) are a broad group of assays that all use an enzyme-mediated colour reaction to demonstrate an antigen–antibody reaction. These assays are relatively complicated and generally need special equipment to perform them. Although there are many EIAs available, all fall into one of four basic types of EIA: antiglobulin, sandwich, antibody capture and competitive. The final results of all EIAs are numerical values which then need to be interpreted as positive or negative results.

The assays are based upon a solid phase onto which either antigen or antibody, depending upon the particular assay, is immobilized; the solid phase is normally polystyrene and either a microwell or bead. Microwells are generally presented in a 96 removable well plate format (12 × 8 wells), into which the sample and assay reagents are added sequentially and the final reaction read. Bead-based assays are performed in a well similar to a standard microwell but larger and in a 12 × 5 well solid plate format, which simply acts as the reaction vessel into which the coated bead is placed and the sample and assay reagents are added sequentially. Depending upon the particular assay, the final reaction is either read in the reaction plate or by transferring the bead into a separate reaction tube and adding the colour development reagents to this tube.

The first stage of an EIA is for the immobilized antigen or antibody to react with any complementary antibody or antigen in the sample resulting in a specific antigen–antibody complex. The sample is added to the well, diluted or undiluted depending upon the particular assay, and incubated at a specified temperature and

for a specified time. At the end of the incubation period the excess sample (plus diluent) is washed away.

The second stage of an EIA is the detection of the reaction. The detection systems used are many and varied, but generally they use a further antibody, such as a broad range antihuman antiglobulin, or an antibody specific to the infectious agent being screened for, or even a specific antigen, but which is chemically labelled with an enzyme. This labelled antibody (or antigen) is called the conjugate, the precise nature of which depends upon the assay and the agent being screened for. The conjugate is added to the well after the sample has been washed away, and incubated at a specified temperature and for a specified time. During the incubation period the conjugate only binds to any specific antigen–antibody complex present, and after binding to the complex it also becomes immobilized. At the end of the incubation period the excess conjugate is washed away. The enzyme part of the conjugate is used in the final stage of the assay in which a synthetic dye known as a chromogen is added and which changes colour, generally from colourless to coloured, in the presence of the enzyme. The chromogen solution, generally known as the substrate, is added to the well after the conjugate has been washed away, and incubated at a specified temperature and for a specified time. At the end of the incubation period the colour reaction is stopped, usually by adding acid, and the OD values (light transmission) of the individual wells read. Thus, a positive result is seen by the appearance of specific colour in the reaction well; and no colour is seen with a negative result.

Included in all EIAs are a set of controls that have to be run with each microplate or set of tests for bead tests. The results of these control samples are used to ensure that the test run has performed satisfactorily, and to calculate the assay cut-off value. The cut-off value is that OD value which is used to decide whether a result is positive or negative (see Chapter 8).

EIAs are technically demanding, need at least some specialized equipment, and are more expensive that particle agglutination assays. However, they are generally reliable and give good quality results with a hard copy which can be retained, indefinitely, if required.

5.3.2 Types of enzyme immunoassay

5.3.2.1 Antiglobulin assay (see Figure 5.2)

The solid phase is coated with purified specific antigen: native, recombinant, or synthetic peptide. Any specific antibody in the sample binds to the immobilized antigen. Bound antibody is detected by adding a conjugate comprising enzyme-labelled antihuman globulin. When substrate is added, any enzyme present will cause the chromogen to change from colourless to coloured. This is probably still the most common type of EIA used to detect markers of infection of infectious diseases.

5.3.2.2 Sandwich assay (see Figure 5.3)

The solid phase is coated with purified specific antigen: native, recombinant, or synthetic peptide; or with purified specific antibody: polyclonal or monoclonal. Any specific antibody or antigen in the sample binds to the immobilized antigen or antibody. Bound antibody or antigen is detected by adding a conjugate comprising enzyme-labelled specific antibody or antigen. When substrate is added, any enzyme present will cause the chromogen to change from colourless to coloured. If this type of assay is used in an antigen assay, e.g. HBsAg, and if a pair of monoclonal antibodies are used, one immobilized and the other as the conjugate, which recognize different epitopes on the antigen, the sample and conjugate can be added simultaneously, as the immobilized and free antibodies will not compete for the same binding sites. This can decrease the assay time and increase the sensitivity.
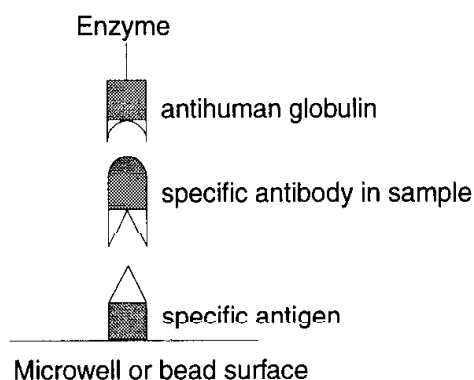
FIGURE 5.2 **Schematic of an antiglobulin enzyme immunoassay**

Enzyme

specific antibody or antigen

specific antibody or antigen in sample

specific antibody or antigen

Microwell or bead surface
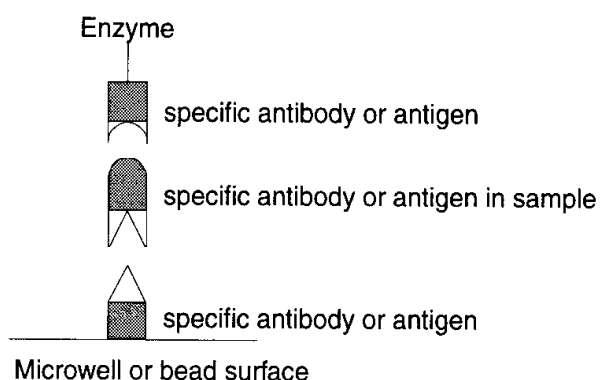
FIGURE 5.3 **Schematic of a sandwich enzyme immunoassay**

5.3.2.3 Antibody capture assay (see Figure 5.4)

The solid phase is coated with purified antihuman globulin specific for IgG and IgM. All IgG and IgM antibodies in the sample, irrespective of specificity, bind to the immobilized antibodies. Any specific antibody present is detected by adding a conjugate comprising enzyme-labelled specific antigen. When substrate is added, any enzyme present will cause the chromogen to change from colourless to coloured. If different specificity enzyme-labelled antigens are combined in the conjugate, more than infectious agent can be detected in the same microwell. This assay type has been used for some time for research and confirmatory work, but has recently been introduced into a routine commercial anti-HIV assay.

Enzyme

specific antigen

nonspecific human globulin in sample

specific antihuman globulin (IgG + IgM)

Microwell or bead surface
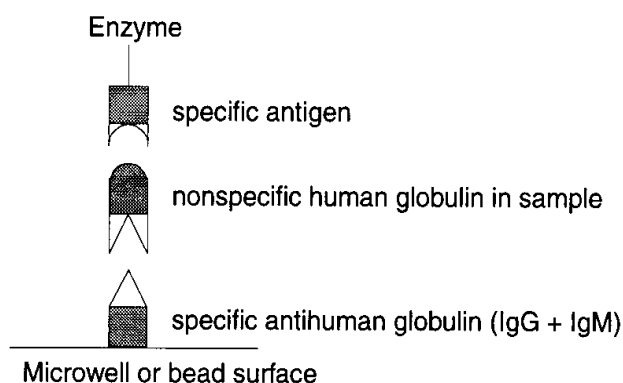
FIGURE 5.4 **Schematic of antibody capture enzyme immunoassay**

Enzyme

specific human antibody in sample competing
with specific enzyme-labelled antibody

specific antigen

Microwell or bead surface
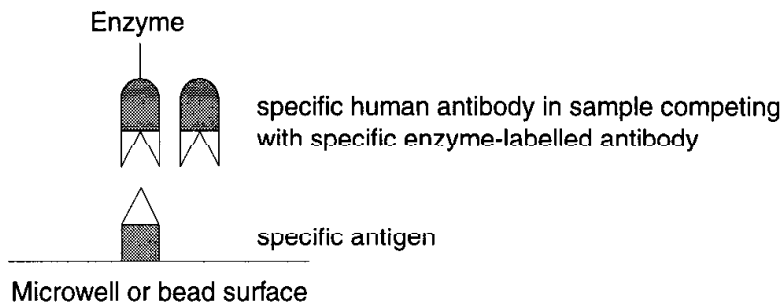
FIGURE 5.5 Schematic of competitive enzyme immunoassay

5.3.2.4 Competitive assay (see Figure 5.5)

The solid phase is coated with purified specific antigen: native, recombinant, or synthetic peptide. Sample and conjugate, comprising enzyme-labelled specific antibody, are added simultaneously. The conjugate and any specific antibody in the sample then compete for the immobilized antigen binding sites, the specific antibody binding preferentially. The specific antibody concentration in the conjugate is carefully set so that the concentration of specific antibody in any sample will nearly always be significantly greater than that in the conjugate and therefore compete successfully for the antigen binding sites. If there is no specific antibody in the sample, the antigen sites will all be filled by conjugate, while if there is specific antibody in the sample the antigen sites will all be filled by that antibody. When substrate is added, any enzyme present will cause the chromogen to change from colourless to coloured, but in this case a positive result is indicated by little or no colour, the antibody in the sample has prevented the conjugate from binding; a negative result is indicated by colour.

5.4 Single use rapid devices

The single use rapid devices are actually large-scale individual EIAs, but are unique in their presentation for use and may incorporate different approaches in the visualization of the specific antigen–antibody reaction sought after. They generally comprise a single use cassette holding a membrane (usually nitrocellulose, to which the antigen or analyte capture system is bound), an absorbent pad under the membrane and a set of ready-to-use reagents. The general principles and procedures of the assay are described in detail in 5.3; only a brief outline of the procedures is included in this section.

The sample (in some cases whole blood is used), is applied to the membrane and during a short incubation period is drawn through the membrane by the absorbent material underneath. Any specific analyte present will be bound to the membrane by the immobilized capture system. Any remaining sample is then washed through the membrane and the appropriate reagents added sequentially according to the manufacturer's instructions.

First, conjugate is added and incubated for a period of time, usually just a few minutes. During this period the conjugate binds to any analyte bound to the membrane. The nature of the conjugate may vary from that normally found in a more conventional microplate or bead EIA. The conjugate may be an enzyme conjugated detection antibody, or a colloidal gold conjugated antibody or colloidal gold conjugated protein A. The use of colloidal gold can be particularly advantageous as no other colour detection system is needed, although it is generally felt that the sensitivity of such an assay may be slightly less than when using a conventional colour development system. During the incubation period any conjugate that binds to immobilized analyte can be seen by the reddish brown "precipitate" that builds up; excess unbound conjugate is washed away and little or no colour is seen. The assay is now complete and the result can be read.

With the classical EIA system, excess conjugate is washed away and the colour development reagent (substrate) is added. The resultant colour reaction (generally colour = positive and no colour = negative) enables the reaction to be read visually and the final result determined. After use the cassette is disposed of appropriately.

Very often these devices incorporate a visual check system to ensure that the test has been performed properly. Such checks often target normal human immunoglobulin in the sample to always give a positive check result irrespective of and separate from the actual test result. These checks serve to demonstrate that sample has been added, each stage of the assay has been performed correctly and that the reagents have worked properly.

At present these single use rapid devices are produced mainly for anti-HIV screening, although a number of HBsAg detection devices are now available in this format, as are one or two anti-HCV tests.

The advantages of these assays are their ease of use and speed, while their disadvantages include price and often slightly lower sensitivity. As they are supplied as discrete tests, single donations may be tested as needed making them

very attractive in situations where small numbers are tested on an irregularly basis. They are generally supplied complete with all reagents and basic disposables needed to perform the test, the manipulations required are all simple and the reagent volumes generally measured in drops rather than microlitres.

5.5 Recent advances in immunoassays

Recent advances have seen a merging of the use of particles together with immunoassay principles. Microparticles are starting to be used as the solid phase rather than a well or single bead. The advantage of microparticles is the significant increase in surface area to which specific antigen–antibody can be immobilized. Sample is mixed with the microparticle solution and incubated. The conjugates used in EIAs are used in the first part of the detection reaction, but in the second part, rather than use chromogens, which are read by measuring light transmission, fluorescence or chemiluminescence are used. Instead of the enzyme in the conjugate catalysing a colour change, it catalyses either the emission of light when examined under ultraviolet light, or the emission of light of a certain wavelength as a result of a chemical reaction. The applications of such technology are now starting to increase and spread across many countries. However, such assays are currently expensive, specialized, need certain equipment and are generally used for confirmation rather than routine screening.

A further advance has been the development and inclusion in EIA of systems that positively demonstrate the addition of sample to the reaction vessel. Such systems generally use a reagent that changes colour when in contact with human serum or plasma (see Chapter 11).

Chapter 6

Screening policies and strategies, and the selection of tests

6.1 Introduction

An appropriate and effective blood screening programme relies upon a clear and well defined national screening policy, the design and implementation of an appropriate screening strategy and the use of appropriate screening tests. Any screening programme should be considered to extend from the selection of the blood donors to the transfusion of the screened blood.

Before an appropriate strategy can be designed, screening policies have to be decided. These are specific to a particular country and are generally dependent upon a number of factors, which themselves are specific to the country or sometimes a region of a country. The screening policy is a key part of the structure of the national blood programme.

6.2 Screening policies

6.2.1 General

Screening policies outline the basic minimum screening requirements for a country to ensure a safe blood supply and encompass the particular agents screened for, the resources available, the expected effectiveness of the screening programme, the commitment of the government to the transfusion service and the provision of safe blood and products to the population. Policies are determined by a number of factors, but primarily by seroepidemiological data, the resources available and financial constraints.

6.2.2 Seroepidemiological data

The seroepidemiological data provide information about the infectious agents present in the population for which the donated blood may need to be screened.

Good seroepidemiological data can be hard to obtain in some cases, especially in countries with a poor health care infrastructure and poor central coordination, where limited data are likely to be available and even these data are unlikely to be collated for the region or the whole country. From the information about the particular infectious agents present in the population, those likely to be found in blood donors can be identified, and the risks of transmission assessed. For each infectious agent the first question is whether there is a significant risk of transmission by blood transfusion, and the second question is whether there is a significant risk of disease in the recipient. Clearly if an agent is not, or is very unlikely to be, transmissible by transfusion, it can be argued that there is very little value in screening for the agent, while if an agent is transmissible, screening should be performed. However, if a transmissible agent is unlikely to cause disease in a recipient, again it can be argued that there would be little value in screening. Indeed there are certain infectious agents, human cytomegalovirus (HCMV) for example, which are transmissible and may cause serious disease in the recipient, but which only cause clinical disease in susceptible recipients; in the case of HCMV susceptible recipients comprise mainly uninfected immunosuppressed individuals who, in many countries, make up only a small proportion of the patients transfused. In addition, the prevalence of HCMV may range from very low level to endemicity. Provision of HCMV-negative blood for this population of patients is usually achieved by the additional screening of small numbers of donations for HCMV specifically for susceptible individuals; unsusceptible individuals may safely receive blood unscreened for HCMV.

The seroepidemiological data can also be used to look at the actual prevalence of the agent in the general population and from that data the probable prevalence in the donor population. This figure should be significantly less than the prevalence in the general population, assuming that donor selection procedures are in place and that they reflect the risk activities of the donor population. This prevalence information is important to be able set up the appropriate screening algorithms, set up the appropriate confirmatory algorithms and calculate the wastage of donations due to the presence of infectious agents. If the prevalence of a particular infectious agent is very low, it may be that screening is not appropriate at that time, but continued surveillance of the prevalence of the agent should be performed so that screening can be introduced as soon as the prevalence reaches the level at which it does become appropriate. Similarly, the prevalence information, either from blood screening or from serosurveillance, can be used to determine the point at which to stop or restrict screening. If it can be accepted that

the prevalence of any infectious agent is higher in the general population than in the donor population, when the prevalence of an infectious agent in the donor population rises to a high level, it is very likely that the prevalence in the general population will be a lot higher, and therefore a significant proportion of the patients receiving blood already is, or has been, infected. In this situation it is important to look critically at the actual effectiveness of the screening performed and ensure that the resources used would not be better used elsewhere within the transfusion service.

6.2.3 Resources available

There are a number of basic resources, not directly financial, the availability of which can have a direct influence on the screening policies developed. These resources include the donors available and the laboratory services available.

The number and types of donors available can have an important influence on the screening policies developed and hence the safety of the blood supply. There needs to be sufficient donors to provide for the blood needs of the country. The particular risk groups of the donors need to be identified and donor selection criteria set to ensure that high-risk donors are properly identified and excluded. How this is achieved is influenced by the types of donor available; whether the donors are voluntary nonremunerated, remunerated, family or compulsory donors. Each type of donor needs a different approach to effectively identify high-risk donors. In addition the stability of the donor base, the provision of panels of regular donors, is important in maintaining a safe blood supply.

The laboratory services available also directly influence the policies developed. The assays available in a country, both the types of assay and range of different assays of each type, can directly affect which agents can be screened for. If, for whatever reason, certain assays are not available, the screening policies have to reflect this lack of availability. The numbers of staff available and their levels of expertise have to be considered. If sufficient competent staff are not available across the whole country, then in order to to make best use of those staff that are available and provide a national service, collection and screening may have to be more centralized, for example on a regional basis rather than on a district or local basis. The same applies to the laboratory equipment available for use in blood screening. Screening policies must take into account what equipment is available, and what is in good working condition and can be maintained in that

condition so that it can be used. However, if specific equipment is not available all suppliers of the screening assays can supply the best equipment needed to perform the assays, and this equipment can be purchased outright if funds are available, or rented or leased for as long as the assays are purchased. In this way all the equipment needed to perform the screening tests in the most reliable and effective way can be obtained, and also maintained in optimal condition.

