# Serology:
General introduction to serological testing

**Authors:** Adapted by Prof M van Vuuren. Originally compiled by Dr RW Worthington (Retired)
Licensed under a Creative Commons Attribution license.

## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can single serum specimens be of value?</td>
<td>3</td>
</tr>
<tr>
<td>Antibody monitoring by means of serological surveys</td>
<td>5</td>
</tr>
<tr>
<td>Establishing the aetiology of virus-induced congenital malformations</td>
<td>5</td>
</tr>
<tr>
<td>Herd health control</td>
<td>6</td>
</tr>
<tr>
<td><strong>Serological tests</strong></td>
<td>7</td>
</tr>
<tr>
<td>Agglutination test</td>
<td>7</td>
</tr>
<tr>
<td>Precipitin test</td>
<td>8</td>
</tr>
<tr>
<td>Complement fixation test (CFT)</td>
<td>8</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>9</td>
</tr>
<tr>
<td>Neutralisation tests</td>
<td>10</td>
</tr>
<tr>
<td>Haemagglutination inhibition tests</td>
<td>11</td>
</tr>
<tr>
<td>Fluorescent antibody tests</td>
<td>11</td>
</tr>
<tr>
<td>General requirements for serological laboratories</td>
<td>12</td>
</tr>
<tr>
<td>General requirements for setting up tests</td>
<td>12</td>
</tr>
</tbody>
</table>
Serology: General introduction to serological testing

Serological tests are *in vitro* tests designed to detect the presence of antibodies in serum (antibody detection tests), or the presence of antigen using antibodies as detection reagents (antigen detection tests). In some cases the mixing of antibody and antigen gives rise to a visible reaction, in other cases some other secondary mechanism is needed to detect whether a reaction has taken place. Some of the commonly used serological tests are briefly described. This submodule is intended as an introduction to the subject for those that have little knowledge of serology.

Serology involves the detection of antibodies in serum, but in practice antibodies can be detected in various body fluids such as milk, cerebrospinal fluid, ascitic fluid, saliva, tears and mucus secretions. Serological tests are performed for various reasons, some of the most important of which are:

- The diagnosis of a recent infection with a particular microorganism or recent exposure to specific antigens, for example drug residues in food animals
- Determination of the immune status of an individual animal or a group of animals
- Sero-epidemiological surveys.
- Testing in-contact animals
- Screening animals before introduction into a household/farm/country
- Pre-mating screens
- Determination of the carrier status of an animal
- Detection of the spread of disease

During the last two decades of the twentieth century, the emphasis on serological testing had moved towards the so-called primary binding assays where the presence of antibody-antigen reactions are demonstrated by means of anti-immunoglobulin antibodies labelled with various markers such as enzymes, radio-active isotopes or fluorescent dyes. The popularity of these tests is based on their ability to be automated for use with large numbers of specimens, as well as their extreme sensitivity. The sensitivity of these tests is the result of efforts during research and development to amplify the signals that indicate specific antibody-antigen reactions.

Serological tests may be expressed qualitatively or semi-quantitatively. In the former the result will appear either as positive or negative. This type of result is especially found with screening tests where large numbers of serum specimens are tested at a specific serum dilution. The serum dilution can vary from 1:10 in an indirect fluorescent antibody test (IFA), to 1:500 or more in various enzyme-linked immunosorbent assays (ELISA). The semi-quantitative method involves serial two-fold dilutions of sera in a diluent buffer. The highest dilution giving a positive result with a particular serological test is then taken as the end point and expressed as a titre. In the case of ELISAs, the amount of antibody is directly proportional to the intensity of the colour produced when the specific substrate is added to the enzyme marker and the amount of antibody may therefore be expressed in terms of international units rather than as a semi-quantitative titre.

For the diagnosis of recent infection, immunoglobulin G (IgG) titres may be determined in paired sera. The first serum specimen is collected as early as possible after onset of disease, and the second, 10-14 days later. These specimens are respectively referred to as acute and convalescent sera. An increase in the titre of two dilutions or more, employing two-fold dilutions of the sera, is significant and
indicative of recent infection, whether it manifests as clinical disease or not. This is referred to as seroconversion. Serological examination is even more valuable as a diagnostic tool when paired sera from several animals in the affected herd are examined. If an IgG increase of two dilutions or more in antibody titres occur, or if some animals free of antibody in the first sample have significant antibody titres in the second sample, you may assume that the infection is active in the herd.

