An introduction to viruses and techniques for their identification and characterisation

Ball B.V.

in

Colin M.E. (ed.), Ball B.V. (ed.), Kilani M. (ed.).
Bee disease diagnosis

Zaragoza : CIHEAM
Options Méditerranéennes : Série B. Etudes et Recherches; n. 25

1999
pages 69-80

An introduction to viruses and techniques for their identification and characterisation

B.V. Ball
IACR-Rothamsted, Harpenden, Herts., AL5 2JQ, UK

Viruses of insects

All forms of life are attacked by viruses and consequently the class of insects, which includes more than a million species, becomes infected with a wide variety of virus types. These are usually host specific or have a limited host range so that they have the potential for selective control of a pest species without affecting natural enemies such as insect predators. Early work in insect virology centred on what are called the "inclusion body" viruses or polyhedroses. The virus particles are "occluded" or embedded in crystals of protein which are anything from 0.5 to 15 μm in size. Because the virus particles are embedded in a large crystalline matrix of protein they may be seen by light microscopy; most other viruses which are not embedded in this way are too small and can only normally be seen by electron microscopy. The virus particles of the nuclear polyhedroses are invariably rod shaped and contain DNA; the cytoplasmic polyhedroses are isometric and contain RNA. Thin sections of nuclear polyhedra, viewed under the electron microscope, reveal the virus rods scattered apparently at random within the polyhedral crystal. The rods may be singly enveloped or enclosed in groups or in bundles of ten or more and there are many hundreds of these within each crystal.

These occluded viruses have only ever been found in insects, most commonly the larvae of butterflies or moths but also in flies, beetles and certain hymenoptera, although none have been detected in bees. Because the virus particles are embedded in protective protein they can persist outside the body of the insect on leaf surfaces and in soil. For this reason they have been used successfully for the control of pest insects.

Comparatively few viruses that have small non-embedded or non-occluded particles, like those that attack most other animals and plants, have so far been identified in insects. Only about 50 non-occluded viruses of insects have been described and about half of these have been isolated from the honeybee.

Virus replication

One characteristic that distinguishes viruses from most other insect pathogens is that they cannot be cultivated on artificial media; they require living cells of their host for multiplication. Bacteria and fungi can digest and assimilate nutrients from their environment and can therefore be isolated and maintained on simple media in the laboratory. Viruses, however, use mechanisms within the cell to make copies of their genetic material and structural proteins. The process of virus replication leads to a loss of cell function and the cell eventually bursts open releasing thousands of virus particles to infect other cells (Fig. 1). It was believed that the presence of a viral pathogen inevitably resulted in a fatal infection, but it is now known that many of the viruses of bees (and of other animals), can persist in individuals without causing apparent damage or reduced longevity. Viruses can remain in a "latent" state within individuals and may spread within bee populations at a low level of inapparent infection. This is one mechanism that has enabled viruses to persist in bees since, unlike the occluded viruses of other insects, the small non-occluded viruses of bees do not remain infective for long outside the body of their host. Under certain circumstances virus replication is triggered or virus transmission increases and overt or apparent infection can spread between bees, leading to outbreaks of disease.

The viruses of honeybees seem unusually specific; they cannot be cultivated in other insects or in insect cell tissue culture. This poses some problems in virological studies and it is essential to fully

appreciate the practical difficulties in virus propagation. Honeybee viruses will only multiply in bees, but as outlined above, individuals in a population may be inapparently infected with any one of a number of different viruses; indeed some viruses, notably acute paralysis virus (APV) and slow paralysis virus (SPV) seem only to persist at this low level and do not normally multiply in individuals sufficiently to cause mortality in nature.