6.2.4 Financial constraints

There is no doubt that in many countries the major initial constraint on the development of an effective transfusion service is financial. This is especially seen when considering the screening of blood for infectious agents, because of the cost of the screening tests. If sufficient money is available only for blood collection and basic red cell serology, this is all that is likely to be undertaken and screening for infectious diseases may not be performed.

A question often asked is: is screening cost–effective? Unfortunately this question is not as simple as it may seem and hard to answer in some circumstances. However, the central issue is: what are the consequences of transmission? The clinical consequences of transmission of infection by transfusion may be severe, if this is the case it is also likely that the financial consequences will be significant. So, although screening may be expensive, the cost of patient care following transfusion-transmitted infection may be as great or even greater, and potentially may also be an important public health issue.

The true costing of blood screening is often underestimated as a number of factors, other than the cost of the screening itself, are often overlooked. The choice of screening algorithm may be a significant factor in the overall cost of screening, the type of assay used and the complexity of the algorithm. Even if the unit assay cost is relatively low, a complex algorithm can lead to an expensive screening programme. Additional factors include the cost of confirmatory testing and the loss of donations—directly influenced by the prevalence of the agent, the numbers of donations tested, the number of staff available, the specificity of the assay used and the number of donors removed from the panel. There is a cost in the recruitment of new donors; in many countries this cost is often overlooked and is rarely calculated. Where there is a large proportion of replacement, or other nonvoluntary, donors, this is common and understandable, but also very short-sighted as true running costs cannot be calculated.

6.3 Screening strategies

6.3.1 General

The strategies selected for donation screening have to reflect the national screening policy and are basically determined by the infectious agents screened for, the number of donations screened and national policies. For any situation there will always be alternative strategies, all of which may have good as well as bad features. No one strategy is likely to be perfect, but recognizing this fact is important in assessing the relative merits of any strategy. However, choice of the most appropriate strategy is vital to ensuring that the screening programme is effective.

6.3.2 Infectious agents screened for

There are a number of issues to be considered when deciding which infectious agents are to be screened for. Although the national screening policy should indicate which agents are to be screened for, for each infectious agent the most appropriate circulating marker of infection needs to be identified. In some cases this is not too much of a problem as the number of markers detectable is limited and it is quite clear which marker should be used for each infectious agent. In other cases, however, this may not be as clear and careful selection of the most appropriate marker is needed. It is also possible that the situation for any infectious agent may change after the screening strategy has been designed and implemented; all strategies must be monitored and changes made when needed. The choice of which marker to screen for needs to reflect the infectivity of an infected donor at any stage of infection, the circulating markers of infection produced by the infectious agent and the predictive value of each marker.

a) Infectivity

Most infectious agents give rise to particular serological patterns of infection, but these can be generalized into four stages: incubation, acute infection, recovery, immunity. In some cases chronic infection ensues, and recovery and acquiring immunity may take years. In other cases infection persists for the lifetime of the individual. However, taking the general four-stage model, the strategy first needs to understand at what point screening is going to identify an infectious donor.

As examples, let us look at the infection profiles of hepatitis B virus and HIV (see Chapter 3 for detailed information on the viruses). If we first look at acute

hepatitis B virus infection, here we can see a relatively simple picture. Following incubation the first marker of infection to appear in the bloodstream is HBsAg. This is produced in very large quantities during acute infection and marks a highly infectious stage of infection. The production of antibodies to viral proteins follows the appearance of HBsAg and ultimately marks resolution of infection and development of immunity. HBsAg assays are widely available and relatively cheap, and have good sensitivity and specificity.

If we now look at HIV infection, the picture is quite different. Following incubation the first marker of infection to appear in the bloodstream is pro-viral RNA, 5–10 days after this HIV p24 antigen and HIV DNA appear, followed 5–10 days later by anti-HIV which persists, probably for the lifetime of the individual. In HIV infection anti-HIV does not represent immunity; rather it demonstrates previous infection and probable persistence of the virus in the bloodstream. Although HIV RNA, p24 antigen and HIV DNA are all produced before anti-HIV, their levels in the bloodstream fluctuate so that screening for only one of them may result in the failure to detect an infected individual, while anti-HIV, once produced, persists, and at a level that is easily detectable. Indeed p24 antigen levels fall below detectable levels very quickly after anti-HIV appears. In addition, assays for RNA and DNA are currently more specialized and not suited to mass screening procedures; p24 antigen assays are available in an EIA format but are expensive and need to be used in conjunction with anti-HIV assays. Thus anti-HIV is the single best marker of HIV infection for the screening of donated blood collected from a population with a low to medium rate of acquisition of HIV.

As can be seen, the marker of infection screened for is dependent on the particular infectious agent and may represent a compromise between as early detection of infection as possible and a practical and workable screening programme. This has to be reflected in the screening strategy selected, but may be affected by the prevalence of the infectious agent in the donor population. Again, HIV is a good example of an agent for which the screening strategy may alter depending upon the prevalence in the donor population (see Chapter 3 for a more detailed discussion on HIV antigen testing). As the prevalence increases it has been argued that the possibility of detecting an HIV-antigen-positive but anti-HIV-negative donation increases and adding HIV antigen testing to the HIV testing strategy may have to be considered.

b) Use of surrogate markers

Screening for surrogate markers has been performed in some countries for many years, while other countries have never used that strategy. Surrogate markers are nonviral specific markers that are thought to provide indirect evidence of viral infection where no specific markers can be found. In some countries serum liver aminotransferase levels, specifically ALT, were used to try to reduce cases of PTNANB hepatitis before anti-HCV assays were available, but are now used to try to prevent cases of HBV transmission from HBsAg-negative individuals and transmission of other hepatic viruses. Generally the introduction of anti-HCV screening has demonstrated how poorly predictive ALT screening is, and a number of countries have ceased or are actively considering ceasing ALT screening. In a similar way, anti-HBc screening was also used before anti-HCV screening assays became available to try to reduce cases of PTNANBH, but this screening now has no value in this context.

6.3.3 Number of donations screened (selection of assay format)

Once the particular agents to be screened for and the individual markers of infection have been decided, the number of donations screened is probably the next major factor to consider. This is important because it essentially determines the most appropriate format of screening assay to use; the major factor in deciding on a particular format is the number of tests performed in a single test run and the frequency of test runs. As previously discussed there are currently three basic formats of screening assay that are commercially widely available: simple/rapid discrete assays, particle agglutination assays (PAs) and enzyme immunoassays (EIAs). There are a number of different approaches that can be taken in relating the number of tests to assay format, but perhaps the simplest is to link the number of donations tested per day or week to assay format. The following schema is a simple and workable approach which could act as a basis for developing more precise individual schemes.

- When testing 1–35 donations per week use a rapid/simple assay.
- When testing 35–60 donations per week use a particle agglutination assay.
- When testing more than 60 donations per week use an EIA.

Such an approach is not only an effective use of resources, but also ensures that the availability of blood is compatible with local needs. On the one hand, a small transfusion laboratory does not need the automated equipment required to

perform EIAs when the workload is small and there are few staff; such equipment would be totally inappropriate and would probably rarely be used. In this situation a supply of rapid/simple tests would be quite adequate to ensure screening prior to use of the blood. In addition, in such a situation it is highly likely that blood is either collected only when needed, or that what is collected is used very quickly; in either case stocks of blood are rarely held. However, using such rapid tests, freshly collected blood can be tested and made available immediately. On the other hand, a large laboratory would have the need and resources to utilize the automated equipment needed to perform EIAs. Because EIAs are slower and more involved procedures, it takes longer to test the blood, but much larger numbers can be tested at any one time. Such a laboratory is, however, more likely to have a more developed blood collection programme and to hold stocks of blood so that blood needs can be supplied from existing tested stock without needing emergency collection and testing.

All three main assay formats have their own advantages and disadvantages, and these can obviously make one format particularly attractive and more appropriate in a certain situation. Table 6.1 lists some of the more important advantages and disadvantages associated with the different assay formats.

6.3.4 National and regional health policies

The national blood screening policy should be part of the general national health policies. However, there may be other policies, national or regional, that affect the screening strategies developed either at national or a more local level. These commonly involve donor rather than donation issues, often to do with the confirmation and notification of positive donors, and may restrict the transfusion service in the management of positive donors.

- It may be policy not to inform donors of a confirmed positive screening result, but only to discard the blood and not recall the donor. Such a policy is uncommon but may reflect the fact that donor identity can often not be confirmed (a donor may give a false name and address). It may also reflect the inability of the health care system to provide appropriate care for such an individual, who may appear to be healthy and well. In such situations the supply of blood is often critical to the patient's survival, and some blood safety issues simply cannot be addressed properly. As long as screening is performed before transfusion and the screening programme is reasonably well designed

TABLE 6.1 **Advantages and disadvantages of different assay formats**

Advantages	Disadvantages
Rapid/simple assays	
All reagents supplied	Eye-read only
No additional equipment required	No hard copy of results
Rapid	Suitable for small numbers of tests only
Simple to use	Expensive
Serum/plasma/whole blood can be used	
Single tests can be performed	
No additional controls required	
Particle agglutination assays	
All reagent supplied	Eye-readable (mechanical read possible)
No additional equipment required	No hard copy of results
Simple to use	High initial dilution
Suitable for larger numbers of tests	Generally suitable for serum only
Relatively inexpensive	Long incubation
Enzyme immunoassays	
Mechanical reading of results	Special equipment required
Sensitive and specific	Multiple stages
Suitable for large numbers of tests	Skilled staff needed
Relatively inexpensive	

and controlled, any risk of infection is at least minimized and such a policy can be largely justified.

• It may be policy not to confirm the screening results, but to inform the donor on the basis of the screening result, either a single result or on the basis of repeat testing. There are a number of reasons why such a policy may be adopted, the main one being lack of confirmatory facilities, but also there may not be a dedicated reference laboratory and the different assays needed to reliably confirm screening results may not be available. It is clear that reliance upon a single screening result to exclude donors is very wasteful of blood as many assays initially give false-positive results, many of which disappear on repeat testing. However, either time or cost constraints can limit the ability to repeat any initially reactive donations, making the use of the initial result essentially unavoidable. A repeatably positive result has a better predictive value than a

single result, depending upon the specificity of the test and the prevalence of the particular agent in the population, and counselling on this basis can be more appropriate.

• It may be policy not to reinstate donors who are reactive on screening but confirmed negative. For many years it is been recognized that some donors will give false-positive results with some assays. When confirmatory testing is performed these donors are shown to be truly negative, just falsely reactive in the primary screening test used. In addition, in many of these donors this reactivity is transient, often only affecting one or two donations; thus, these donors may be deemed only temporarily unsuitable. Some national policies, however, may permanently defer such donors on the basis that any repeatably reactive screening result should be considered relevant. This is very wasteful of donors and is not really an appropriate policy with the range of assays now available to confirm the initial screening results and the wealth of knowledge available about such donors.

6.3.5 Specific screening strategies

Taking into account all of the previous comments and discussion, some specific screening algorithms are now examined and their specific applications discussed.

a) WHO strategies

In 1992 WHO published some basic screening strategies that could be applied to HIV testing in a number of different circumstances depending upon the reason for testing (WHO *Weekly epidemiological record*, 1992, 20:145–149). Three basic strategies were presented and suggestions made about the suitability of these strategies for different testing scenarios. Table 6.2 suggests possible uses of the testing strategies outlined below.

Strategy I. Samples tested with one EIA or rapid/simple assay. Any reactive sera considered to be anti-HIV-positive.

Strategy II. Samples tested with one EIA or rapid/simple assay. Reactive sera retested with second assay but based upon different antigen of test principle. Sera reactive in both tests considered to be anti-HIV-positive, sera reactive in only first test considered to be anti-HIV negative.

TABLE 6.2 **HIV testing according to strategy**

Testing objective	Prevalence of infection	Testing strategy
Safe blood	All prevalences	I
Surveillance	>10%	I
	<10%	II
Diagnosis	All prevalences	II
	>10%	II
	<10%	III

Strategy III. As strategy II but a third test is used for sera reactive in the first two tests. Sera reactive in all three tests considered to be anti-HIV-positive, sera reactive in only the first two tests are considered to be anti-HIV indeterminate.

Although strategies I to III can be made to work in blood screening, they were designed on a more universal basis to provide common strategies to cover different screening rationales. There are other slightly different approaches that are more specific to blood screening and commonly used by many transfusion services.

Strategy 1. Use WHO strategies I/II. Discard all reactive donations.

Strategy 2. Repeat any initially reactive samples in duplicate using the same test. Discard any donations reactive in either of the repeat tests.

Strategy 3. Repeat any initially reactive samples in duplicate using an alternative test of equal sensitivity and the same principle. Discard any donations reactive in either of the repeat tests.

Of these, strategy 2 is the one most commonly used in developed countries. Strategy 3 is increasing in acceptability; it is most useful if the specificity of the initial assay is not optimal; and a number of donations would be repeatably reactive, although subsequently confirmed negative, if strategy 2 were to be used, and discarded. Use of strategy 3 would reduce significantly wastage of blood.

b) Pooling

All of the six strategies discussed in item a) above are based on the testing of single discrete samples. The pooling of samples prior to testing is a very controversial issue. Despite the argument of cost, the disadvantages of pooling

include potential loss of sensitivity and the requirement of individual laboratory validation. Additionally if the prevalence of the infective agent in the donor population is too high the number of positive pools becomes too high and the amount of additional testing needed to identify and confirm the positive samples exceeds the savings in cost that would be gained.

The health of an individual is the highest priority, and all optimal measures must be taken to minimize the risk of a transfer of a disease. It is a common rule that a specimen from an individual received by a laboratory for diagnostic testing for diagnosis or exclusion of a disease should never be pooled with another specimen. It would be against ethical and legal principles to mix specimens for testing knowing that there is a potential loss of sensitivity. The courts of justice in some countries in Europe (e.g. in France and Germany) have condemned several laboratories because they deliberately ignored this rule. The argument of those who pool, that it is better to pool the samples and accept the weaknesses rather than not to screen at all, does not justify recommending pooling. Policy-makers may take it as a standard and acceptable rule in all situations that pooling should not be practised. The following are among the technical arguments against pooling:

1. Each method (even the most sensitive one) has a limit of detection. The limit of detection is the lowest concentration at which an agent can be positively identified. The limit of detection of a method is determined by diluting the concentration of an agent to be measured. Pooling a specimen with other specimens in clinical routine or for blood transfusion will automatically result in the same effect. This means that an agent that may be present in a specimen at a concentration near to the limit of detection of a method, will not be detected by the test when the sample is diluted to a concentration below this limit. This also means that diluting a specimen might create a condition of measurement that is less than optimal.

2. Each test system that is used by a clinical laboratory is more or less specific. Enzyme immunoassays (such as for HIV antibodies and hepatitis B surface antigen) measure an agent by means of an immunological reaction. Such a reaction can be interfered with by nonspecific interferents that might be added when pooling blood or a serum specimen with other blood or serum specimens. For example, certain immunoglobulins (such as rheumatic factors) can interfere with an immunological test. The interfering substance can therefore inhibit the test for diagnosis. A negative test result caused by such interference

is not caused by a dilution and may occur at a concentration of an agent where one would normally expect a positive test result.

3. Testing blood for transfusion as well as for the preparation of blood products for therapy of patients requires the highest possible standard, and optimal conditions of investigation must be chosen to exclude even a minimal risk of infection. This is why the WHO Expert Committee on Biological Standardization (ECBS) has recommended that each donation be individually tested for infective agents. Moreover, when blood donations are pooled for the preparation of blood products, the pools must be tested during the manufacturing process in addition to the first testing of each individual donation.

Pooling might be acceptable for epidemiological studies of population.

6.4 Screening procedures

6.4.1 General

Screening procedures can be considered under two headings: nonlaboratory based and laboratory based. The nonlaboratory based procedures are those of donor preselection and all the issues associated with the identification of suitable "safe" donors before venepuncture. The laboratory based procedures are mainly the performance of the specific screening tests used to identify potentially infectious donations, but also include procedures not based upon specific screening, such as leukodepletion or viral inactivation. It is not the remit of this manual to specifically discuss donor preselection or nonscreening procedures; however, some comments need to be made about the relationship between donor preselection and donation screening, and some information given about leukodepletion and viral inactivation procedures.

6.4.2 Donor preselection

Donor preselection is as vital to providing safe blood as the screening tests used identify potentially infectious donations. All transfusion services have to look very carefully at what policies and strategies they need to adopt to provide safe blood. No transfusion service is in a position to specifically screen the donations for every possible infectious agent that may be present. Rather, the most significant agents are specifically screened for, and the donors are questioned and examined to try to minimize any other risks. Laboratory screening should never

be relied upon solely to provide safe blood; it is just one part of the whole process by which transfusion services provide blood that is as safe as possible. Donor preselection is the first half of the screening process; laboratory screening is the second half. On their own the two halves cannot provide the degree of safety that the whole process can. Donor preselection is therefore just as important as laboratory screening, and the checks and controls placed upon laboratory screening equally apply, and must be applied, to donor preselection.