It is advisable for clinicians to submit the 2 specimens together following collection of the second specimen. This will in a way force the laboratory to process both specimens at the same time, with the same procedure and reagents (although most responsible technologists will do this in any case). This will minimise analytical variation associated with technologist expertise, quality of reagents, malfunction of instruments, different assay protocols, etc.

Serological tests alone are seldom clinically diagnostic. Serological results must be interpreted in conjunction with the clinical evaluation of the animal and other laboratory tests designed to isolate the causative organism or detect antigens directly in the tissues.

**Can single serum specimens be of value?**

It stands to reason that the method of testing paired sera is a slow method with its concomitant disadvantages. A diagnosis made on a single serum specimen will be of greater value for the management of the infected animal. This approach cannot commonly be employed, but there are indeed situations where serological tests on single serum specimens can be of value, and a number of those will briefly be discussed.

**Detection of specific immunoglobulin M**

Detection of IgM to diagnose recent viral infections has not been practised to the same extent in veterinary science as it has in human diagnostics. In the 1960s the different behaviour of the various antibody classes was elucidated. Antibodies of the primary response belong to the IgM class, and diminish rapidly after 2-3 months. The detection of specific IgM antibodies normally indicates a recent infection while IgG antibodies are more useful to indicate previous exposure and possible immune status.

There are various methods for the determination of specific IgM antibodies of which direct and indirect solid phase immuno assays such as IFA and ELISA are currently the methods of choice. The lack of the widespread use of these methods in veterinary science was due to the poor availability of commercial anti-animal IgM reagents. More recently however, this situation has been reversed, and IgM antibodies conjugated to either fluorescent dyes or enzymes are readily available for all domestic animal species.

It must be emphasised however, that the possible diagnostic use of specific IgM detection has to be established for each microorganism. The procedure however can be very helpful in infections where the causative agent is difficult or expensive to isolate in the laboratory, e.g. bovine rotavirus, or where the logistics of submitting specimens in appropriate transport media, for example for virus isolation is impractical, e.g. bovine respiratory syncytial virus.
Abortion

Until recently, serological diagnosis of infectious causes of abortion was performed with paired sera where an attempt was made to indicate a significant increase in the antibody titre for a specific abortigenic agent. The publication in 1992 of the 10-year surveys of the aetiology of bovine and ovine abortions conducted by C. A. Kirkbride and co-workers provided a new perspective on the subject. They argued that examination of paired serum samples seldom contributed additional significant diagnostic information. In most cases of abortion, the dam's serum antibody titre against the abortigenic agent had reached its maximum by the time abortion occurred. It is therefore unlikely that the serum antibody titre will rise following an abortion. Exceptions to this observation include Chlamyphilia-induced abortions in cattle and Toxoplasma-induced abortions in sheep. In addition, negative results may effectively rule out the abortigenic agent as the cause of the abortion.

Persistent viral and bacterial infections

Retroviruses can by virtue of their ability to express the enzyme reverse transcriptase, become incorporated into the host's cell nucleus as a provirus, thereby causing a persistent infection. The provirus is protected from immune clearance and can co-exist with antibody in the host. Demonstration of antibodies can be used to identify infected animals (provided the animal did not receive colostrum/milk with antibodies against the virus). Interpretation of the serological assays is thus based on the assumption that once an animal is infected it will remain infected for life.

Other diseases for which the presence of antibodies indicates a persistent infection include Johne's disease and brucellosis. The incubation period of Johne's disease is very long, and it can take up to 18 months before clinical signs are observed. Similar to tuberculosis, infection does not necessarily lead to recognisable clinical signs, but may result in subclinical carriers in which clinical manifestations of the disease may develop some time later. Although the serological screening of herds has so far not proven to be reliable to detect carriers, a positive serological test in an animal with clinical signs compatible with Johne's disease can be regarded as indicative of a persistent infection. Brucella abortus infection in adult cattle can likewise lead to a persistent infection.