Identification and detection of honeybee viruses

Most of the viruses of bees are small icosahedral particles about 30 nm in diameter (Fig. 2). When viewed in the electron microscope they appear as featureless spheres and they cannot be distinguished morphologically. Some have descriptive names, for example, black queen cell virus (BQCV) and deformed wing virus (DWV), which suggests that specific symptoms are associated with infection that could be useful in their recognition or field diagnosis. Unfortunately, with a few exceptions, symptoms which could be reliably used to confirm the presence of a particular virus are entirely lacking. It is true that queen larvae or prepupae that have been killed by BQCV infection often cause a melanisation of the interior of the queen cell which is visible to the naked eye. However, it was not until the virus had been isolated and an antiserum produced, that BQCV was found to be a very common infection of adult worker bees and was specifically associated with the Microsporidan gut parasite *Nosema apis*. Although the virus shortened the lives of these individuals, infection produced no visible symptoms. Similarly, the symptoms of wing malformation associated with DWV infection are only produced if honeybee brood is infected at an early stage of development. The virus multiplies slowly so that the pupae continue to develop but emerge with incompletely developed or malformed wings. Adult honeybees may also become infected with the virus but because these are fully formed individuals, no similar symptoms are produced. Although these two viruses were first recognised because of the symptoms they produced in natural infections these are unreliable as aids to diagnosis and their descriptive names are misleading.

Whilst electron microscopy cannot be used to distinguish the 30 nm honeybee viruses it is a useful aid to recognition if the virus has a distinctive size or shape. The filamentous virus can readily be detected by this means in the extracts of diseased bees and this is the method of choice for routine analysis (Fig. 3a). The large size of the particle also makes other detection methods difficult. Chronic paralysis virus (CPV) also has distinctive shaped particles which is useful for diagnosis (Fig. 3b). However, these are not always easy to see in crude extracts of bees because the negative contrast of electron dense stains is poor under these circumstances, and the particles are difficult to recognise as they are very variable in size and have irregular outlines. The iridescent virus of *Apis cerana* may also be readily detected by electron microscopy but when infection is well established it can be reliably diagnosed by eye. The virus particles are very large, about 150 nm in diameter, and they form crystalline aggregates in infected tissue, especially the fat body which lines the abdomen (Fig. 3c). These crystals of virus refract light so that infected tissues iridesce a bright blue colour when illuminated with a bright white light. Individual infected cells may be clearly distinguished from the surrounding creamy white tissue.

The physical properties of viruses may also be useful in differentiating particles of the same size. The associate particle which often accompanies CPV infection (CPVA) and cloudy wing virus (CWV) are both extremely small, approximately 17 nm in diameter (Fig. 3d). They may be readily distinguished, however, by their different stability in buffer of low molarity. CPVA remains in suspension when centrifuged at low speed in 0.01 M potassium phosphate buffer at pH 7.0 whereas CWV aggregates and sediments. The same technique may also be applied to differentiate the sacbrood virus of *Apis mellifera* from that of *Apis cerana*. Sacbrood virus of the European bee (SBV) is stable and remains in suspension in dilute buffer, whereas Thai sacbrood virus (TSBV) is unstable and sediments when centrifuged at low speed.

Apart from the examples cited above and CPV and SBV, which produce specific symptoms of infection, the detection and identification of virus infections currently relies upon the production of specific antisera and serological techniques. These antisera are not commercially available but the basic techniques for virus isolation, propagation and purification are outlined below to enable individual researchers to produce their own antisera for diagnostic tests. The dependence on serological testing makes detailed descriptions of the diagnosis of the majority of the honeybee virus diseases academic and the remaining two sections of this chapter deal only with the viruses that produce reliable diagnostic symptoms of infection. Further information on the natural history and epidemiology of the remaining honeybee virus diseases may be obtained from Bailey and Ball (1991).