6.4.3 Selection of specific screening assays

6.4.3.1 General

The choice of basic assay format has already been discussed. Once the most appropriate format has been selected, there remains the selection of the most suitable assay of that format. Assay choice is a very complicated and important process; selection of an assay which actually provides the level of performance required is essential. There are many parameters by which assay performance can be measured but no matter how good a particular assay is, the critical factor is how well it actually performs in each laboratory using it. The overall performance of any assay is totally dependent upon the quality of the performance of the assay procedure—**a good assay performed badly gives bad results**. In the same way—**a bad assay, even performed well, gives bad results**.

6.4.3.2. Assay performance

There are three main factors to take into account when considering assay performance: sensitivity, specificity and ease of use; in addition a number of other factors need to be considered. Sensitivity and specificity are probably the most important, although, as previously mentioned, the absolute sensitivity and specificity of any assay depend upon its performance in each laboratory, how easy it is to perform and how well it is actually performed. Unfortunately sensitivity and specificity tend to be inversely related; as one increases the other decreases. For blood screening it is clearly essential that the assays used are as sensitive as possible, but at the same time poor specificity may result in the unnecessary loss of too many donations. A balance is therefore needed between the two which favours the sensitivity but still provides acceptable specificity.

a) Sensitivity

The sensitivity of an assay can be defined as its ability to identify as positive all samples from individuals truly infected with the agent being screened for. There are a number of ways in which this sensitivity is quantified, some of which are more accurate and meaningful than others. Antigen assays, such as assays for HBsAg, can more easily be quantified as viral antigen, can be relatively simply isolated, purified and quantified, and can be designed to show a linear response curve at the lower end of their sensitivity range. Dilution of the antigen can be used to assess and compare directly the sensitivities of different assays.

Unfortunately the same is not true for antibody assays. Although the assays themselves can be designed in a similar way, dilution of antibody solutions is not simply a matter of gradually decreasing the amount of antibody present. That part of the immune response to any infectious agent that gives rise to the circulating antibody molecules does not simply recognize the agent as foreign and produce an antibody against it; a number of antibodies may be produced all of which recognize individual parts of the agent, or proteins produced by it in the infected individual, as specific individual antigens. Thus, a single antibody response actually comprises the production of a whole range of individual antibodies (the antibody profile), all of which recognize different parts of the infectious agent or proteins produced by it, and all of which may have different characteristics, such as titre, avidity or Ig class. In addition, the antibody profile may vary depending upon the particular stage of infection, and can change gradually over a period of time.

The sensitivity of any antibody assay can accurately be determined only using true weak samples, normally those from seroconverting individuals (see Chapter 3 for more details about seroconversion) or, rarely, those few individuals who mount only a very weak and/or abnormal antibody response following infection. The use of endpoint dilutions of strongly positive sera may appear to allow comparison of assays but such comparisons are seriously flawed, as most assays have not been designed to work optimally with diluted sera and therefore do not give the linear responses needed for true comparison. Most sensitivity studies are performed using panels made up of sequential samples from seroconverting individuals. If such sera are not available locally or nationally from patients previously identified, they are available from commercial suppliers such as Boston Biomedica Inc. (BBI) in the USA, although such panels are relatively expensive. In addition, panels of one-off weak and unusual samples are also used

to assess the general sensitivity performance of assays against a range of different samples. Again such panels can be put together locally or nationally or purchased from commercial suppliers. It should be noted that, at this time, there are only a few commercial suppliers who can supply good quality and well characterized panels of such sera. The formula for calculating sensitivity as a percentage is given as:

$$\text{sensitivity} = \frac{\text{no. of true-positive detected}}{\text{no. of true-positive detected} + \text{no. of false-negative detected}} \times 100$$

Specificity

The specificity of an assay can be defined as its ability not to identify as positive any samples from individuals who are not infected with the agent being screened for. The specificity of any assay is assessed by testing the assay against a large number of samples, generally at least 2000, that are known to be negative for the agent being screened for. Any initially reactive samples are retested and from these data the initial and repeat reactive rates obtained and the specificity calculated. The formula for calculating specificity as a percentage is given as:

$$\text{specificity} = \frac{\text{no. of true-negative detected}}{\text{no. of true-negative detected} + \text{no. of false-positive detected}} \times 100$$

When considering the sensitivity and specificity of assays, two other measures are also important: the positive and negative predictive values. These values give an indication of the accuracy of any given screening result and are based not only upon the assay itself, but on the performance of the assay by a particular laboratory and the prevalence of the particular agent being screened for in the donor population. Thus the positive predictive value gives an indication of the probability that a positive screening result is a true-positive, and the negative predictive value gives an indication of the probability that a negative screening result is truly negative. Of the two, the negative predictive value is more critical and is influenced by the sensitivity of the assay. To ensure blood safety its value should be 100%, i.e. it is certain that a negative screening result is a true result. The positive predictive value is more influenced by the specificity of an assay and is not so critical. We know that in many blood screening situations the majority of repeatably reactive results are falsely positive, and a low positive predictive value is therefore acceptable.

c) *Ease of use*

The ease of use of any assay, while not being the first consideration, does have a significant impact on assay performance and can lead to bad results being obtained from an otherwise very good assay. Consider the situation of a new assay being released that is clearly significantly superior, in terms of sensitivity and specificity, over existing assays. If this assay is particularly complicated or not very robust in routine use, it is very likely that the improved sensitivity and specificity will never actually be seen because the level of technical performance needed to ensure optimal results from the assay would rarely be achieved under routine testing conditions. Indeed, in some circumstances it could be argued that not only would the increased benefits of such an assay not be seen, but the working sensitivity and specificity actually achieved could be lower than that obtained with the assay in use previously, i.e. changing to the new assay actually resulted in a decrease in the safety of the blood supply because it could not be performed properly. Thus, ease of use, specifically ease of use within individual laboratories, must be considered early on in the selection process as it may actually be more appropriate to select an assay with an apparently slightly lower sensitivity, but a sensitivity which can routinely be achieved in the working laboratory.

Factors which make an assay easy to use vary tremendously according to the equipment available, the numbers and expertise of the staff, the numbers of samples screened and the time-scale for screening. However they include: sample volume, number of steps, incubation times and temperatures, wash requirements, stability of reagents and overall robustness.

6.4.3.3 Assay support

A final issue when considering assay selection is the support and back-up provided by the kit supplier. Whether the assay kits are supplied directly by the manufacturer or are supplied by a distributor, good customer support is vital. This support should include both assay support and the provision of any equipment needed to perform the assay.

Assay support should include: full training in the use of the assay including basic troubleshooting, rapid response if problems are encountered later on when the assay is in use, the facilities to refer problem samples back to the manufacturer for full investigation, and regular visits of a representative to monitor assay performance and encourage user feedback on the general performance of the assay.

If the equipment needed to perform the assay is not already available in a laboratory, or if whatever is available is old and unreliable, it is well worth considering getting the supplier to provide all the equipment needed, either as a free loan, or through rental, lease or purchase. This can increase the cost slightly, but can be a very good way of obtaining all the equipment needed and of ensuring that it is maintained and serviced properly and regularly, and that back-up equipment is available in extreme circumstances.

6.4.4 Strategies not involving specific laboratory screening

Although it is not the function of this manual to cover in detail leukodepletion and viral inactivation procedures, it is important that the role of these procedures in providing safe blood is understood.

Filtration of blood, primarily for leukodepletion, is now common. Although this was initiated to prevent adverse reactions due to histocompatibility antigens, it was also realized that the removal of white blood cells could also remove some viruses that were present in these cells. Leukodepletion can be used to reduce any risk of transmission of viruses such as HCMV, *Toxoplasma* and HTLV I/II. Although not appropriate for all patients, selective leukodepletion does have a place as a means of reducing any risk of transfusion transmission.

Viral inactivation procedures are largely confined to the inactivation of plasma pools or the products prepared from them. These procedures are now becoming very important to the commercial fractionators of human plasma and large sums of money are being spent on developing procedures that can inactivate any virus present in a product without affecting the efficacy of the product. Although performed in addition to the screening of blood, it is mainly aimed at those infectious agents potentially present but not normally screened for; these are mainly viruses and include hepatitis A virus and human parvovirus B19, both of which may cause problems to recipients of infected products.

Chapter 7
The performance of screening tests

7.1 Introduction

Although part of a larger screening system, the laboratory screening of blood donations is the main way of specifically identifying infectious or potentially infectious donations. However, it is important that no laboratory screening assay should be considered to be totally infallible. While modern assays are very reliable, their overall effectiveness is dependent upon the actual performance of the assay, variability in operator performance and laboratory conditions or practice. In this chapter assay performance is broadly defined as the reliability and reproducibility of the results obtained, and the chapter will be more concerned with those factors that can have a direct effect on the reliability of the assay results obtained, rather than the inherent performance characteristics of the assays themselves. A key issue here is staff performance; in all areas it is important that checks are made on key operations to ensure that the operation is being performed correctly, no matter how well trained the staff are. A lot of problems encountered when performing assays are simple problems resulting from obvious mistakes or clerical errors rather than more complicated reasons.

7.2 Staff training

Staff training is an important issue in the performance of the assays. Poorly trained staff, even using the best and simplest assay, are likely to produce poor results, results that are unreliable, inconsistent and unreproducible. Even if automated systems are used, the operator can still influence the results if they have been poorly trained and do not know how to run the equipment properly. An important question to ask is: what does staff training actually mean? As far as performing the screening tests is concerned, it is not enough to train staff just to perform an assay. To be able to perform an assay properly, achieving reliable and reproducible results, the training must include a thorough understanding of both the principles of the assay and where problems are most likely to occur. Staff who

understand how and why an assay can go wrong are very unlikely to make the mistakes or take shortcuts likely to adversely affect the reliability of the assay.

Do not just teach staff what to do, also teach them what not to do

The best way to train staff to perform assays is to allow them to run assays in parallel with the routine testing. However, this is expensive and time consuming, but is by far the best way to train them. If this is not possible, the next best way is by assay sharing: performing the assay with, and under the supervision of, another member of staff who has good experience of the assay and is able to teach. Whatever training is given needs to be properly recorded, noting the dates, the aim of the training sessions, precisely what training was done and, if appropriate, a statement of competence in the particular task, including the signatures of both the trainer and trainee. Full training records must be kept and training files gradually built up for all members of staff.

7.3 Samples and sample preparation

7.3.1 General

Sample type and quality can have a significant effect on assay performance. Most commercially available assays clearly define the sample type required to obtain optimal results and also give basic sample quality requirements. Failure to use the correct type of sample or a poor quality sample may significantly affect the quality of results obtained and, importantly, may also invalidate any responsibility that the manufacturer has for the performance of the assay.

7.3.2 Sample type

Serum is the universally accepted preferred sample type for infectious disease serology. Serum contains fewer nonspecific proteins and other soluble high molecular weight compounds than plasma and is therefore a "cleaner" substance to use and potentially likely to give fewer spurious nonspecific results. However, most good quality assays can now use either serum or plasma, the exception being some particle agglutination assays which only work properly with serum; plasma samples nearly always give a high level of false-positive results.

Serum samples must be **fully clotted** before use. Overnight storage at 4 °C is ideal, although if clotted samples need to be tested soon after collection,

incubation at 45 °C for 45–90 minutes followed by centrifugation gives good quality serum.

7.3.3 Sample condition

Any sample used for infectious disease testing, either serum or plasma, must be in good condition to get good results. Samples should be collected into clean, dry, acid washed glass tubes, or new plastic tubes (serum samples); or into plastic or glass tubes containing anticoagulant (plasma samples). Plasma samples must be well mixed as soon as the tube has been filled and capped to ensure that the anticoagulant is distributed through the whole tube and that localized clotting does not occur. The anticoagulant must be the right type and concentration, prepared using good quality chemicals and water, sterile and particle free. Ideally, ready prepared anticoagulated tubes should be purchased, ready-to-use, from a reputable supplier. Plastic tubes should not be re-used.

Collected samples should be stored at 4 °C as soon as possible after collection until testing takes place to ensure that there is no loss of the antibody or antigen being tested for, and to minimize any risks of contamination. All samples should be centrifuged before use, whether manual or automated testing is performed, and in some cases, where appropriate sample identity systems can be maintained, the samples may be separated before use. Samples must be as fresh as possible when tested, but if they need to be kept for extended periods before testing, the serum or plasma should be separated and stored frozen (ideally at −40 °C, although −20 °C is usually suitable for short term storage). Haemolysed or lipaemic samples should not be tested, nor should any other samples which have a strange appearance or where the sample identity is not certain.

7.3.3 Test kits and their storage

To ensure optimal performance, test kits must be transported and stored properly. Most test kits need to be kept at 2 °C to 8 °C, although not all the individual components need be stored at this temperature. Some should be kept at room temperature (e.g. wash buffers) and some kept frozen. It is important that the manufacturer's instructions are read and followed as soon as a delivery arrives. One important question is whether kits need to be shipped at 2 °C to 8 °C. In temperate countries, chilled transportation is generally not necessary. However,

in hot countries, especially where lengthy periods before customs clearance may occur, there is always the risk that kits may be adversely affected by the high temperatures encountered. In the same way, they may also be adversely affected by very cold conditions. It is therefore very important that the complete delivery schedule is known in advance so that the laboratory has a good idea as to whether the kits may have been exposed to extremes of temperature during their transportation before arrival in the laboratory.

Depending upon space, the main stock of kits should be kept in a suitable lockable cold store or refrigerator. Sufficient kits for 1–2 days testing can be kept in the laboratory and replaced as necessary. If more than one batch (manufacturer's lot number) of kits is in storage at the same time, the current batch should be used up first, and the other batch(es) clearly labelled and put to one side.

7.4 Equipment

7.4.1 General

The subject of automation is covered in some detail in Chapter 9; however in this chapter the direct effect that stand-alone equipment can have on assay performance is discussed. Even manually performed assays use some equipment, and generally this equipment is used at critical steps where equipment failure, or more importantly and commonly, poor equipment performance, can adversely affect overall assay performance. Such equipment includes EIA plate washers (critical), incubators and plate readers. With all equipment the aim should always be prevention and not cure. It is always worthwhile spending extra time each day incorporating preventative procedures into the daily start-up and shut-down routines. In this way the risk of breakdown or declining performance is minimized and time is not wasted resolving a problem that has built up unnoticed and uncontrolled over a period of time. The use of equipment is usually associated with the use of EIAs; while this is generally true, particle agglutination assays may also involve the use of incubators and plate readers.

7.4.2 Plate washers

Plate washers are critical to assay performance. Many problems with an assay can be traced back to poor or incorrect washing between assay stages. Most plate

washers work on a relatively simple principle, and comprise wash fluid and waste fluid reservoirs, pressure and vacuum pumps, a dispense/aspirate manifold and a moving plate carrier. The wash fluid is pressurized, and a valve opens and allows the fluid through a manifold and into the microwells or other assay wells as appropriate. The waste fluid is under a vacuum and is aspirated back through the manifold to the waste container. A number of cycles of dispense and aspiration comprise the washing of a plate. At the end of the wash procedure the microwells must be essentially empty of fluid.

There are a number of problems that could occur with a washer, and it is essential to perform a defined start-up procedure on each washer at the start of each day and a shut-down procedure at the end of each day. Such a procedure includes, at the start of each day, washing any overnight cleaning solution out of the fluid lines, checking that all the dispense and aspirate nozzles on the manifold dispense and aspirate fluid cleanly and evenly and none are blocked, that the plate carrier moves smoothly, and that there is no fluid left in the wells after running a wash cycle. At the end of each day the washer should be shut down by flushing through with distilled water, flushing with a solution of deproteinizing agent (Decon 90 is a very effective agent) and leaving the manifold and fluid lines soaking in the deproteinizing agent (cleaning solution) overnight.

7.4.3 Incubators

Although incubators are rather simple pieces of equipment, they can still affect the quality of the assay results. The incubation temperatures stated by the assay manufacturers must be adhered to in order to perform the assay properly, and any incubators set accordingly. However, there is usually some flexibility in the temperature needed, with an acceptable range being given in most cases. Incubator temperatures should be monitored regularly using calibrated temperature monitoring devices, and the effect of frequently opening and shutting the incubator door determined. For smaller incubators, where opening the door may have a significant effect on the chamber temperature, alloy plate blocks, which are designed for standard EIA plates to sit on, may be useful. These blocks, absorb the heat in the incubator and ensure that the heat is distributed evenly across the bottom of every microwell and, importantly, minimize any localized temperature changes within the incubator, caused for example by opening the door, so maintaining an even temperature throughout the incubation period. Such blocks can be purchased through assay suppliers.

7.4.4 Plate readers

While and all particle agglutination assays can be read by eye, most EIAs cannot, and it is clearly better to automate reading of any assay results if at all possible. Most plate readers need very little specific maintenance, apart from being kept clean and in as dust-free an environment as possible to minimize dust build-up on the optics and filters. However, depending upon the particular reader and the set-up of the optics, problems can occur, with low OD values being obtained, for example, on just one row of wells. Such problems are sometimes very obvious, but sometimes not, especially if there is a problem with just one filter and that filter is either not used frequently or is used in dual wavelength measurements. A simple check that can be performed regularly is to use a standard coloured plate for which there is an expected OD range; if the OD values across the plate are not within that range the reader should be checked. Such a plate can be purchased from the plate reader supplier or made by carefully fixing a sheet of good quality coloured acetate or acrylic sheet onto a standard empty microplate frame. The colour chosen should reflect the filter wavelength(s) most commonly used.