Failure of passive transfer of colostral antibodies

A well-known concept that is not often exploited by large animal practitioners is that of the efficient or poor transfer of colostral antibodies to the neonate. Failure of passive transfer (FPT) is the cause of most losses among neonatal calves from gastro-enteritis, pneumonia, sepsicaemia, omphalophlebitis and polyarthritis. It occurs even on well-managed farms, and every valuable calf should preferably be tested. In commercial herds, regular spot testing will provide information on the adequacy of passive transfer of maternal immunoglobulins in the herd.
Radial immunodiffusion tests are commercially available for the accurate measurement of immunoglobulins in the serum of neonates. These commercial kits are however expensive and practitioners may rather want to choose a cheap test such as the serum turbidity test. The latter is technically not a serological test, as there is no antigen-antibody binding. Immunoglobulins are precipitated by low concentrations of metallic ions in solution. By comparing the precipitation reaction of the neonates’ serum in for example zinc sulphate, with that of its dam under the same circumstances; it is possible to detect complete failure of passive transfer of antibodies. This test cannot accurately measure partial transfer of colostral antibodies, and specimens with low readings should preferably be quantified with the radial immunodiffusion test.

Indications for the use of single serum specimens for clinical diagnosis are few, and reliance on the results of such tests may lead to confusion. There are however, situations where such results can be very helpful, but requests by practitioners for laboratories to perform such tests must be based on an insight into the limitations of such procedures.

**Antibody monitoring by means of serological surveys**

Disease surveillance is defined as the continuous observation of a population aimed at early case finding. Important serological applications of disease surveillance programmes include:

- To monitor vaccination programmes, e.g. the Pan African Rinderpest Campaign (PARC) that was part of the Global Rinderpest Eradication Programme (GREP). This programme succeeded in enabling the eradication of rinderpest in the year 2011.
- To detect spread of disease
- To detect the incidence/prevalence of disease

The regular detection of the humoral immune response in herds to pathogens of importance is an approach that has not been fully exploited by large animal practitioners as it has been for example in the poultry industry. Regular monitoring of the serological status of groups of animals for selected pathogens will provide information on the infectious agents that are active on the farm in both healthy and diseased animals. Serological monitoring as practised in the poultry industry must be applied on a regular basis to obtain a base line against which subsequent results can be measured, even in cases where vaccinated animals experience a breakdown in immunity.

**Establishing the aetiology of virus-induced congenital malformations**

The first reports of congenital malformations of the central nervous system of domestic animals were related to the vaccination of the dam in early pregnancy. These effects were seen with classical swine fever virus, bluetongue virus, bovine viral diarrhoea virus, Rift Valley fever virus and Wesselsbron virus.

The natural silent infection of the pregnant female by certain viruses and the teratogenic consequences observed months later in their off-spring was first reported in the mid-seventies in
Japan, Australia and Israel. In cattle, sheep and goats epidemics of foetal death, abortion, stillbirth, premature birth, congenital arthrogryposis and hydranencephaly were linked to two viruses of the Simbu serogroup of the genus *Bunyavirus* in the family *Bunyaviridae*, namely Akabane virus and Aino virus.

Establishing the aetiology of virus-induced congenital malformations poses some difficulties, the most important of which is related to the isolation of the causative agent from the aborted foetus or the neonate. In the past when no definitive aetiological agent could be associated with the congenital malformations, the condition was regarded as an inherited malformation. To make a diagnosis or come to a meaningful conclusion, one will have to consider the history, clinical signs, pathology and serological test results.

The teratological effects on the foetal brain caused by the above-mentioned viruses are lesions for example, which develop over a period of time, with the result that the virus cannot be isolated from the brain or other tissues following abortion or birth of the neonate in many cases. For this reason, indirect evidence such as the presence of specific viral antibodies can be of benefit.

The detection of antibody is the most practical method to study congenital malformations. On a regional or provincial basis this can involve selected and strategically placed sentinel herds of cattle and flocks of sheep. This is especially true for viruses such as Akabane for which the only known effects have been on the foetus, and has been successfully implemented in for example Australia.

**Herd health control**

The economic implications of serological testing constantly stimulate a search for quick, simple, sensitive and specific diagnostic tests. The primary binding tests such as ELISA and FA partly fulfils these needs and can be used for both the detection and quantification of virtually any antibody and antigen. The ELISA test especially, lends itself to quantitative and automated reading. In addition, it is quite economical in the use of reagents by virtue of its performance in microtitre plates. In the last few years the ELISA method has become an important assay in serological diagnosis of especially infectious diseases in production animals.

The wide variety of vaccine combinations that are available commercially for use in the prophylaxis of bovine respiratory disease, as well as the multifactorial nature of the disease lend itself to serological monitoring of selected groups of cattle on arrival at feedlots. Detection and quantification of specific viral antibodies provides veterinary consultants information on the efficiency of their vaccination schedules, as well as the susceptibility of various groups of calves as they arrive from different parts of the country at the feedlots. Such a programme will eliminate the chances of unnecessary vaccination or inefficient vaccination.

A monitoring programme in a feedlot should make provision for strategic bleeding of cattle during their stay in the feedlot. Animals can for example be bled during processing prior to immunization. This may provide information about the immune status of various groups of animals as they arrive at the feedlot.
A second group of serum specimens can be collected between days 15 and 25 following arrival when cattle are provided with high-energy rations. This period is marked by an increase in respiratory tract infections. A third period in which respiratory tract infections may increase is at approximately 45 days when re-implantation is performed. By regularly evaluating the antibody titres and comparing them with a standard that is progressively built up for a particular feedlot, one can keep your finger on the pulse of the efficiency of your vaccination programme, as well as breaks in the immunity of different groups of calves throughout the year.

**SEROLOGICAL TESTS**

**Agglutination test**

In agglutination tests the antigen is a suspension of insoluble particles, usually bacteria. When serum and antigen suspensions are mixed antibodies attach to the antigen at specific binding sites (epitopes). Since each antibody has two binding sites and the antigens have multiple binding sites the antibodies react with the antigen to form large lattices of cross-linked particles. If serum and antigen have been mixed in a test tube the large agglutinated complexes sink to the bottom leaving a clear supernatant when there is antibody present in the serum. The particles are left in suspension if there is no antibody present. Agglutination tests are done by mixing serum and a standardised antigen suspension in a test tube, leaving it to incubate for the required time (usually overnight), and observing to see whether agglutination has occurred.

Rapid slide or plate agglutination tests are performed by mixing a drop of antigen suspension and a drop of serum on a glass slide or ceramic tile, gently rocking and observing to see whether agglutination takes place. Agglutination will occur in a few minutes with positive sera. Agglutination is recognised because the even suspension forms large clumps of agglutinated particles that have a typical granular appearance. To make observation easier the antigen suspension may be coloured with a distinctive dye.

The buffer in which the antigen suspension is made up may be modified in several different ways to reduce the number of cross-reactions or non-specific reactions occurring with the test. Such modifications may include the use of acidic buffers, high salt solutions, buffers containing chelating agents such as EDTA etc.

When an antigen is soluble or invisible due to its very small size, the antigen particles are sometimes attached to large inert particles so that an antigen suitable for use in an agglutination test is formed. Examples of this are latex agglutination tests where antigen is attached to inert particles of latex and indirect haemagglutination tests where particles are attached to red blood cells. Indirect haemagglutination tests are seldom used and are mainly of historical interest, but a number of latex agglutination tests are still in use.

Tube agglutination tests were at one time commonly used for the diagnosis of brucellosis in cattle. Several countries eradicated brucellosis using this test as the main diagnostic test in their eradication campaigns. Due to problems with specificity, the complement fixation test and ELISA have generally
replaced the agglutination test for brucellosis, but it is still used in some countries and laboratories. Rapid plate agglutination tests are used for several applications.

**Precipitin test**

When a soluble antigen is precipitated from solution by an antibody this is a precipitation reaction, and the antibodies causing these reactions are known as precipitins. Precipitin tests can be performed in tubes or in slabs of gel in which the antigen and antibody diffuse toward each other. Tube precipitin tests are rarely used in disease diagnosis but gel diffusion precipitin tests have in the past been useful in several applications in veterinary diagnostics and biochemistry.

**Complement fixation test (CFT)**

Complement is not a single factor but a complex pathway (cascade) involving a series of interacting proteins found in the blood. The end products of the complement cascade are involved in various ways in the body’s defence mechanisms. They can play a role in the lysis of some protozoal parasites, kill some Gram-negative bacteria in conjunction with lysozyme, can coat (opsonise) some foreign particles and assist in the process of their being taken up by phagocytes. Those interested in immunology will find descriptions of the classical complement cascade and the alternative complement pathway and the roles they play in defence of the body, in textbooks on immunology. However, for our purposes complement can be regarded as a single substance. It is found in comparatively high concentration in fresh or suitably preserved unheated guinea pig serum. In practice, the reagent referred to as complement is fresh whole guinea pig serum or freeze-dried or preserved frozen guinea pig serum.