7.5 Sample addition

The first stage of most assays is the addition of sample. Sometimes the sample is diluted with a sample diluent, and sometimes the sample is used undiluted. There are a number of specific aspects of sample addition: sample identity and plate position, volume, volume of any sample diluent, and confirmation of sample addition.

The sample identity and plate position are important issues which can either be handled by using automated sample handling, or, if manual handling is used, can be handled by the use of a master sample plate, which is the same format as the assay plates and contains sufficient volume of each the samples and in the same position as intended in each assay plate. The advantage of such a master plate is that pipetting of serum (or plasma) from the original sample tubes is performed only once, and thus all the checks required must be performed only once. A master worksheet can be prepared, listing all samples pipetted and their positions in the master plate. Subsequently, direct transfer from master plate to each assay plate can be performed very simply and quickly.

Sample volume is clearly important, although most manufacturers allow at least 10% variation on their stated required volume. Accuracy of sample volume can be achieved by regular calibration and validation of automated sample handlers and manual pipettes. It is important that manual pipettes are used with the correct disposable tips and that these tips are only used once and then discarded. Although the tips can be washed and reused, their accuracy and precision would no longer be assured. The accuracy of the sample volume added is also affected by whether any sample diluent is required. Generally, sample addition, especially by automated systems, is more precise if sample, especially small volumes, is dispensed into liquid (sample diluent). However, if diluent is required, it is then vital that the sample and diluent are well mixed. A simple way to achieve a well mixed sample when sample diluent is required is to add the diluent in two stages; add half of the required volume of diluent, add the sample and mix and then add the remaining diluent and mix well.

Over the last few years a number of manufacturers have introduced specific sample addition check systems (see Chapter 11). These systems demonstrate a specific colour change when sample is added to sample diluent. In this way whether or not sample has been added to the microwell can be seen easily, although such a colour change is qualitative rather than quantitative, giving a simple yes or no answer to the question—has any sample been added? Although the use of automation has improved greatly the accuracy and precision of sample addition, even automated systems can and do fail occasionally so that any sample addition check system has equal relevance to automated as well as manual systems.

7.6 Assay washing

There is little doubt that the washing of the microwell, or bead, between assay stages is the most critical factor affecting the specificity and overall performance of any assay. Although actually very simple in concept and operation, the wash procedure is always well defined by the assay manufacturer, and to achieve the expected results the procedure must be followed carefully; poor washing gives poor results. Poor washing, however, is normally due to one of three things, insufficient washing, too much washing or problems with the wash buffer.

Insufficient washing is ineffective washing, the result being an increase in the number of false-positive results seen when using the assay. For whatever

reason, the wash procedure is not being performed properly or the wrong wash procedure is being used. Depending upon the particular assay, the wash may thus leave residual sample or reagent from the previous step. For example, small traces of sample remaining when conjugate is added may, in a truly negative sample, cause conjugate to bind nonspecifically; similarly any conjugate remaining when the substrate solution is added will cause some colour formation, even in a negative sample.

Too much washing may reduce the sensitivity of an assay because of the risk that any truly bound specific antibody or conjugate could be washed free. Normally this would not be expected to have any effect on the assay results, but in the case of a very weakly positive sample, for example a seroconversion sample, the loss of just a little bound specific antibody may be sufficient to reduce a weak final assay result to a negative result. In addition, if the wash procedure leaves too much wash fluid in the well, the effect may be the same; to dilute the reagents added and potentially reduce the sensitivity.

Finally, the wash solution itself is important. The composition of wash solutions varies tremendously from plain distilled or deionized water to complicated pH critical solutions. In virtually all assays using a specific wash solution, the solution is supplied as a concentrate which needs to be diluted with distilled or deionized water. There are three critical points here: the use of good quality distilled or deionized water, the correct dilution of the concentrate, and the correct storage of the diluted solution (time and temperature). Any problems due to one or more of these three may cause problems with assay performance because the wash solution is not as required for the assay. With the preparation of any assay component, wash solution or reagent, checking of the procedure by a second member of staff can be a significant means of prevention of such problems. Once prepared, the diluted wash buffer should be labelled with its identity, its expiry date and the temperature it should be stored at.

7.7 Reagent preparation and addition

The amount of reagent preparation needed varies a great deal between assays. Some assays have some ready-to-use reagents which are just added to the assay at the appropriate times, while others may have concentrated reagents that need diluting or two or three part reagents that need mixing. However, most EIAs have substrate solutions which require some preparation.

The preparation of reagents, as for wash solutions, usually needs to be checked at all stages and records kept. All reagents should be prepared in preferably new disposable containers, although if these are not available, clean glassware may be used. Glassware should be washed thoroughly using a good laboratory detergent, acid washed, rinsed in good quality distilled or deionized water and then dried. If possible it should be autoclaved before use and then stored in clean conditions. All pipetting must be performed using calibrated pipettes with new disposable tips. As well as checking the actual preparation, the volume of prepared reagent needs to be considered, especially when only part of a test kit is being used, to ensure that a reagent is not used up before the test kit itself is used up. Some of the reagents used are provided only in quantities sufficient for the number of tests in the kit; preparing excess reagent is wasteful and may result in fewer tests being performed with a test kit than expected. All prepared reagents must be clearly labelled with the contents, date prepared and, if appropriate, the expiry date. After addition any prepared reagent left, unless suitable for long-term storage, should be discarded.

Addition of reagents should again be performed carefully and checked. Generally all reagents should be added in the same way and added to the tests in the same order as the samples were initially added.

7.8 Reading of final assay results

The final assay results may either be determined by eye (eye-read) or by machine (machine-read).

If the results are to be eye-read, as in the case of rapid assays, particle agglutination assays and one or two EIAs that allow eye-reading, a system must be put in place to check that the results are read correctly and that the correct results are recorded, and against the correct sample identification. Clearly this needs two people to ensure mistakes are not made; one person to read and the other to score. They should then exchange roles and recheck the results.

If the results are to be machine-read, although the results themselves do not need to be rechecked in the same way, the identity of the plate needs to be checked, and the reader set-up needs to be checked at some stage. The plate identity must be checked before reading, and the identity clearly marked and checked on the printout obtained; if a software package is being used some of these checks may not be so critical as they may be handled within the software.

The correct filter(s) must be used otherwise the OD values will not be correct and the plate may fail validity checks, and/or positive results may not give the OD values expected and could appear as negative.

After the tests have been read and the results have been recorded or printed automatically, the plate or tests should be put to one side and the results checked before they are discarded. These checks should include a check to ensure that any test that is clearly positive has been read correctly and has given an appropriate OD value, and a check to ensure that the OD values across the plate or run are consistent and that there are no obvious trends or drifting of the OD values across the plate or run.

Chapter 8
Data handling

8.1 Introduction

The initial raw results of most EIAs, and of particle agglutination assays read on plate readers, are simply numbers—optical density (OD) values obtained from the plate reader. Once these numbers have been obtained, three key steps then have to be followed:

1. The validity of the particular assay run has to be determined;
2. The actual individual sample results have to be determined; and
3. These individual results have to be linked to the individual sample identifications.

The automated linking of result to sample identification is covered in Chapter 9; this chapter will thus only include a small section on the manual linking of result and sample identification. Whether the data handling is automated, as in most developed countries using commercial data reduction software, or calculated manually, the same basic calculations and criteria are used to determine assay validity and sample results.

8.2 Assessment of plate validity

The first step in data handling is to determine the validity of the results, whether a single microplate, or batch or run of bead tests. All assays include control sera which the manufacturers supply and for which they usually provide acceptable OD values and/or ranges, usually positive and negative sera, although some assays may include specific weakly positive sera. In addition, in most cases these control sera are used to calculate the assay cut-off value; that is, that OD value which is used to determine whether the individual sample results are positive or negative.

Most assays specify precisely how many of each of the individual controls need to be set up with each plate or run. The individual control OD values and the

mean values of each set of controls are then used as part of the assessment of run validity, although these are essentially just basic run pass/fail criteria. In addition, some assays require a blank well, usually known as a substrate blank, which is used to control the background OD value of the substrate solution itself. The blank OD value is then subtracted from the test and control OD values before any further calculations are performed. The actual value of using a substrate blank with most modern assays is unclear, as all the subtractions and calculations are relative, with a constant value—the blank OD value—being subtracted from all the test and control OD values. However, a number of commercial assays still incorporate the use of a substrate blank.

Once the OD values have been obtained, the control sample OD values can be examined and the manufacturer's calculations performed. The acceptable ranges given by manufacturers are usually based upon minimum values for the individual and mean OD values of the positive controls, maximum values for the individual and mean OD values of the negative controls, and a minimum difference between the mean positive and negative control OD values. Also, where two or more replicates of a control are used, the elimination of one abnormal OD value (significantly higher or lower than the other control values) is normally allowed. Although these criteria are very broad, they can provide a framework upon which more precise criteria can be applied. For each control type a very strict OD range can be defined and applied which should not be affected by kit batch. Such a range can easily be defined by simply looking back over previous run results and calculating the normal range of OD values within which the particular control sample falls. Over a period of time, as assay performance gradually improves, the width of such a range can usually be decreased gradually. If the manufacturer's criteria are met, and any other validation criteria applied are met, the results of the plate or batch of tests can be considered to be valid.

While the manufacturer's controls are the main criteria for plate validity, these are not always sufficient and often it is beneficial and scientifically sound to use additional, ideally external, criteria. Such additional criteria can include: external quality control or control samples, the OD values of the negative test samples or the number of tests requiring repeat testing; any number of additional criteria may be applied according to the particular laboratory's policy. External quality control or control samples will be discussed in more detail in Chapter 11, but will be covered briefly here. If good quality external quality control or control samples are available, they can be incorporated into the routine testing procedures and possibly used as part of the test run validation procedure; for example, the

sample(s) must be positive or reactive at a certain level for the run to be valid. For this to work properly the external samples must be good quality, stable and available in large quantities.

The negative test results can themselves be used to assess test run validity, but to do this data must be accumulated from previous test runs to be sure that any parameters set are valid and reliable and actually work. A simple validation tool, which depends upon the prevalence of the particular marker in the donor population, is to set a maximum acceptable number of initially positive repeat samples per plate or run. If a plate/run has more than this number, the plate/run has failed and all the tests are repeated. Additionally, the OD values of the negative test samples can be used, an expected range defined for these values and the mean OD value of all the negative samples in each plate/run calculated and checked to ensure that each lies within the expected range. This sort of calculation can help to achieve consistency between testing runs but really needs a suitable computer and software to simplify the calculations.

8.3 Cut-off values

The cut-off value is that OD value which is used to determine whether a particular result (the sample OD value) is to be interpreted as positive or negative. Probably the majority of commercial assays use the negative control OD values to determine this value, although some use specific weakly positive cut-off control OD values, some use the positive control OD values, and a few may even use combinations of the positive and negative control OD values. The actual calculation and setting of the cut-off value of any assay is based upon the assay's reactivity with panels of weak and seroconversion samples. The cut-off has to be set to ensure that the weakest possible truly positive sample is detected and interpreted as positive, while false-positive reactions are not interpreted as positive. This level of discrimination is very hard to achieve and it is accepted that, in blood screening, sensitivity is paramount and a slight loss of specificity is thus acceptable.

An important issue concerning the calculation and use of cut-off values is the use of a "grey zone" or "query zone" around the cut-off value. The problem with a single cut-off value is to decide whether, for example, a sample that has an OD value that is below the cut-off, but clearly raised above the OD values of the rest of the negative samples, is just a negative sample with higher than usual

background reactivity in the assay or a very weak but truly positive sample. Thus the use of such a zone can serve to widen the range of samples which could be interpreted as neither negative or positive, but are equivocal and need to be retested. Most manufacturers who recommend the use of a "grey zone" state that any sample giving an OD value within 10% of the cut-off value should be repeated.

There are other ways of using the principles of grey zones, but these depend upon local circumstances and practices. One effective way is similar to that described in the assessment of plate validity above, and is to define an acceptable OD range for the negative sample OD values to fall within. Any sample OD values that fall outside this range, above or below, are considered to give initially unclear results, and the test needs to be repeated.

8.4 Determination of sample results

Once the plate has been validated and the cut-off value, or other appropriate criteria calculated, the OD values can be translated into the final results. This is very straightforward and, depending upon the assay, all samples giving OD values below the cut-off, or below the cut-off grey zone, or within the acceptable sample OD range, whatever criteria are being used, would be considered to be negative for the marker being screened for. All samples giving OD values either above the cut-off, within the cut-off grey zone, or outside the acceptable sample OD range would be considered to be initially reactive or initially unclear. In blood screening all such samples would normally be repeated, usually in duplicate, to confirm the initial reactivity, before any donation was considered unsuitable and discarded. In clinical laboratories, however, this may not be the case and the initial screening results could be considered to be final.

8.5 Linkage of result to sample identification

Once the sample results have been determined, they can be linked back to the appropriate sample identification. With a manual system this has to be done by simply comparing lists—sample plate/run position results against sample identification plate/run position details—and writing in the result against the sample identification. With a computerized automated system this is performed automatically with little or no manual intervention. However, not all laboratories

have such systems and there is always a significant risk that errors may occur when linking final result to sample identification. It is important to remember here that most fatal errors in blood transfusion practice are due to clerical errors and so all manual recording and transcription should be performed very carefully and with the appropriate checks at all stages.

Chapter 9
Automation

9.1 Introduction

The introduction of automation to transfusion screening has improved significantly the quality of the screening of donations. Automation is not, however, without its problems. The more complex nature of the assay procedures and the need to integrate very large amounts of data have meant a radical change in approach by the manufacturers of these automated systems.

In recent years many changes have occurred in the field of transfusion microbiology. The number of infectious agents screened for has increased, the complexity and sensitivity of tests have increased and the need for a more controlled and quality oriented approach to transfusion microbiology has been emphasized. For many years most of the work performed in transfusion microbiology departments was essentially manual: manual pipetting of samples and reagents, manual recording and monitoring of incubation times and temperatures, manual entry of donation numbers onto worksheets or into computer systems, in some cases manual reading of assay results, and manual calculation of plate control and cut-off values. The only automated procedures involved plate/bead washing with stand-alone assay washers and the reading of the final OD values using stand-alone readers. Although there is no doubt that most laboratories strive to maintain reasonable levels of quality and security in the performance of assays and collation of results, it is clear that the potential for human error in such systems is great.

To reduce the risk of such errors, the use of automated systems is now becoming common in developed countries, where the supply of blood is generally sufficient, the pressures on the transfusion services are more regulatory and legislative, and formal comprehensive quality systems are in place. Such systems comprise automated sample handling, assay processing and data handling, and can ensure security, consistency and reproducibility in the performance of the assays and results obtained. These systems are most suitable for use with enzyme immunoassays, and consequently are primarily designed for them. However,

although they are inappropriate for the processing of rapid or simple assays, they do have some use with haemagglutination and particle agglutination assays. Modern plate readers can be used to scan and interpret the agglutination patterns and store the results, so removing the reliance upon eye-reading, which is subjective and does not provide archivable data. At present most of the systems used comprise separate but linked computer-controlled sample handling devices and assay processors, together with specialist dedicated software, both for sample handling and result handling. Some people use the term "front end" automation to refer to sample handlers and "back end" automation to refer to assay processors. This does however mean that some manual intervention is required, most often in the manual transfer of assay plates from the sample handler to the assay processor. The sample handling systems are generally very flexible, being able, for example, to manipulate and dispense a single sample into a number of different assays, while the assay processors can process a large number of samples at the same time under strictly controlled and reproducible conditions. Although it is clear that small transfusion centres may have neither the funding or the throughput to justify such automation, there are increasing numbers of smaller integral dedicated processors, often designed using experience gained from the larger equipment, that can only process small numbers of samples but that combine sample handling, assay processing and result data handling in a single piece of equipment.

Thus, although automation may seem to many to be a distant event, some transfusion laboratories and services in developing countries already use dedicated assay washers and readers, and in some cases have access to smaller integrated automated systems. The specific advantages of these systems for blood screening are gradually being recognized.

Currently there are two basic forms of automatic immunoassay available for blood screening: those performed in a microplate and those performed using a bead. Single use rapid tests are not included here because, as previously stated, the use of automation for them is largely inappropriate. The microplate-based tests include both EIAs and particle agglutination assays, which are available from a range of manufacturers globally; the bead tests mentioned refer to the EIAs produced mainly by Abbott Diagnostics, which use a polystyrene bead as the solid phase. Most of this chapter on automation applies equally to both microplate-based and bead-based assays; although Abbott Diagnostics produce their own range of equipment, which is based upon the same principles as the microplate handling equipment but is specifically designed to work with their bead assays.

9.2 Positive sample identification

An important concept when looking at the whole automated system is that of positive sample identification. The concept of positive sample identification is that of ensuring a totally reliable and precise computerized data linkage between sample identification and final assay result(s). It is this feature that is at the heart of all good automated systems. This concept requires a defined computerized data link throughout the whole assay procedure: assay plate identification-linked with sample identification and plate position; recording of individual plate processing events linked to plate identification; final result reading linked through plate identification back to sample identification; and result interpretation linked to sample identification. In this way it can be ensured that the correct final assay result is applied to the correct initial sample identification.