For some antibody/antigen reactions three factors are required: the antibody, the antigen and complement. Complement is involved in reactions that help to destroy antigens. When some antibodies combine with antigen in *in vitro* tests, complement is also involved or “fixed” in the reaction. However, the reaction between complement fixing antibodies, antigen and complement cannot be visualised, and a second indicator system is required to show whether a reaction has occurred. The indicator system makes use of the haemolysis of red blood cells to make the reaction visible and is known as the haemolytic system. The indicator haemolytic system consists of sheep’s red blood cells, antibodies generated against sheep’s red blood cells and complement. Sheep’s red blood cells are injected into a rabbit and rabbit antiserum known as haemolysin or amboceptor is harvested. The antibodies in this serum will react with sheep’s red blood cells, but will not haemolyse them. However, if a mixture of sheep red blood cells, complement and haemolysin are mixed together the red blood cells will be haemolysed. This is a suitable system for indicating the presence of complement.

In a CFT, sera to be tested are first heated to about 60 °C to inactivate any complement they contain. The exact inactivation temperature and time differs according to the animal species being tested and the test protocol. Test antigen, serum being tested and a carefully titrated amount of complement are mixed and allowed to react. If there are antibodies in the serum the complement will be fixed in the antibody/antigen reaction and will not be available. If there were no antibodies present the complement will still be available for use by the indicator system. At this stage a carefully titrated amount of red blood cells and haemolysin are added. If complement is present indicating that no
reaction has occurred between antibody and antigen the red blood cells will be haemolysed. If antibody was present in the serum and no complement is available then the cells will not lyse.

The CFT is a valuable test and is still widely used. Sometimes smaller laboratories and those that do not use the test regularly tend to prefer other tests because the CFT is more complex and requires careful titration of reagents. However, once the test and reagents have been standardised testing can be done rapidly and efficiently. At the brucellosis laboratory in New Zealand during the brucellosis eradication campaign up to 30,000 tests were done per day.

The CFT is a valuable test still used for the diagnosis of many diseases most notably the diagnosis of *Brucella abortus* infection in cattle and *Brucella ovis* infection in sheep.

**Enzyme-linked immunosorbent assay (ELISA)**

The ELISA is probably the most commonly used of the serological tests and has replaced many of the older tests. In this test antigen is fixed to a solid surface, usually the wells of a polystyrene microtitre plate. A wide variety of protein, polysaccharide and lipopolysaccharide antigens will adsorb directly onto the plastic. It is necessary to find the best conditions (pH, buffer type, temperature, time etc.) for adsorption of antigens but most antigens adsorb readily in either phosphate buffers at a pH slightly above 7.0 or bicarbonate buffer at a pH above 9.0. Methods are also available for binding antigen covalently to plates. The amount of antigen adsorbed to the plate is carefully titrated to give the most sensitive and easily read results. Once antigen has been adsorbed the plates can usually be dried and stored for later use. In this way large batches of plates can be prepared and used for many tests without the need for re-standardisation. Having a solid phase antigen is useful because serum can be added to the wells according to the test format and antibody, if present, will attach to the solid phase antigen. Plates are then washed to remove other serum components and tested to demonstrate whether antibody has bound to the plate.

Detecting antibody bound to a plate is usually done by using a second antibody against the primary antibody. If bovine serum is being tested, a second antibody is used, that was raised against bovine immunoglobulins in another species of animal such as a rabbit. The rabbit immunoglobulin is purified and a marker enzyme is covalently attached to it. The enzyme used is one that will react with a substrate to produce a coloured product. Horseradish peroxidase is a commonly used enzyme, but several other suitable enzymes are available. Horseradish peroxidase catalyses the oxidation of ortho-phenylene-diamine (OPD) by hydrogen peroxide to from a brownish oxidation product. The purified second antibody that is covalently bound to enzyme is generally referred to as the conjugate.

To detect antibody attached to antigen on the plate, the enzyme labelled second antibody is pipetted into the washed wells and given time to react before they are again washed to get rid of any unattached second antibody. Finally substrate consisting of OPD and hydrogen peroxide is added and the colour allowed to develop for a carefully controlled time before the reaction is stopped by addition of acid. In the last phase of the test the amount of colour that develops is proportional to the amount of enzyme present and thus to the amount of primary and secondary antibody that is attached to the well. The colour that develops is measured by spectroscopy, using an ELISA plate reader.
Within the limits of linearity of the test the optical density is assumed to be proportional to the concentration of antibody in the serum being tested. Therefore, the test gives a quantitative estimate of antibody concentration from a single dilution of serum tested. It is also possible to read tests by eye, but definition is not as good and quantitation of the reaction strength is not as accurate.

The ELISA method is extremely versatile. It can be adapted for the detection of antigen, or for use with monoclonal antibodies in competitive tests of very high specificity.

All the equipment for doing ELISA tests including diluters and dispensers, plate washers and ELISA plate readers, is available from commercial suppliers. ELISAs can thus be done rapidly and efficiently.

A disadvantage of ELISA tests is that it is difficult to repeatedly get the same optic density (OD) readings if sera are tested on different plates and on different days. This problem is overcome by relating all results to the negative and positive control sera on the plates. For this reason results are generally expressed as being relative to the standards and whichever method is used to achieve this requires a calculation to be made for each serum tested. This is time consuming unless the ELISA plate reader is directly on line to a computer that calculates and prints out the results.

Neutralisation tests

The most commonly used neutralisation test is the serum neutralisation (SN) test or virus neutralisation (VN) test. This test is used for virus diseases and it is necessary to have the capability and facilities for doing tissue and virus culture work. Therefore, it is often carried out in fully equipped virology laboratories. Tissue cultures are grown in microtitre plates and a carefully standardised amount of virus added to the wells. Before the virus is added to the wells it is mixed with serum and incubated to allow the serum to neutralise the virus if antibody is present. Where the serum contains neutralising antibody the virus fails to grow. Doubling dilutions of serum are tested and the highest dilution of serum that inhibits viral replication (end titre) indicates the quantity of antibody in the serum. The actual characteristic that is visualised to determine whether virus is growing in the culture may vary from test to test and include such things as cytopathic effects of virus on the cells, reduction in the number of viral plaques, peroxidase linked antibody detection of virus and fluorescent antibody detection of virus.

Neutralisation tests are also used to detect toxins. In the past clostridial toxins were often identified using neutralisation tests done in mice. Injecting these toxins intravenously into mice killed the mice. However, mixing and incubating the toxin with antiserum before it is injected will neutralise the toxin and the mice survive. Toxins can be identified by testing suspected toxin-containing material with batteries of standard antiserum toxins to identify the types of toxins. The test can also be used to measure the amount of neutralising antibody in a serum, if varying dilutions of serum are tested against a fixed amount of standard toxin.

Toxin tests using live animals are increasingly regarded as being unethical and are being replaced with in vitro tests. For instance E. coli Vero toxin is cytopathic to Vero cells in culture and can be assayed using a Vero cell culture system to detect the presence of toxin.
**Haemagglutination inhibition tests**

Several viruses will agglutinate red blood cells from various species of animals. For example avian influenza and Newcastle disease virus agglutinate avian red blood cells. This agglutination by the virus can be inhibited by antibody to the virus thus providing a mechanism for a serological test. Doubling dilutions of a serum to be tested are made in a microtitre plate. A carefully titrated amount of virus suspension is added to each well and the plates are incubated. A suspension of chicken red blood cells is then added to each well. Where antibody is present agglutination of the red blood cells is inhibited. The highest dilution of serum that inhibits haemagglutination (end titre) gives an indication of the antibody content of the serum.

**Fluorescent antibody tests**

Antibodies can be conjugated to a fluorescent compound such as fluorescein isothiocyanate and used as reagents to locate an infectious agent in sections of tissue, tissue culture, blood smears etc. For example tonsil or other tissues from suspected cases of swine fever can be sectioned and stained with anti-swine fever immunoglobulin conjugated to fluorescein isothiocyanate. Fluorescent virus-containing cells and structures can then be identified when examined under UV light with an UV microscope.

Indirect fluorescent antibody tests (IFA) are used to identify antibody in serum. A smear of a suitable standardised preparation of the antigen is fixed to a glass slide. The smear is then flooded with the serum being tested and incubated for a suitable time, washed and flooded with a second antibody that has been conjugated with a fluorescent marker. After a suitable incubation time the slide is washed, dried and examined with a UV microscope. A typical example would be a test for *Babesia* antibodies. The antigen would be a blood smear containing a standardised number of *Babesia* parasitized cells. If the sera being examined were from cattle then the second antibody would be an anti-bovine immunoglobulin serum prepared in a heterologous species and conjugated to fluorescein isothiocyanate.
General requirements for serological laboratories

Probably the single most important requirement for a laboratory doing serology is to have a source of highly purified water for making up reagents. This is particularly important where CF tests are being done as small amounts of organic contaminants in the water can be anti-complementary and variations in levels of these contaminants can make results unpredictable and unreliable. Water should be purified by glass distillation, or by deionisation and reverse osmosis. In either case the water should be continuously checked to ensure that the conductivity meets specified standards.