One of the key elements needed to build a system of positive sample identification is the use of bar-codes. The use of bar-coded sample numbers and assay plate numbers, together with equipment capable of reading and storing those numbers, is essential. Nowadays many consumer goods have bar-codes on them and their use is spreading throughout world. The advantages of using bar-codes are numerous, but one of the main ones is that of reducing clerical transcription errors (still the biggest cause of adverse transfusion reactions globally). Being able to automate sample reading and logging the positions of samples in assay plates is a major step forward in assuring quality and improving the safety of the blood supply. There are many international guidelines and standards now available for use in blood transfusion services. Two important principles are to use a sample identification bar-code with a check digit encoded into it and to always have the coded number present in an eye-readable format. Clearly, a computer system is required, together with bar-code readers; however, such items are becoming less expensive, and many diagnostic assay suppliers are in a position to supply suitable systems for use with the kits they supply.

A further and more recent development is the concept of total process control. This seeks to extend data tracking to all aspects of the test process for each sample: the incubation temperatures and times, wash parameters and reagent addition checks after each addition stage. Such a system can provide a complete data profile for each sample tested, increasing the security of the testing system and again the confidence in the results obtained. Quite whether this level of control is either really necessary or justified is not yet clear. What is clear,

however, is that regulatory authorities see such a system as the way forward, whether or not it will actually increase blood safety.

9.3 Sample handling

The first critical area in laboratory automation is the handling of the samples. Correctly identifying the individual samples and controls, and adding the correct volumes to the correct wells in the correct assay plate is a key requirement to ensure the accuracy of all test results and to ensure positive sample identification.

Although sample handling devices have been available for over 20 years, the early instruments were very limited in their application and effectiveness. They were relatively inflexible items, often designed for a specific test only, and essentially only capable of pipetting simply from sample to reaction vessel without any sample identification. Often the samples were placed in a fixed carousel or other holder and only relatively small numbers could be handled at one time. The appearance of dedicated robotic sample handling devices, designed to be used with virtually any type of assay system, has opened the way for more effective sample pipetting. It offers greater flexibility in the type of pipetting actions performed, the ability to positively identify the sample being pipetted and the plate (or other sample receptacle) into which it is being pipetted, improved accuracy in the volumes delivered, and performance at an appropriate speed. These robotic devices are also now produced by companies who specialize in this area and not, as was the case previously, by diagnostic companies who saw them simply as a means by which their own assays could be performed. The currently available dedicated robotic sample handling devices, such as those produced by Tecan, Hamilton, Hewlett Packard, and Kemble, are all controlled by microprocessor and are now capable of flexible pipetting from sample tubes (and control vials), identified by bar-codes, to a number of individual assay plates, each requiring different sample volumes. Plate maps detailing sample identification numbers and plate positions for each plate can be produced and presented as data files directly transferable between laboratory computers. A high degree of complexity in pipetting patterns is now achievable together with the ability to pipette accurately sample volumes from 1 ml to as low as 10 µl, and in a remarkably short period of time.

The advent of fully automated sample handling has significantly reduced the number of errors that previously occurred regularly when manual sample

pipetting was performed routinely. Over the years many cases have been cited of posttransfusion infection where failure to detect an infectious donation was due to incorrect sampling rather than the inability of the test used to detect the infected donation. In addition, clerical errors, when recording the identity and position of the samples tested, has also similarly contributed to instances of posttransfusion infection.

More recently, the development and inclusion of sample addition monitors in some diagnostic assays has added a further level of security and confidence that correct sample transfer has taken place. Sample addition monitoring is a recent innovation to mass microbiology screening and provides a means by which a positive indication of the addition of sample to the assay well is produced (see also Chapter 11). Two systems are currently in use, chemical and electronic. Chemical systems use a dye system incorporated into the assay sample diluent, in which the presence of sample causes the dye to change colour. This colour change is generally a qualitative change (although some claims for semiquantitative changes have been made) which can then be seen by eye and/or monitored automatically. Electronic systems are an integral part of the sample handling device and sense sample addition in a number of ways, including conductance and pressure changes within the fluid system of the sample handler. Again, as in the chemical system, predetermined changes in the measured parameters are expected, and any failures are indicated automatically. The chemical systems have the advantage of simplifying the equipment, but have the disadvantage of currently being limited to certain tests and manufacturers. Both systems subsequently flag for error any well in which sample does not appear to have been added. Such sample monitoring systems are important additions to the total automated packages available because incidents are now being reported in which automated sample handlers have appeared to fail to dispense sample into the assay well. Unfortunately, even though they have provided a significant improvement in quality terms, robotic sample handlers are not infallible and can still make errors.

9.4 Assay processing

The majority of assays currently performed in transfusion microbiology laboratories, at least in developed countries and certainly for the mandatory testing of blood donations, are EIAs. Such assays are generally of good quality and well

standardized, but are still relatively complex procedures with a number of stages and specific actions required at each stage (see Chapter 5).

Following sample addition, the assay requires processing through the required stages to produce the final set of results for those samples. Typically these stages will comprise incubation, washing and reagent addition, all of which need to be performed in a specific and standardized manner. Although it is true to say that manual assay processing does not have the same potential for errors as manual sample addition, errors can occur and, importantly, variability can arise in the processing of different batches of tests, resulting in significant and unacceptable variability in the final results obtained. One of the main advantages of automated assay processing is the standardization of assay performance so that any variation between testing batches is reduced to a minimum. Such variation during manual assay processing may result, for example, when incubation times are altered slightly by the operator to fit in with other plates or assays being performed, or when consecutive reagent additions are performed in slightly different ways or even when a different operator performs some of the assay steps. All these factors can introduce subtle changes during assay performance that can result in significant differences between individual assay plates processed during a single testing period.

The automated assay processors currently available offer a variety of capabilities depending upon the numbers of samples to be tested and the level of complexity required. They all comprise incubator(s), a wash device, reagent addition device(s) and a plate reading device, although the precise formats and designs obviously vary. These processors are all capable of virtually "hands-free" processing of assay plates, requiring only the addition of the appropriate reagents by the operator. Once a plate has been loaded the processor tracks each plate knowing precisely which stage each plate has reached, what reagent to add and how much, how long to incubate the plate and at what temperature, how many washes the plate needs at each stage, and finally when to read the final OD values and at what wavelengths. There are differences in some additional features offered, such as the continual active monitoring of incubation temperature and additional active checks on the identity and volume of each reagent added, although such facilities are now becoming standard features on more recent systems. In a similar manner to the use of sample addition monitoring during initial sample handling, some assays now use coloured reagents that enable the systems to monitor their own addition of reagents to the assay wells at every stage of the assay (see Chapter 11). Again, after each reagent is added OD values are

obtained and compared to an expected range for that particular reagent. If any well gives an OD value outside of the range an error flag is highlighted on the system. Such systems have the advantage of taking some of the monitoring burden away from the machine itself, thus simplifying to some extent the way it works. However, not all reagent manufacturers currently have this facility available.

As previously mentioned, increased regulation in many countries is starting to pressurize laboratories to monitor all stages of assay performance as closely as possible and retain all the data generated. This is put together with sample addition data to build a complete record of the conditions maintained throughout the whole of the assay procedure. In the past the real value of this total process control has been very hard to determine. Although it was considered important to be able to demonstrate that every assay had been performed correctly, the final test has always been the quality of the actual results obtained. If these conformed to the required standards laid down by both the manufacturer and the user, they were considered acceptable and valid and used appropriately. However as operating requirements become ever more stringent, some means to prove that all the steps of an assay have been performed correctly is needed; this checking can also provide the means to check for gradual assay drift. It is possible to perform such checks in a manual system but this is usually very time consuming and labour intensive, while most automated systems would be able to provide such data as part of their normal process tracking systems. A key feature in the total quality approach is thus the ability to link all the data from the sample handling device with the data from the assay processor. These data are then linked to the data reduction software used to obtain and interpret the final assay results. This maintains the link from start to finish of the procedure so again helping to maintain the concept of positive sample identification. Total process control then adds a new level of security ensuring that not only does the right data go to the right sample but that the data are valid and has a relevance to the process.

9.5 Assay result reading and interpretation

Once an assay has been completed, the OD values need to be read at the appropriate wavelength and the raw results require interpretation and subsequent linking with the individual sample identifications to produce a final output of sample identification and assay result. Most assay processors include a plate reader that performs this function. The final assay result output is then usually handled automatically by specific data reduction software which is capable of

converting the raw assay data, provided by the plate reader as numerical optical density values, into result interpretations—positive, negative or query, depending upon local procedures. There are many commercial software packages available that will perform this function, although their complexity and hence additional features vary tremendously. This software can be used not only to calculate basic assay parameters, but also to perform more complex procedures, such as storing individual sample history data, providing statistical and graphical analysis of data, and packaging and transfering data to major computer systems.

The automated calculation of basic individual plate parameters, such as cut-off values and mean control values, is essential to reduce the errors that are likely to occur when manual calculations have to be performed. Once these calculations have been made for any plate or batch of tests, the validity of the test run can be determined and the results accepted or rejected. The results from a valid run can then be interpreted, and linked to the original sample identification. The data can then be directly transferred to a mainframe computer system or other destination as required. The automation of these steps is another vital area where in the past errors have occurred; for example the incorrect identification and retrieval of a positive donation because of a failure to link the sample identification with the correct assay result. Ultimately such systems are used for computer-controlled donation-status assessment and release of only donations that have been found to be suitable for issue after microbiology testing, blood grouping and any other screening that is required prior to release for clinical use.

9.6 Selection of automated equipment and systems

Any laboratory that is faced with the task of selecting a suitable automated or semiautomated system needs to consider its options very carefully before selecting such a system. A number of basic questions need to be answered before any conclusions can be arrived at.

Why is automation required? What is the reason for automating a process? and What is this going to achieve for the laboratory? are the first two questions to ask. Apart from the obvious reasons of quality there are other reasons for introducing automation; staff shortages may be relieved, variation in assay performance may be reduced significantly, and wastage due to poor assay performance may be reduced. The overall efficiency of a laboratory may be increased by the careful introduction of automation. However, in smaller laboratories, possibly testing

only a few donations per day, automation may not be appropriate; indeed if rapid/ simple tests are used there is very little automation available, whereas if particle tests are used, automation would at least standardize the reading of the agglutination patterns and facilitate storage of the raw data.

What automation is required? Is a fully automated system needed or a semiautomated system? The choice system may depend upon other factors such as cost, reasons for automation and the number of samples tested. There are no fixed definitions of semiautomated systems and such systems may be whatever combinations of equipment are appropriate for the laboratory. For example, appropriate automation for particle agglutination assays may simply involve the use of an automated reader linked to a microcomputer.

What assay format is to be used? If the intention is to use the same assay and simply automate it, the equipment needed must be compatible with that particular assay format. If a totally new package is being considered the choice between bead-based and microplate-based EIAs must be made, each having its own specific advantages.

What systems are available? Although there are numerous systems available, not all systems are available in all parts of the world. In some areas choice of system may be limited to what is available locally. However, an attractive option may be to approach the assay suppliers to see what systems they can provide that can be used to process the particular assays used. Advantages with this approach include the ability to lease the equipment or use a reagent rental deal which provides the equipment needed at a reasonable price and for as long as the assays are purchased from that supplier.

Are appropriate support and service available? This question is linked with the previous question. One major advantage of leasing or renting equipment is that the supplier can be made to maintain the equipment. In this way problems often faced by laboratories which have equipment for which there is no local or even national servicing or spare parts available are overcome. The supplier would be in a position to maintain the equipment and provide local service very quickly.

What are the drawbacks? Although the advantages of automating blood screening are significant and generally far outweigh any drawbacks, there are some issues of which potential users need to be aware. Automation is a big step and one which, if well planned, will quickly show great benefits. At the same time, once that step is taken it is very hard to remove automation from a laboratory.

There is a need to extend the skills of staff to be able to maintain the equipment and possibly an ongoing financial issue if regular supplies of disposable items are needed.

Thus the selection and introduction of any automated system are major steps for most laboratories, one which will inevitably cause some initial disruption, need careful control and staff retraining and change working patterns forever. However, there is no doubt at all that automation is a significant factor in the improvement and maintenance of quality within screening laboratories and is a step that laboratories should consider taking if it is appropriate for that laboratory/ country, if the resources are available or could be made available and if there is suitable equipment available locally or nationally.

9.7 Maintenance of automated systems and equipment

Although most modern equipment is now very reliable, there is clearly a need to maintain any piece of equipment in optimal condition. This obviously applies to any mechanical or electrical item which is used in the laboratory. The increasing use of automated equipment in laboratories has increased the maintenance workload and has meant that laboratory staff have been required to broaden and increase their skills to encompass a full understanding of the equipment they use; the principles of operation, routine use, routine maintenance, troubleshooting, and preventative measures to ensure a consistent high-quality output.

There are a number of specific and critical maintenance issues associated with automated systems, as well as more general issues concerned with the overall general performance of the systems. These general issues are more concerned with ensuring that the equipment does what it is supposed to do, and does it well, and should be reflected in daily maintenance procedures. The most effective maintenance programme is that of continuous preventive maintenance—prevent breakdowns rather than repair broken equipment. Although such a programme may increase slightly the amount of maintenance work performed on a daily basis, a carefully thought out and designed programme can remove the need for regular maintenance shutdown periods and significantly decrease equipment breakdowns.

Probably the most critical specific maintenance aspect is that of the calibration of the equipment. Any equipment that dispenses specified volumes of

liquid needs a regular programme of calibration checks. To ensure accurate dispensing, especially of the very small volumes of sample required by modern assays, calibration must be performed regularly and in a way that accurately reflects the way in which the equipment is actually used. There are a number of ways in which calibration can be performed but probably the best way is gravimetrically, accurately weighing volumes of liquid dispensed and, by using the specific gravity of the liquid, calculating the actual volume dispensed. However, any calibration procedure must be performed by the same procedure used to dispense the samples or other reagents. The calibration procedure must take into account factors such as whether multiple pipetting is performed, whether there is any liquid already in the well (e.g. sample diluent) and the specific gravity of the liquid dispensed. Other critical issues include calibrating the temperature of any incubator modules and the accuracy of the timing system used to time the incubation periods during assay processing.

In general, to ensure the optimum performance of all equipment at all times a very proactive approach has to be taken. There is no doubt that this requires a great deal of effort to maintain, but, in order to be able to rely upon automation to provide the level of quality and consistency both expected and required, such efforts have to made.

9.8 New developments in automation

Currently, a number of new developments in automation are appearing; these include both new technology and improvements to existing technology.

At present, as discussed in 9.6, the automated systems suitable for the testing of large numbers of donations comprise separate sample handlers and assay processors with the consequent need for manual transfer of plates between the two. New systems are addressing this issue by combining sample handling and assay processing in one piece of equipment without the need for any manual intervention, except for the loading and unloading of samples, and which can process the numbers of samples required by large transfusion services. Such systems are basically either a single enclosed box type of system, such as the Abbott Prism system, where everything is enclosed in a relatively compact space using dedicated new technology, or a robotic arm type of system, such as the Tecan Track system, where a robotic arm is used to integrate existing sample handling and processing systems. Both types of system have their own specific

advantages and disadvantages, but both have the overall advantage of providing a hands-free walk-away automated total system.

Image analysers, capable of more effectively reading red cell or latex agglutination patterns in microwells, are now becoming more readily available, so making it possible to incorporate haemagglutination and particle agglutination assays into automated systems previously dedicated only to EIAs. Although to some this may appear to be more of a sideways step, the value of agglutination assays has been proved many times over and they have a very important role in blood screening, especially in developing countries where the number of samples tested does not warrant using EIAs or where the cost of using EIAs is prohibitive, but where the security and level of quality afforded by an automated EIA is needed.

Chapter 10
Confirmatory testing

10.1 Introduction

The performance, role and uses of confirmatory testing vary tremendously from country to country and from service to service, and depend on the resources available and local and national policies and strategies. The purpose of confirmatory testing in blood screening, however, is constant: to determine the true status of the donor for the marker of infection being screened for. As previously discussed, after the patient the second responsibility of all transfusion services is to the donor. If the results of screening tests suggest that a donor is infected, it is the responsibility of the blood transfusion service to determine whether or not the donor is truly infected. This area is the interface between the blood transfusion service and public health and is generally not a clear area. However, it is clear that in many cases infected individuals would probably not have been identified if they had not given blood. How relevant or significant this is is uncertain, and it depends upon the prevalence of the agent in the population and the health care facilities available.

10.2 Reference laboratories

Normally, and preferably, confirmatory testing are performed in a specialized reference laboratory. In many countries however, there may only be one or two such laboratories, and these are generally the main public health laboratories where expertise is concentrated and the necessary resources are available. These resources include a variety of test kits, equipment, advanced techniques, effective record systems and the finance needed. In some cases it is possible to include a reference laboratory within the blood transfusion service or even individual centres; however this is only possible if all the resources needed are available, including the expertise, and if the reference work can be completely separated from the routine screening.

10.3 Initial screening results

All samples which react positively on screening should be referred for confirmation. Normally the initially positive samples should be retested at the original centre, ideally in duplicate, so that the initial reactivity can be confirmed or dismissed. National policy and strategy usually require discarding of the blood units donated once blood screening has shown a positive result. The donor, however, should be retained as a donor if confirmatory tests prove the unit is in fact negative.

10.4 Samples for confirmatory testing

A critical factor for any reference laboratory, as for primary screening, is the quality of the samples it receives. All initial confirmatory testing should ideally be performed on the original sample giving the repeatable reactivity. Unfortunately that sample, when received by the reference laboratory, is usually a lot older than when used for primary screening. In addition, the sample has probably sat for a long time in nonoptimal conditions—at the BTS and then during transport to the reference laboratory. There are two problems that this may give rise to: first, the degradation of weak specific antibodies which may have been detected by primary screening and be the reason for referral, and second, the microbial contamination of the sample. Samples referred for an antigen investigation, for example for HBsAg, are not quite so susceptible to poor storage as the antigen is not so degradable; however, microbial contamination can have adverse effects.

Some antibodies are more unstable than others and degrade rapidly in a blood sample. This degradation can be even quicker when the sample is stored under nonoptimal conditions (such as a high or widely fluctuating temperature), has been frequently frozen and thawed, or has been kept as an unseparated sample, i.e. the serum or plasma has not been separated from the clot or red cells. A weak antibody (weak because it was initially present at a low level) may therefore degrade to such an extent that it becomes completely undetectable, or its reactivity in an assay drops to below the cut-off value. Similarly, microbial contamination of a sample may also result in a false result due either to enhanced degradation of any specific antibody caused by microbial by-products, or to the masking of specific antibody by the bacteria or their by-products.

A further issue concerning sample quality is the identity of the sample. It is important, first, to ensure that the correct sample for referral has been identified and, second, to ensure that when that sample is separated prior to despatch, the separated serum or plasma is labelled properly with the correct sample identity.

10.5 Confirmatory algorithms

The confirmatory algorithms used need to be appropriate for the confirmation of reactivity in blood donors and appropriate for the particular marker of infection being screened for. It must also be remembered that the confirmation of clinical results and blood donor screening results follow very different routes.

There are many algorithms that can be used to confirm reactivity, all of which must take into account the primary screening results and assay used. In this chapter the principles behind confirmatory algorithms will be considered together with basic algorithms to confirm infections with HBV, HCV and HIV. Appropriate confirmatory algorithms are based upon a number of specific options:

- the use of different screening assays, i.e. based upon different principles and/ or antigens
- specific neutralization
- the testing of the sample for additional markers of infection
- the use of specialized serological assays, e.g. Western blot, line assays
- the use of molecular techniques to detect nucleic acids.

a) Use of different screening assays

The most common approach to confirmation taken by reference laboratories is to test referred samples with different assays, usually based either on different principles or using different antigens (or antibodies for an antigen assay) and generally from different manufacturers. These assays need to have a sensitivity at least equal to that of the original screening assay used, and a specificity similar to that of the original assay, although a slightly poorer specificity may be acceptable depending upon the particular infectious agent and the number of different screening assays available. Where reactivity is still seen with the different assays, it is highly likely that the sample is truly positive and the donor is infected. Depending upon policies and resources available, additional testing may then be performed using more specialized assays or for additional markers of infection.

Where reactivity is not seen with different assays, or reactivity is seen with some but not all assays, it can usually reasonably be concluded that the donor is not infected, and the original positive screening result, although repeatable, is nonspecific—a biological false-positive.

b) Specific neutralization

The classic confirmatory assay is neutralization of antigen by specific antibody, for example neutralization of HBsAg by anti-HBs or of HIV p24 antigen by anti-HIV. The principle of neutralization is that with a true-positive sample, the positive assay result can be prevented by the neutralization of the antigen in the sample by specific antibody added to the sample. If an HBsAg-positive sample is tested using an HBsAg screening assay, a positive result is obtained. If the same sample has high-titre anti-HBs added to it and is incubated for a short time and then retested using the same HBsAg assay, the assay result will now be negative. This reaction pattern is conclusive of the presence of HBsAg, and the same principle can be applied to any antigen detection assay.

c) Testing of the sample for additional markers of infection

In some confirmatory investigations there is the ability to test samples for additional markers to confirm infection. The most obvious example here is HBV infection and HBsAg screening (see Chapter 3). Although a number of other markers are present, the marker most commonly used in addition to HBsAg is anti-HBc (total IgG + IgM), and reference laboratories may often include an anti-HBc assay in their initial investigation of any referred HBsAg-positive samples. A positive anti-HBc result with a positive HBsAg result is a good indication that the donor is truly infected with HBV. Other HBV markers, such as HBeAg/anti-HBe and anti-HBs, can then be performed to build up a full serological profile to determine the stage of infection. Depending upon local/national policy, a positive anti-HBc result with a negative HBsAg result may be investigated further to determine whether the donor is an "anti-HBc only" donor (see Chapter 3), chronically infected, or immune, having recovered from acute HBV infection.

d) Use of specialized serological assays

Specialized serological screening assays are generally used as the final confirmatory steps when the original reactivity has been confirmed with different assays (as described in item a) above). These assays generally give more information than a simple "yes" or "no" answer, as they give a picture of the

antibody profile of the samples tested and reactivity against individual epitopes, enabling determination of the true antibody status by examining the antibody profile against that expected for a truly positive sample. The most well known of such assays is the Western blot, which used to be most commonly used to confirm anti-HIV reactivity. The Western blot comprises a strip of (usually) nitrocellulose membrane onto which electrophoretically separated viral proteins are transferred. The proteins are separated on the basis of molecular weight, the smallest proteins moving furthest down the electrophoresis gel, the largest moving the shortest distance. The test sample is incubated with the strip and any specific antibody present binds to the viral proteins immobilized on the strip. The bound antibodies are detected in a similar way to an EIA, except that the colour reaction produces an insoluble coloured compound which is deposited on the nitrocellulose strip at the point where the antibody is bound. The pattern of bands resulting is then examined and the specific viral proteins recognized by the antibody in the sample are identified and recorded.

Other similar assays are now available, for example for confirmation of anti-HCV reactivity by RIBA (recombinant immunoblot assay, Chiron Corporation, USA), in which the viral proteins are not separated by electrophoresis, but are individually purified and then each applied to nitrocellulose strips at discrete and specific positions on the strip. Samples are incubated with the strips and any specific antibodies binding are detected as described above. Again, a specific pattern of reactive bands is looked for to confirm the presence of specific antibody.

Unfortunately, even these assays have their problems. Sometimes the banding patterns obtained do not fit with any expected specific patterns and a conclusive result cannot be obtained; the results are termed indeterminate. Many such samples are most likely to be demonstrating nonspecific reactivity rather than unusual but true reactivity, and additional testing is needed to obtain a conclusive result.

e) Use of molecular techniques

Increasingly, molecular techniques are being used to provide additional information to aid interpretation of confirmatory results and to follow up cases of posttransfusion infections. Although of value, there are two important factors which limit their usefulness: 1) the techniques are not yet available in a format which is appropriate for the routine screening of blood donations, and 2) in many

instances the information obtained is only relevant to the infectious state of the donor and does not help in the identification of infectious donations. The main value of molecular techniques over serological techniques is in the case of recent acute infections when a donor may donate in the window period of an infection (see Chapter 3). If, as in the cases of HCV and HIV, viral nucleic acid can be detected in the bloodstream before any serological markers, there is clearly a theoretical value in using a molecular technique to detect the nucleic acid. However, the problem then arises as to whether the molecular technique can replace the serological technique, or be used in addition to the serological technique. In most cases the molecular technique cannot replace the serological technique because the serological markers, after they have appeared in the bloodstream, persist, often for a lifetime, virtually permanently identifying a potentially infectious individual. Nucleic acid on the other hand will usually disappear very quickly after serological markers have appeared, and an infected individual, cannot then be detected. The value of using both molecular and serological techniques also has to be questioned because, depending upon the prevalence and infection rate of any infectious agent in the donor population, very few individuals would be detectable only by a molecular technique; most would be detected by serology only, with just a small number being detected by both. However, as stated at the beginning of this section, molecular techniques do have value when used in the right situation and are helping transfusion microbiology to develop further in its ability to identify as early as possible infected donors and donations and remove them from the system.

Chapter 11
Quality in transfusion microbiology

11.1 Introduction

The high profile of blood safety and the transmission of infectious agents has highlighted the need for ensuring and maintaining quality in transfusion microbiology. The essence of this is to ensure that all screening results obtained are accurate and reported against the correct sample identification. This applies equally whether a manual simple rapid test is being used or a complicated automated EIA. The quality system essentially starts with the selection of the donor and extends to the issue of the donation to a patient. The basic definition of quality in blood transfusion services is "safe blood fit for transfusion", and this is achieved by the generation of reliable and consistent results, and the identification and disposal of unsafe donations. Generally, quality can be considered to be both simple and complicated. If the simple principle of "doing it right first time" is always adopted, the whole concept of quality can be simplified. However, there are so many issues to consider that the whole subject eventually becomes very complicated. "Doing it right first time", although a very correct and proper principle, is no longer sufficient and the principle becomes "demonstrating and proving comprehensively that it has been done right every time".

A number of different terms are used when talking about quality, and these need to be clearly understood as they are often used incorrectly.

Quality assurance (QA) is the whole package of procedures and actions that together ensure the quality of a service or product reaches a specified level. In transfusion practice many larger transfusion services have QA departments whose function is to develop and maintain quality systems that cover all the activities taking place in a transfusion centre.

Quality control (QC) involves the use of specific parameters to monitor specific actions or activities to ensure that set quality levels are attained. Specifically in transfusion medicine QC often involves the use of external standardized samples that are tested on assay runs to ensure that a minimum level of sensitivity is maintained.

Quality monitoring (QM) involves regular checks on the products or services provided to ensure that what is actually produced is of a certain quality. Specifically in transfusion medicine QM is generally concerned with ongoing monitoring of all aspects of assay performance to ensure that the testing performed is of a consistent quality with minimal run–run variation.

Many specific aspects of quality have already been covered in previous chapters and will be only briefly, if at all, included here. Rather, in this chapter, in addition to some key aspects of any laboratory quality system, the concept of process control will be examined in some detail as a particularly effective way of assuring quality in the screening of donations for infectious agents.

There are many elements to any quality system: all are interlinked and all are important in building and maintaining the system. However, there are some key elements that should be part of every quality system, and without which assuring quality can be difficult.

11.2 Key elements of laboratory quality systems

11.2.1 Standard operating procedures (SOPs)

Whatever test or procedure is being performed, it is essential that it be performed in the same way by everyone performing it, and in the correct way. Although good training is an important part of ensuring that all staff perform procedures in the same way, a written procedure that is always followed is the only way to ensure this. Unfortunately some written procedures are just scraps of paper stuck to refrigerators and walls. Often handwritten, they become out of date, get altered and sometimes contain only the briefest of instructions. Formal SOPs are necessary to prevent such situations. These not only describe fully the way a procedure should be performed, they also describe the only way a procedure is to be performed. In addition, because they are controlled documents, unauthorized alterations to procedures are not made and therefore all staff have a clear set of instructions to follow. An SOP should be written to cover each procedure used in the laboratory and then used by all staff when performing the procedure.

SOPs need to be written carefully and in a specific way to ensure that they are consistent and easy to follow; they must be written in a clear manner using simple language so that the individual steps can be read as step-by-step instructions. Each SOP should cover only one procedure but be comprehensive and cover it from

start to finish; however, an SOP must not be too long or it will not be followed properly. SOPs ideally should be written (at least in basic outline) by staff routinely performing the procedure, and the final copy should be checked and authorized by the head of the laboratory. Each SOP should be made a controlled document which is formally prepared, numbered and issued, and must not be altered or changed in any way except when it is formally revised and a new edition issued. Once issued SOPs must be followed precisely at all times and incorporated into staff training programmes, ensuring that all staff performing any procedure have read and understand fully the SOP for that procedure before being allowed to perform it.

11.2.2 Standard forms

In a similar way to SOPs, any forms used in the laboratory as part of the setting up, testing and result reporting systems should be formalized so that the correct forms are used always, and are not altered. Many aspects of transfusion microbiology involve the use of forms, especially for the recording of sample identification, the preparation of reagents and the recording and interpretation of test results. Such forms and their contents are critical and must be designed carefully and then controlled to prevent improper changes. This is particularly important as the test results and donation identifications need to be kept for many years and so have to be clear, legible and capable of being understood by someone who may not have performed the original testing that they represent. As with SOPs, all forms used should be initially drafted and approved by all staff using them; then numbered and dated; and finally made controlled documents which cannot be altered without a formal review. Normally a master copy is produced from which working copies can be made by photocopying, although in some laboratories the forms may be printed, for example at a local print works.

11.2.3 Control of change

As has been made clear in 11.2.1 and 11.2.2, uncontrolled changes to procedures or documentation are dangerous and must be avoided. The same also applies to other changes, such as the introduction of a different assay or algorithm. Changes regularly need to be made, but these changes must be made in a controlled way through a strict procedure. In this way unnecessary changes are avoided while necessary changes can be introduced, but are checked before being

formalized. A fully documented change procedure is essential to control change properly. There are many ways in which such a procedure can be implemented, but the important issues are that change is made through a process that allows all the implications of the change to be considered and that everything is documented so that the reasons for the change are clearly stated and the date of the change is recorded. As part of the change procedure, validation of any new procedures or processes can be required so that the procedure is not just a control measure, but a comprehensive means of improving the performance of processes and procedures. All obsolete forms must be removed and destroyed.

11.2.4 Training records

The training of staff is an important part of any quality system. Whether those staff are skilled technical staff who perform assays, or basic laboratory aides who assist and perform the simpler and more routine tasks, they need to be well trained if they are to produce results of the required quality. The records of that training are an important part of the training programme. These records are of direct value to the trainee and the trainer as both can see what training has been given and what needs to be given. The records also enable both to be able to review the training and identify areas of need. In addition, they provide a good framework for consistency in the training of different individuals in the same activity. Indirectly they indicate to others, both internally and externally, the general level of training of staff and therefore the overall quality of the laboratory, both the management and quality of investigations performed.

Ideally the training records should be clear and concise, and divided into specific activities or procedures, each linking the activity or procedure with the detailed training given and the SOP(s) which cover the activity or procedure. In this way all trainees can be trained in the same way and to the same level, even by different trainers. As the training for each activity is completed, both the trainer and trainee should sign the training record: trainers to indicate that they consider trainees have been trained properly and can perform the particular activity, and trainees to indicate that they have evaluated the training course and offered suggestions for future improvements.

11.2.5 Audits

No matter how good a quality system is, and no matter how conscientious the laboratory staff are, there is always the need for external independent audit of the system. An audit is a planned assessment of the quality system in place. It is performed to ensure that such a system is actually in place, that the system is appropriate, and that it complies with any regulatory requirements. By its nature, an individual audit can never cover adequately all aspects of the work of a laboratory, but regular auditing can gradually address the quality aspects of most activities and procedures. Precisely what areas an audit covers varies greatly, but often an audit will follow a particular process through from start to finish, including related issues. Any deficiencies or other issues raised may also apply to other processes, and any additional changes can also be made.

An important issue concerns who performs audits. In large and well organized services there may be national or even local QA staff available to audit. In some countries the ministry of health or other government body may be responsible for the auditing of health care services. In other countries it may be appropriate for colleagues from other laboratories or specialties to audit. If there are no other alternatives, self-audit may be appropriate. Self-audit is not ideal, but still useful, and entails taking a full and critical look at the procedures used in the laboratory and asking questions such as the following:

- Are all the procedures fully documented and being performed correctly?
- Can any of the procedures be improved to increase reliability and hence confidence in the results?

All auditors should have received some sort of formal training to ensure that they can audit effectively, but they do not need to have extensive specific experience in the particular specialty; rather they need to have good all-round laboratory experience and common sense.

Unfortunately some staff feel that audits are designed solely to monitor and criticize staff performance and tend to fear them, rather than to see them as an opportunity to improve their quality systems and to put right things that they may have not noticed due to familiarity. Sometimes the apprehension about being audited comes from senior staff who fear criticism, something that will be prevented if all staff understand properly the principles of QA; in turn they will benefit from the audit procedure. These things are essential in all laboratories to ensure absolute confidence in the results generated.

11.3 In-process control

11.3.1 General

Diagnostic screening assays are all biological systems, and, no matter how well made or performed they are, many factors can affect the assay and thus affect the reliability of the final results. In some cases total failure may occur, but this is always obvious and the assay run can be repeated. However, in other cases the effects may be more subtle; although total failure does not occur, at least immediately, a gradual deterioration in assay performance may occur with the consequent risk of failing to detect weakly positive samples because of the loss of sensitivity. Less critically, assay specificity may also suffer and the number of false-positive results may increase. In-process control is thus the provision of a system of integral and continual monitoring of assay performance, which then enables an appropriate response when required. The aims of such control are to ensure quality by producing reliable and reproducible results with optimal sensitivity and specificity, ensuring results conform to internal and external specifications, and to improve efficiency so minimizing waste. The prevention of the gradual deterioration of assay performance can be achieved by the introduction of procedures to continuously monitor assay performance. Some of these procedures detect any change in performance as soon as it happens while others monitor results over longer periods. Such procedures may include the monitoring of individual assay steps, the use of QC samples or the setting of defined ranges for the final results.

At a level higher than in-process control, pre-process control is an important part of process control. This level of control is concerned with deciding on the suitability of assays for blood screening before they are actually used. There are two specific issues in the assessment of the suitability of assays for blood screening: a) formal licensing or approval or evaluation of individual assays; and b) individual batch validation and release. Control at this level is quite general and does not control the actual use of an assay. However, it does have the important function of ensuring that the individual assays used are actually suitable for blood screening. The formal licensing of assays, if undertaken, is generally a national activity, and any of the licensed assays are then available for use within a country. Batch validation is performed to check that the minimum sensitivity of the batch is as expected and that batch-to-batch variation is minimal. However, validation can be difficult to perform in some countries where only one batch is

shipped at a time and replacement kits are not available within a reasonable time. Although desirable, validation may therefore not be possible.

11.3.2 What to control

Effective control depends upon the control of every action during the performance of an assay. It is also important to try to identify the critical or weakest area of each procedure or process and to ensure that this is adequately controlled or monitored, as appropriate.