Facilities for preparing clean glassware are less important in modern laboratories because of the almost exclusive use of disposable plastic pipette tips and microtitre plates. However, wherever glassware is used for reagent preparation it should meet high standards of cleanliness including washing with a suitable detergent and thorough rinsing in distilled water. Generally for serology laboratories it is not necessary to use sulphuric acid and oxidising agent baths for glassware preparation.

Checking of volumetric glassware is generally unnecessary, provided it is obtained from a reliable supplier. Hand pipettes and dispensing equipment, incubators, water baths, pH meters, balances, spectrophotometers and all other measuring equipment should be checked and maintained according to specifications laid down in a quality manual.

Many modern laboratories purchase most of their reagents and many use test kits from commercial sources. However, smart packaging and high price tags do not always guarantee the quality of product and all purchased reagents and kits should be strictly quality controlled. There should be adequate facilities for production of antigens that are produced in house, including the capability to culture infectious agents where this is required. Where production of simple bacterial antigens is required this is usually easily accomplished with comparatively simple equipment. However, for viral antigens where tissue culture facilities and perhaps ultracentrifuges are required, antigen production may be undertaken in a dedicated virology laboratory.

General requirements for setting up tests

Equipment

Most serological tests are performed in microtitre plates. A standard microtitre plate contains 96 wells arranged in eight rows of twelve wells. The vertical rows of wells (columns) are numbered 1-12 and the 8 horizontal rows identified alphabetically as A-H (Figure 1-1). Several suppliers offer microtitre plates with round or flat-bottomed wells. The numbering of microtitre wells that may be used is shown in Figure 1-1.
Many serological tests are performed on doubling dilutions of serum. The lowest dilution of sera being tested is usually put into wells in the first row (horizontal row H) of a microtiter plate and buffer is put in all other wells of the plate. Serial transfer of serum across the plate results in doubling dilutions of serum being made across the plate. A typical protocol for the serial dilution of sera could be:

1. Pipette 50 µl of a 1 in 4 dilution of each of 11 sera to be tested into wells H1 to H11, and 50ul of positive control serum in well H12.
2. Place 25 µl of buffer in all other wells.
3. Transfer 25 µl from each well in row H to the corresponding well in row G, mix well and transfer 25 µl from each well in row G to the corresponding wells in row F.
4. Continue this process to row A and discard 25 µl from each well in row A.

Serum in row H has a dilution of 1 in 4, serum in row G is diluted 1 in 8 etc. until finally in row A the dilution is 1 in 512. This type of dilution protocol can be modified as required to suit different situations and test methods. This serum dilution step is common to many of the methods and is simply described as "making doubling dilutions from row X to row Y".
Dispensing reagents into the wells can be done with single or multiple tip hand held pipettes of the Oxford type (Figure 1-2) or by sophisticated automated dispensers/diluters. The system used will depend on the number of sera to be tested and the financial resources.

Figure 1-2: Single and multiple head dispensers used for dispensing and diluting sera in microtitre plates.

Test formats

For every plate that is set up one of the sera tested should be a positive control serum. This serum is a standard positive reference serum of known end titre. It should always give the expected result within the allowable limits defined for the particular test, otherwise all the results for all the sera on the plate are invalid and the tests should be repeated. It is preferable to have a negative control serum on each plate, but when large numbers of mainly negative sera are being tested negative control sera are tested on only a few plates for any batch of tests. Protocols for some tests require the testing of a number of positive controls of different expected end titres. This is especially common for tests where sera are tested in single wells and thus large numbers of sera are tested on each plate. In ELISA tests it is common to have up to 6 negative sera and duplicate tests of 3 or 4 dilutions of the positive control serum on each plate. Alternatively a low medium and high positive serum may be included on each plate. Positive control sera should have been standardised against an international or national reference serum.