Sample/reagent addition. Both qualitative and quantitative control are needed. The correct samples must be added to the appropriate wells and the sample positions recorded. Similarly the correct reagent must be added at the correct stage of the assay procedure. Ideally both sample and reagent addition, if not performed using automated procedures, should be checked by a second member of staff. The volumes of both samples and reagents added must be as required by the assay. The calibration of automated equipment, including handheld mechanical pipettes, is very important (see 9.7) and must be performed regularly and recorded.

Incubation. Control of both time and temperature is needed. All stages of every assay have specific requirements for incubation times and temperatures. Any incubators or waterbaths used need to have their temperatures checked regularly and recorded, and normal working ranges should be established for each piece of equipment and for each temperature the equipment may be used at.

Washing. This is probably the most critical factor in ensuring the cleanliness of any assay. The tests must be washed strictly according to the manufacturer's stated wash method. Where an automated washer is used, this must be maintained properly and checked every day to ensure that the tests are washed properly and that the reaction vessel is left completely dry at the end of the wash programme. All the wash programmes used must be correct, and in the case of microplates the washer set up must reflect the type of well used, i.e. "U" well or flat-bottomed well.

Reagents. Many commercial assays contain ready-to-use reagents and, with the exception of preparation of the wash buffer, no further action is required. Where reagents need to be prepared, this must be done properly according to the manufacturer's instructions and checked by a second member of staff. If prepared reagents can be stored, they must be labelled clearly and stored appropriately until the expiry date indicated by the manufacturer is reached. In some cases the pH of

the wash buffer is important, and may need to be measured, and the buffer may need to be stored under certain conditions to maintain the correct pH range.

Results. The final assay results can be controlled in a number of ways. Manufacturers give clear information about the expected reactivity of the kit controls which must be met for the results to be valid. In addition, the results may be examined externally using a tool such as SPC (statistical process control) to monitor change in performance.

11.3.3 How to control

The in-process control needed can be achieved in a number of ways: automation of sample handling and assay processing; setting performance criteria at certain stages during testing; and use of tools provided by the manufacturer within the assay. Ideally a combination of all three is adopted, but in many cases this may not be appropriate, for example if automation is not available or generally not suitable.

Automation. This has been covered in detail in Chapter 9 and is only mentioned briefly here in the context of using automation to control variability. There is no doubt that automation can help to improve quality by introducing a degree of consistency and reproducibility, but on its own it does not provide all that is needed to provide effective in-process control. Automation also has its own problems. While a machine may perform a particular operation in a more consistent and controlled manner than a human, the results obtained may not be noticeably better. A machine can only do what it has been instructed to do, and while this is an advantage in some areas, its inflexibility may be a disadvantage in others. However, while humans are generally more flexible, often they do what they *think* they were told to do, not what they were actually told to do. Thus both automated and manual procedures have their advantages and disadvantages, but both can achieve certain levels of quality if controlled appropriately.

Performance criteria. A number of criteria can be set locally once experience with any assay has been gained and sufficient data have been generated and analysed. The value of using such parameters is variable, but what is important is that they can be set to apply to both manual and automated procedures and they generally provide information immediately, which can then be used to assess performance on the spot. The criteria used depend upon the assay used, the procedure used and the resources available, but can include: semiquantitative

checking of reagent addition by reading the OD values of the wells used; checking the mean and range of the OD values to ensure they are within a defined set range (best suited to microplate systems); and defining expected final OD values for assay controls. The final results (OD values) can be used in a number of ways as a measure of assay performance, by using the manufacturer's kit controls, external QC samples (see 11.3.4), and the test sample results themselves. The handling of final result data has already been discussed in Chapter 8.

Manufacturer's control tools. A number of assay manufacturers are now providing effective tools to aid in-process control and increase confidence in assay performance. These tools centre on the use of a sample addition monitoring system which positively indicates the addition of sample into the assay vessel. The first system suitable for blood screening was the SAM system produced by Murex Biotechnology Ltd (Dartford, UK) and from this the term SAM is now used widely to describe any sample addition monitoring system. The majority of these systems are based upon a colour change system whereby the sample diluent contains a detection system that changes colour on contact with human serum or plasma. The colour change can be seen by eye as well as being permanently recorded, if required, by reading the colour change OD values using a standard plate reader. Such systems have proved to very effective in the monitoring of the performance of automated systems, but can also be valuable when manually pipetting samples. Unfortunately SAM systems only work when sample diluent is used in the assay. However, if the first step of an assay requires the addition of only sample, this is clearly visible by eye, and the change in OD value can easily be measured using a plate reader if necessary.

Following the development of SAM systems, the inclusion of dyes in other reagents is becoming standard; thus some assays now include SAM together with a full set of coloured reagents. The advantage of this is that it becomes very clear that the correct reagent has been added and at the appropriate stage of the assay. Again, this is of value for both automated and manually performed assays.

While the introduction of SAM and coloured reagents have been significant advances in the in-process monitoring of assay performance, they are qualitative rather than quantitative procedures which are designed simply to demonstrate that sample has been added or that the correct reagent has been added, but not to demonstrate that the correct volume of sample or reagent has been added. The calibration of sample and reagent dispensers is still a separate, but linked, issue

and must be performed separately using the protocols and procedures supplied or suggested by the equipment manufacturer.

11.3.4 Quality control samples

Independent monitoring and control is an essential part of effective in-process control. The use of the manufacturer's kit control samples is the basis of determining assay validity because of the calculation of the assay cut-off values. However, such samples are atypical samples designed to work in a particular way with a particular assay and are usually highly diluted samples in a solution containing a number of other substances to preserve reactivity over the shelf-life of the kit. While external QC samples may still be diluted, they are generally as near native in composition as possible, often being prepared by diluting positive material in pooled human serum. QC samples can be considered in a number of ways, depending upon their intended use. Some samples are meant to be used as simple monitoring tools, assessing assay performance on a run or batch or daily basis, or even more infrequently, depending upon numbers tested and frequency of testing. Other samples are designed specifically to be used to release batches of results as testing is completed. The best use of QC samples often combines both uses, but depends on such samples being available and being of good quality.

There is an increasing interest in the use of QC samples as release tools. Such samples are commonly referred to as go–no-go samples and have to be positive for that batch of test results to be released. They do not replace the manufacturer's validity checks, but are used in addition as an extra level of assurance that the batch of tests has been performed correctly.

A major problem with all QC samples is in their preparation because they are invariably high dilutions of strongly positive samples. The preparation of any QC sample needs experience and expertise, and even then it is not easy to prepare QC samples that give similar results with all of the assays that they may be used with. This can be quite a serious problem, especially if go–no-go samples are being prepared, as many assays are sensitive, but this sensitivity is aimed at detecting early seroconversion samples, these generally being the weakest samples likely to be encountered. This does not necessarily mean that the assay will perform well with diluted samples, which virtually all QC samples are. However, a QC sample need not be positive with a particular assay to be of use. If the sample always gives results that are negative but with OD values clearly above the negative test

samples, the consistency of the results obtained is just as valuable in monitoring assay performance. Indeed the same can be applied to all QC samples as long as the OD values are not so high that they are above the range of the plate reader. The signal/cut-off ratio for all QC samples should be calculated and recorded to monitor assay performance both for each batch of tests and for all the batches tested over a period of time.

11.3.5 Statistical process control (SPC)

As mentioned in 11.3.1, most in-process control measures are used to ensure the quality of the particular batch or run of tests being processed at that time. However, there are tools available that can use those same data to monitor the processes over long periods of time to look for gradual changes that may go unnoticed on a day-to-day basis. However, such tools are not appropriate in all situations. For example, it is very hard to monitor the use of the simple rapid assays except by including a QC sample with every set of tests performed, but statistical process control (SPC)would not be possible as the final results can only be positive or negative.

SPC is a tool which is used in many different environments and has proved to be very useful in the analysis of screening data produced on a regular basis. SPC involves the recording of certain data on simple charts which then show visually any trends or sudden changes—*what you see is what you control.*

Statistical—an analysis of data in a standardized numerical approach

Process—applicable to a process or procedure

Control—a visual representation of each work process.

Using SPC to monitor assay performance continuously, overall control and quality are improved by removing the problems caused by: guesswork (making the wrong changes based upon wrong data); uncertainty (is there a problem with an assay or not?); surprises (suddenly an assay fails for no obvious reason); setting unclear targets (no one is very sure what results to expect and therefore how to judge the results actually obtained); overreaction (to an apparent problem which is actually a normal and acceptable variation in assay performance); or wasted time (trying to resolve the wrong problems).

In blood screening SPC is most appropriate in the monitoring of daily initial and repeat reactive rates for each assay, together with the sample OD/cut-off ratio

for a specific and appropriate QC sample for each assays used. The QC sample data can be monitored in different ways depending upon sample usage and the amount of data available, but this could be per plate, per test run, or by daily mean.

An example of a standard blank SPC chart is given in Figure 11.1. The monitoring period or unit is set on the x-axis, and the test result value is plotted on the y-axis. The y-axis is marked with the mean value and the range ±3 standard deviations (SD); the range is calculated from historical data (if available).

SPC tries to show a system under control, that is, a system that has some variability, but which is only that expected in any such system and is always within a tightly defined range, i.e. ±3 SD of the mean expected value. The mean and range are calculated from historical data, either directly from the previous SPC chart, or if this is the first chart used, from previous testing data.

The rules used to interpret SPC charts are the same whatever parameter is being measured and whatever assay is being monitored (assuming that data suitable for SPC can be obtained).

For a system to be considered to be under control the following criteria must be met:

- all points must be within ±3 SD from the mean
- two-thirds of all points must be within ±1 SD from the mean.

Figure 11.2 shows an SPC chart for an HIV QC sample which is tested daily. The y-axis represents the daily signal/cut-off ratio for the sample, and the x-axis represents each day. The chart obeys the above rules and the system is seen to be under control.
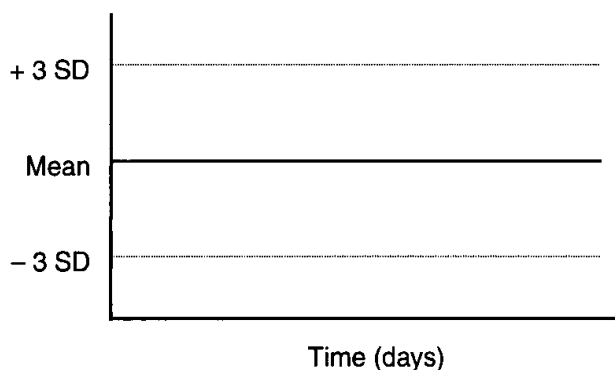

Time (days)

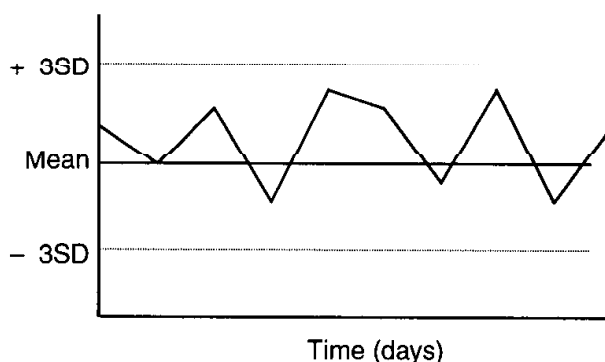FIGURE 11.1 **Example of a standard SPC chart**

FIGURE 11.2 **Example of in-control SPC chart**

A system that is out of control is indicated by the following:

- runs of 7 or more consecutive points up or down (daily readings)
- runs of 7 or more consecutive points above or below the mean
- cyclic patterns.

If one or more of the above criteria are seen on an SPC chart, the system can be considered to be out of control and the problem(s) must be identified and resolved.

Figures 11.3, 11.4 and 11.5 depict SPC charts which demonstrate out-of-control patterns.

As already noted, SPC may not be appropriate for all laboratories, depending upon the tests performed and numbers of samples tested. In addition, there are problems if suitable QC samples are not available, although plotting the initial and repeat reactive rates, or just the initial reactive rate if repeat testing is not performed, will still give a good SPC chart which can indicate problems giving rise to changes in reactive rates. An important point about SPC is that the charts should be completed by the assay operator, as this will help to involve all staff in the procedure. This allows them to see for themselves any problems that may be occurring, or that the process is under control and the laboratory is performing the assays correctly. SPC is a very simple yet powerful tool that can play an important part in in-process control. There is no doubt that depicting performance visually is of great benefit to all staff.
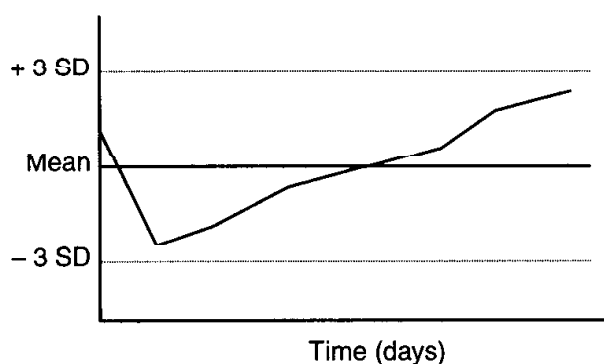
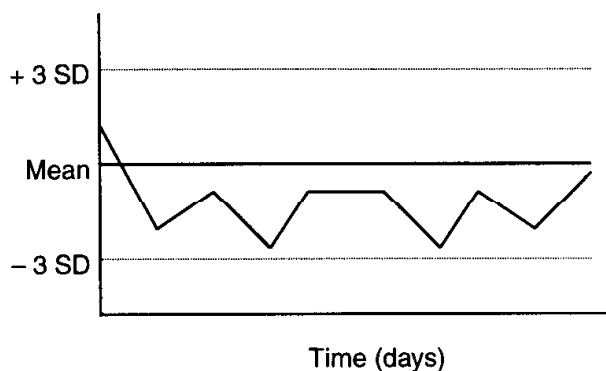FIGURE 11.3 **Example of an SPC chart which shows an upwards run of 7 points**

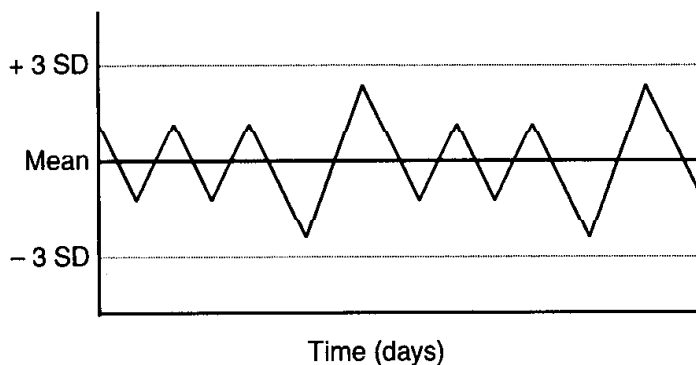FIGURE 11.4 **Example of an SPC chart which shows a run of 7 points below the mean**

FIGURE 11.5 **Example of an SPC chart which shows a cyclic pattern**

Chapter 12

Bacteriological and environmental monitoring

12.1 Introduction

The subject of bacterial contamination has been covered to some extent in Chapter 4; however, this chapter will look at the theory and principles behind the application of bacteriological and environmental monitoring to aspects of transfusion practice. For some reading this text, the whole idea of bacteriological monitoring may be very far removed from the reality of the daily running of the laboratory that they work in. As with the rest of this manual, however, the subject is covered in some detail to try to provide a comprehensive overview, thereby enabling readers to get what they need from the text. However, even if just one unit of blood is handled and processed, there is still a risk of bacterial contamination if an effective and appropriate cleaning programme is not in operation. Bacterial contamination may be intrinsic or extrinsic (see 4.1). Intrinsic contamination is hard to prevent and can really be dealt with only through the donor selection process, although product sterility testing is felt by some to be an important procedure. Extrinsic contamination, however, is more easily preventable, and is dealt with by developing and implementing a good environmental monitoring programme, including the monitoring of the blood collection process. An exception to this, however, is the prior contamination of products supplied as "sterile" and ready for use. These may include blood packs, blood collection and giving sets, and grouping reagents or other solutions. Although it is always assumed that products supplied as "sterile" are indeed sterile, this has often not been the case. Failures during the manufacturing process have resulted in the bacterial contamination of a number of products used in transfusion medicine that were supplied as "sterile", including *Serratia* spp. in blood packs and *Staphylococcus* spp. in blood giving sets. In such cases even strict QC of the goods supplied may not identify "contaminated" products as the contamination may be sporadic and/or low-level making it very hard to detect.

The value of any such monitoring programme is therefore to: determine the level of bacterial contamination in any work area; understand the significance of bacterial contamination to the work being performed and the way it is performed; determine the acceptable level of contamination (the level up to which any risk of contamination of the work undertaken is minimal); decide on how the level of cleanliness agreed upon is to be achieved and maintained: and agree the monitoring process that will ensure that the standards set are maintained.

Very often staff who are bleeding donors or preparing blood products or reagents, especially in larger hospitals or services, do not understand fully the significance of bacterial contamination and the risks to the work that they are doing. An important point with the design and implementation of any monitoring programme is that both the staff who perform the monitoring and who work in the areas being monitored need to understand fully what they are doing and why they are doing it. In many cases this understanding alone can prove beneficial in improving standards. For example, if staff understand precisely why the blood product processing area needs to be kept clean, they will be more willing to clean both properly and regularly, and any risk to the products will be thus reduced. The important issue here is that of raising the awareness of staff of the risks.

12.2 Monitoring programmes

12.2.1 General

Monitoring programmes must be very carefully thought out and designed before they are implemented. There are a number of basic issues that must be addressed and questions that must be asked before an effective programme can be agreed upon. If a programme is not going to be effective, there is no point in implementing it. Indeed an ineffective programme can often do more harm that good. If monitoring is being performed of an area or procedure for which no action can be taken whatever the results, it is often better not to monitor rather than to simply produce data which of no use.

12.2.2 Aim of monitoring

Probably the first question to ask is: what is the aim of monitoring? This question needs to be asked generally and then specifically for each area or process to be monitored.

The general aim of bacteriological monitoring in the transfusion service is to minimize any risk of contamination of the blood or blood products. This is achieved a) by proper and appropriate active monitoring of work areas and processes, and b) by raising the general awareness of staff to the problems of bacterial contamination and means by which those risks can be minimized. Clearly the prevention of intrinsic contamination centres on donor selection, consideration of which, as noted in Chapter 1, is not the prime aim of this manual. Prevention of extrinsic contamination, however, is the main aim of bacteriological monitoring, and, as noted in 12.1, is an area where the awareness of staff is critical.

However, when considering the monitoring of specific areas or activities, the aim of the monitoring performed can differ in subtle ways. For example; the primary aim of donor arm monitoring is to ensure that the arm is as clean as possible before venepuncture; in a donation processing area the primary aim is to ensure that the level of background contamination does not rise above a certain level; and in a reagent preparation area the primary aim is to ensure that all products are sterile. The primary aims thus vary according to what the needs of that particular area or activity are and depending upon what results are reasonable to expect and achieve for that area.

12.2.3 Work areas

Not all work areas need to be included in every monitoring system, although Good Laboratory Practice and health and safety requirements would generally require all work areas to be appropriately clean and safe. Those work areas that either need or would benefit from active monitoring programmes include donor collection, blood processing and storage, and reagent preparation (including any basic fluids such as distilled water and saline).

12.2.4 Monitoring activities to be performed

The monitoring activities will also vary according to the work area, and what cleaning programmes are already in place. The activities themselves must be defined together with how frequently they are performed and whom they are to be performed by.

A number of different tools are available for use in the monitoring programme, each giving different information and being appropriate in different situations.

Atmospheric settle plates are agar plates that are exposed to the atmosphere for a set amount of time and at specific sites within the work area. Bacteria and fungi in the atmosphere settle on these plates in the same way as they would settle on the work surface and their numbers and types can then be seen. It is very useful to use a "split" settle plate for atmospheric monitoring. This is a standard 90 mm Petri dish which is split into two equal size compartments; a bacterial specific medium is poured into one half and a fungal specific medium in the other. Both media are then exposed at the same time, and both bacterial and fungal levels can be quantified.

Swabbing any surface, including donor arms and equipment, is used to determine the total amount of contamination on that surface. Usually an area of a certain size is swabbed, and the swab sites are specifically identified, even if exactly the same spot is not continuously monitored. The surface is swabbed using a sterile cotton wool swab moistened with sterile distilled water, and then inoculated onto a general purpose medium. The information obtained from this technique gives a specific indication of the actual bacterial load at the sites swabbed. Such data can then be collated over a period of time, and in relation to cleaning and usage, to monitor the bacterial load as it builds up during use of the bench or piece of equipment and as it is reduced by cleaning.

Contact plates are an alternative to direct swabbing, although more expensive and not suitable for restricted areas, such as corners inside equipment. Contact plates are specialized agar plates (generally half the diameter of standard agar plates) that contain a general purpose medium which has been poured so that it stands slightly above the rim of the plate and can be "touched" onto the surface to be monitored. Such plates are suitable for a wide range of surfaces and equipment, as long as there is enough room to be able to handle the plate properly, but are not generally suitable for donor arms. Although they can be used on arms, these plates are best suited to hard flat surfaces. Donor arms often have a slight hollow where the venepuncture is to be performed and thus direct contact with the skin cannot be guaranteed. The information from these plates is essentially the same as that from cotton wool swabs, except that when using swabs a much larger area can be swabbed.

Filtration of pure fluids which should be sterile is the best way to demonstrate sterility. A known amount of fluid is passed through a sterile nitrocellulose or nylon membrane filter. The filter is then removed from its holder and placed onto a good general purpose medium. Any contaminating bacteria will be seen growing on the filter. This is a good method of demonstrating sterility and also of quantifying the bacterial load of a fluid which should be as free as possible from bacterial contamination but does not need to be sterile. Because a known amount of fluid is used, the contamination per millilitre of fluid can be calculated.

Whichever techniques are used, it is essential that they are standardized so that the results obtained from any technique by different individuals performing the procedure are totally comparable. This means that the actual procedures themselves must be very clearly defined and written up so that they are performed in an identical manner by all staff, even if the same member of staff usually performs the monitoring. Thus, all plates are exposed for the same length of time, and swabs are taken covering the same surface area, swabbing for the same length of time and with the same amount of pressure on the swab. Good training and very clear and precise SOPs are needed to achieve this. If standardization is not achieved, the monitoring results are unlikely to be comparable and no real conclusions can be drawn from the data.

12.2.5 Results and their interpretation

At the simplest level, the monitoring results are best quoted as colonies counted per plate. Thus each plate inoculated gives a count of viable organisms (total viable count—TVC), and this can be used directly or related back to the area swabbed or volume of fluid monitored. If a more scientific approach is needed, the morphology and growth rates of the organisms can be studied. This can be done by looking at the plates and checking to see if there is an obvious growth of one particular organism or general mixed growth, determining the rate of growth, or identifying the major organisms present. In most cases such additional information may be of limited value and total counts are sufficient. However, if there is pure growth of one organism this may indicate a potentially serious contamination problem and from a specific source, and should be investigated further. Therefore, when reading the monitoring plates, as well as counts, staff must be taught to look for other simple indicators of potential problems. One important factor, often overlooked, is that there is always a time delay between the monitoring activity and the production of the results. In some

cases this is as long as 5 days, and in that time many hundreds of donations could have been processed. A delay is inevitable but needs to be as short as is practically possible.

To make best use of the data, acceptable levels and specifications should be set for the numbers of organisms present at each monitoring site. These levels will clearly vary from site to site, but will simplify the interpretation of the monitoring process without losing any vital information. Outside the normal limits two levels are generally best in this situation: an alert level and an action level. The normal limits are those in which it is recognized and accepted that the background bacterial contamination (TVC) will normally lie, and which is acceptable for the work activity performed in that area. The alert level is that level at which the TVC is becoming higher than would be expected for that area when cleaned properly. Although not so high as to require urgent action, it is an indication that levels are gradually rising and may soon exceed the maximum allowable for that area. The action level is the level above which the TVC is too high and unacceptable, requiring urgent action to identify and rectify the problem. The idea of the alert level is to ensure that action is taken before it becomes too late and the action level is exceeded, when work may have to be stopped in the affected area. With a good cleaning programme and an effective monitoring programme, all work areas should be easily kept within the normal limits.

12.2.6 Response to monitoring

The problems of effectively responding to monitoring results should not be underestimated. What action should be taken if the results are out of specification? In some instances the cause of the raised levels is clear and the solution is straightforward. However, in other cases this is not so, and there is great difficulty in identifying the cause of the raised levels and hence a means of rectifying the situation. Whatever the cause, it is important that the response is at least consistent with the unacceptable results obtained and is a reasonable way of trying to reduce the levels back to within the normal limits, at least until a cause for the high counts can be established. As with all monitoring situations, there are always going to be occasions when the results are out of limits. It is, however, the response to this that matters in maintaining an effective monitoring programme.

However, the main outcome of monitoring is that of checking and providing the impetus to improve the cleaning procedures used. At the instigation of any monitoring programme it is highly likely that the cleaning procedures already in

use will be shown to be relatively ineffective in many areas. This may be because the cleaning procedures are not appropriate, cleaning is not performed as frequently as needed or the cleaning is performed at the wrong time in relation to the work performed. Because of the infinite variability of the individual laboratory set-ups and the complexity of cleaning programmes it is inappropriate to be too specific about individual cleaning programmes. However, there are some general principles that can be applied to cleaning programmes. An appropriate and effective disinfecting agent must be selected and then changed, perhaps every year, to prevent build-up of resistant organisms; separate equipment must be used for cleaning each work area and should not be used for any other purpose; and very clear SOPs should be produced.

12.3 Blood collection and donor arm cleansing

The collection of blood should always be performed in an area that is as clean as possible. If there is a mobile team that visits places like schools and factories, it is not always possible to control the environment of the collection area, but there are always some measures that can be taken to at least minimize any risks of contamination. Where there is a permanent collection area or room, it is reasonable to expect that it is kept clean and generally in such a way as to minimize any risk of bacterial contamination during blood collection. All equipment and hard surfaces that are used in the collection process should be cleaned regularly, using an appropriate validated method. Such cleaning is very important to prevent the build-up of bacterial contamination that may result in the contamination of the donations being collected.

The cleanliness of the donor's arm is an important factor in the prevention of extrinsic contamination due to bacteria entering at the time of venepuncture. At the very least, as matter of good practice, the venepuncture site should be cleaned to prevent infection of the site as a result of the needle taking bacteria from the surface of the skin underneath it. More important, however, bacteria on the skin of the donor can enter the blood pack or bottle and lead to gross contamination of the pack with possible posttransfusion sepsis and subsequent death of the recipient. This situation has been reported to have happened on many occasions and most probably has happened on many more occasions that were not reported.

Donor arm cleansing is therefore a very important first step in the prevention of the bacterial contamination of blood and blood products. There are many ways

in which the arm can be cleansed before venepuncture, but the aim is to ensure that the bacterial load on the skin is reduced by both bacterial killing and physical removal. Cleansing of the skin is normally carried out with the use of a specific anti-bacterial agent, or mixture of agents, such as alcohol, industrial methylated spirit (IMS), chlorhexidine or cetrimide; choice of agent really depends upon local availability and policy although they are all generally available in most countries. However cetrimide is generally not as effective in this particular situation as the others, and alcohol and IMS should be used at a concentration of 70% *V/V* for maximum effectiveness. There has been considerable discussion on the rotation of disinfectants in hospital environments to prevent the build-up of resistance. As far as blood donor arm cleansing is concerned, there is probably no real advantage in rotating the arm cleansing agents, but if one particular agent is not proving to be as effective as it used to be, changing the agent should be considered.

The method of using the cleansing agent is the next important step. Any cleansing process is improved by increasing the number of applications of the cleansing agent. This applies equally to donor arm cleansing, and two or three applications of the agent can improve significantly the effectiveness of the cleansing procedure. The most effective procedures use either individual swabs impregnated with cleansing agent, or an aerosol containing the agent which is either sprayed directly onto the arm and the arm wiped with a sterile gauze swab, or is sprayed onto a sterile gauze swab which is then used to wipe the arm. Whichever method is used the venepuncture site must be thoroughly cleaned and then left for about 30 seconds to allow the cleansing agent to kill any bacteria left on the skin.

Ideally the effectiveness of the cleansing method should have been validated prior to use, and then monitored "in use" on a regular basis. Such monitoring can be performed by checking the cleanliness of the venepuncture site after cleansing and just before venepuncture. This can be achieved simply by swabbing the site with a sterile cotton wool swab moistened with sterile water and then plating this out onto a good general purpose medium such as tryptone soya agar. The number of remaining colonies can then be counted and the number recorded and checked to ensure that it is within acceptable limits. The results obtained can then be used not only to monitor the performance of the cleansing procedure by the donor collection staff, but also to develop further the cleansing procedure in order to increase its effectiveness.

12.4 Environmental monitoring

In most transfusion centres that have a routine bacteriological monitoring programme, environmental monitoring is the main component of that programme. While it may not be practical to monitor every venepuncture performed, it is quite possible to design and implement an environmental monitoring programme that covers comprehensively those work areas where donations are processed and stored. The aim of such a programme is to ensure that the overall background bacterial levels are as low as possible and also to try to prevent sporadic build-ups of bacteria; all surfaces and equipment used for the processing, storage and transport of blood and blood products need to be monitored regularly.

The first step in developing an environmental monitoring programme is to identify the specific areas or equipment to be monitored. This will include all work surfaces and walls, floors and ceilings of cold stores and refrigerators, centrifuges, plasma extractors and blood transport boxes. The type of monitoring to be performed needs to be decided next, followed by the frequency. Environmental monitoring is usually most effective if a range of techniques is used. This may include atmospheric settle plates, swabs and contact plates; and depending upon the procedures performed, monitoring frequency may vary from day to day or from week to week.

As with donor arm monitoring, the cleaning procedures used are an important part of the environmental monitoring programme. In effect, the monitoring checks the effectiveness of the cleaning procedures and provides the information needed to improve these procedures if necessary. It is important, however, to be able to identify, if high bacterial counts are being obtained, whether the cleaning procedure itself is inadequate, or whether the procedure is fine but is not being performed correctly. Again, the monitoring process serves to increase the awareness of staff with regard to the importance of cleanliness.

The general levels of airborne microorganisms are best monitored using atmospheric settle plates which are sited at strategic, defined places in the work area and exposed for a set length of time during each work period. The benches and equipment should be swabbed regularly, again defining the particular area/ item of equipment to be swabbed. Alternatively, hard surfaces, but probably not some items of equipment, may be monitored using contact plates rather than swabs.

12.5 Product sterility testing

It is important to decide whether product sterility testing is needed or whether bacterial monitoring of products is more appropriate. Sterility testing is a formal procedure that is part of product release; the release would require the demonstration of the sterility of each individual product. However, products may range from an individual red cell donation to specialized manufactured plasma derivatives, such as albumin solutions, produced from large pools of plasma. Clearly, sterility testing would be appropriate for manufactured products where samples from the manufactured batch could be tested for sterility, but not for individual single donor products. Currently, sterility testing of all single donor products is not performed by any transfusion service.

Sterility testing of individual single donor products is very hard to perform to a level that makes the testing effective and leaves an uncompromised product that is suitable for clinical use. Indeed, even the testing of large pool products is not always a simple and straightforward matter. A major issue is the sensitivity of the testing procedure used, i.e. whether bacterial contamination can be detected. Taking a simplistic scenario: if, at the time of sampling, a donation of 450 ml is infected with a bacterium that is present at a low level of 50 viable bacteria in the pack, this would mean that the organism would be present at a concentration of 1 bacterium/9 ml of blood. If sterility testing is performed by blood culture and an inoculum of 5 ml is tested, there is only a 50% to 60% chance of one bacterium being present in the 5 ml sample and thus only a 50% to 60% chance of detecting the presence of the bacteria in the product. Even then, a single bacterium may not be detectable in such a testing procedure, although the level of contamination in the pack may be sufficient to cause significant illness in the recipient of the product. Such testing would then need to be performed on all individual single donor products before release for clinical use. Thus sterility testing, at least of individual single donor products, is not really an appropriate approach for ablood transfusion service. Therefore the general monitoring of the levels of bacterial contamination may be a more appropriate approach, and give an overall indication of the general level of bacterial contamination of the individual products available. However, the problem that arises then concerns the action needed if a contaminated product is identified. Is the finding a genuine indication of a contaminated product or was the sampling itself the source of contamination? Do all the other individual products from the original donation need to be discarded?

12.6 Reagent sterility testing

Reagent sterility testing is a far simpler process. The sterility of any noncellular reagent can best be checked by filtration through a 0.2 µm sterile membrane filter followed by the incubation of the filter on a suitable agar medium. Any contaminating bacteria can be identified from colonies growing on the filter and, in particular, quantified by reference back to the original volume of sample passed through the filter. This is a very sensitive technique which effectively concentrates the bacteria, even if only present originally in low numbers, from virtually any volume of fluid, onto a single surface. The bacteria can then be grown on the most appropriate medium. With some reagents, checks can be performed throughout the production process to ensure sterility at all stages and also to identify both those steps at which contamination is most likely to occur and those which result in a significant decrease in any contamination levels.

The sterility of cellular reagents, such as red cell panels for serology, is a little more complicated. Red cell suspensions can be plated out directly onto appropriate media, but this is not a particularly sensitive approach. Alternatively, the red cells can be lysed, the stroma filtered out using a coarse filter and the remaining solution passed through a sterile 0.2 µm filter. However this approach is not always reliable as bacteria present in the original reagent may be trapped on the coarse filter with the red cell stroma, and the free haemoglobin and other large molecular weight proteins may block the 0.2 µm filter quickly, so reducing the volume of fluid that can pass through it and therefore reducing again the sensitivity of the technique. The best way of ensuring the sterility of cellular solutions, or other solutions that cannot be sterilized by filtration or other nondamaging means, is by ensuring that all raw materials, equipment and disposables are sterile before use, and that all work is performed under sterile conditions.

Transfusion microbiology practice

The provision of blood and blood products free from contamination with any infectious agents is globally the aim of all blood transfusion services and the staff working within them. Currently in many countries there is great concern about the safety of the blood supply. Many infective agents are potentially transmissible through the transfusion of blood and its derivatives. Although the relative importance of different agents may vary in different countries and even within different areas of the same country, transfusion microbiology practice is a topic of a global importance.

This publication covers theoretical and practical aspects of modern transfusion microbiology and considers a wide range of infective agents, including those that may be of special significance for few countries only. This publication is intended for blood transfusion specialists, technologists and technicians, who must strive to ensure the provision of blood and blood derivatives free from contamination with infective agents.