According to the type of test there may be several other control wells needed, to demonstrate that each of the reagents is working properly. In ELISA protocols there are some wells with no antigen, some to which all reagents except test serum are added, some with no conjugated second antibody, and some with no substrate. In this way faulty test results are easily seen and
the source of the problem can be quickly identified. In complement fixation tests there is an additional control well for each serum tested. This is a well that contains no antigen and is known as the anti-complementary control. Some sera contain unknown anti-complementary substances that will destroy complement activity. Because these sera destroy complement in a non-specific manner they will give false positive results unless there is a control well to demonstrate that the serum is anti-complementary. The anti-complementary control well contains no antigen and thus will give a negative result unless the serum contains anti-complementary substances.

**International and national reference sera**

The primary reference point for a test is the international standard reference serum for the particular disease. For example the WHO/FAO *Brucella abortus* reference serum can be used as the primary reference for brucellosis tests. Whenever international reference sera are available for other diseases, particularly from OIE reference laboratories, these should be used. Generally international reference laboratories will only supply small amounts of reference sera. National reference laboratories should produce national standards that are carefully standardised against the international standard. National reference sera should be produced in sufficient quantities to ensure an unchanged source of reference serum for at least 10 years. Ideally national reference laboratories should supply the reference serum as a working control serum to testing laboratories. However, if national laboratories are only able to supply small amounts of national standards, testing laboratories must produce their own working control sera and standardise them against the national standard.

National reference sera can be prepared from naturally infected animals or from experimentally infected or vaccinated animals. The use of naturally infected animals is preferred. For a national standard a high titre serum is usually preferred, and this serum can then be suitably diluted for routine use. Serum from several animals should be pooled where this is possible. In order to obtain the amount of blood required it is often necessary to buy suitable animals and sacrifice and exsanguinate them. To do this, animals should be anaesthetised and the carotid artery canulised and the blood collected directly into sterile glass centrifuge buckets. After the blood has clotted, the clot is loosened from the side of the container and it is allowed to stand for several hours for the serum to separate from the clot. If necessary the centrifuge buckets are again centrifuged and the serum pooled, centrifuged to clarify it and stored at 4 °C. Aliquots of serum are given to at least three operators to test. Each operator determines the exact end titre of the serum in the appropriate test. For example in the CFT the serum dilution that gives 50% haemolysis in the standard CFT test is determined. This may involve several tests, firstly to determine the approximate dilution giving 50% haemolysis and then by using closer spaced dilutions making an accurate estimate of the end-point. Each operator should determine the end titre at least in triplicate. On the same day and using the same reagents the end point of the international standard and where available a previous national standard are also determined.

Where the reference serum antibody is given in international units (IUs), the antibody content of the serum should be calculated in the same units. For example if the international reference
serum contains 500 IUs per ml and has an end titre of 1/230 and the serum being prepared has a titre of 1/320 then the serum being prepared contains 500 x 320/230 = 696 IUs per ml.

The reference serum should then be aliquoted and bottled. However, if preferred it can be diluted with negative serum to give a convenient number of IUs per ml e.g. in the above example to dilute to 500 IUs per ml, 196 ml of negative serum is added to 500ml of serum. If no international serum is available for comparison national units can be allocated arbitrarily to the standard. In this case it may be convenient to declare the number of national units per ml of serum to be equal to the reciprocal of the end titre for the serum.

The serum must be accurately aliquoted and freeze dried under nitrogen or vacuum, preferably in glass vials but rubber stoppered bottles may also be used. So as not to be wasteful, only a small amount of serum is dispensed to each bottle. If the serum is of reasonable high titre it will be considerably diluted when used as a reference standard. In the above example serum had an end titre of 1/320. If it is required to be used at a dilution that gives an end titre of 1/16 it will be diluted 1/20 for use. Therefore, one vial containing 0.5ml will be made up to 10ml. If 11 sera are tested per plate this will be enough to serve as standard on 200 microtitre plates or 2,200 tests.

Once a standard has been aliquoted and freeze dried, a suitable number of randomly selected bottles must be tested to make sure that the serum titre has not declined during the freeze-drying process. If necessary the number of units of antibody allocated to the serum can be reallocated.

Once freeze-dried serum has been reconstituted it can be stored at refrigerator temperature for two weeks or aliquoted and frozen for several months. However aliquots of frozen serum should not be re-frozen once thawed.

For some tests several reference sera may be needed e.g. a high positive, a moderately positive and a borderline positive control. In addition negative control sera are useful for several tests. Production, standardisation, storage and distribution of positive control sera require a significant commitment of time and resources by reference laboratories.