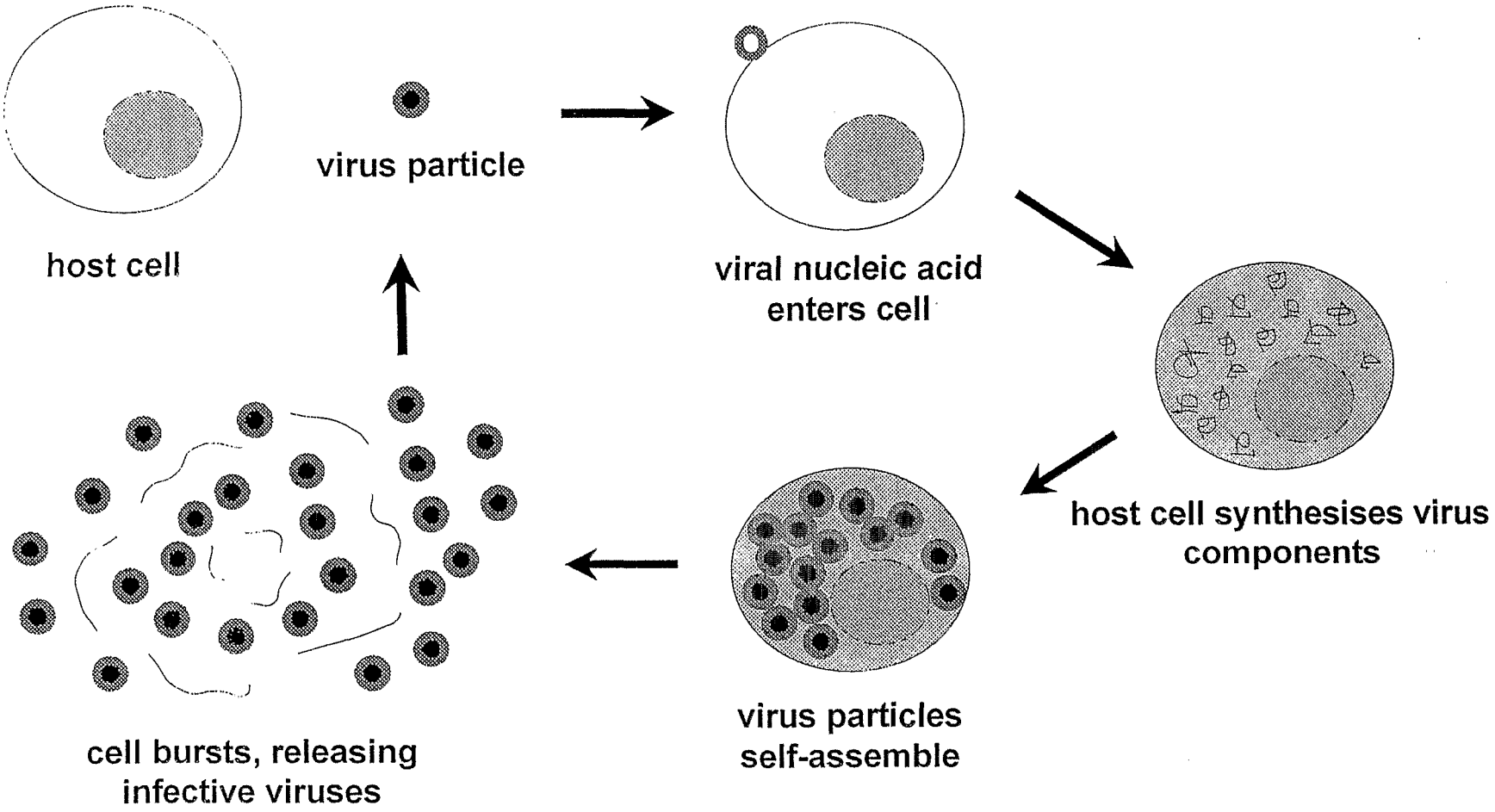


Fig. 1. A typical virus replication cycle. The virus binds to a host cell receptor after which the genetic material enters the cell. The infected cell loses its normal functions as it synthesises thousands of copies of virus components. The cell eventually dies, releasing the next generation of virus.

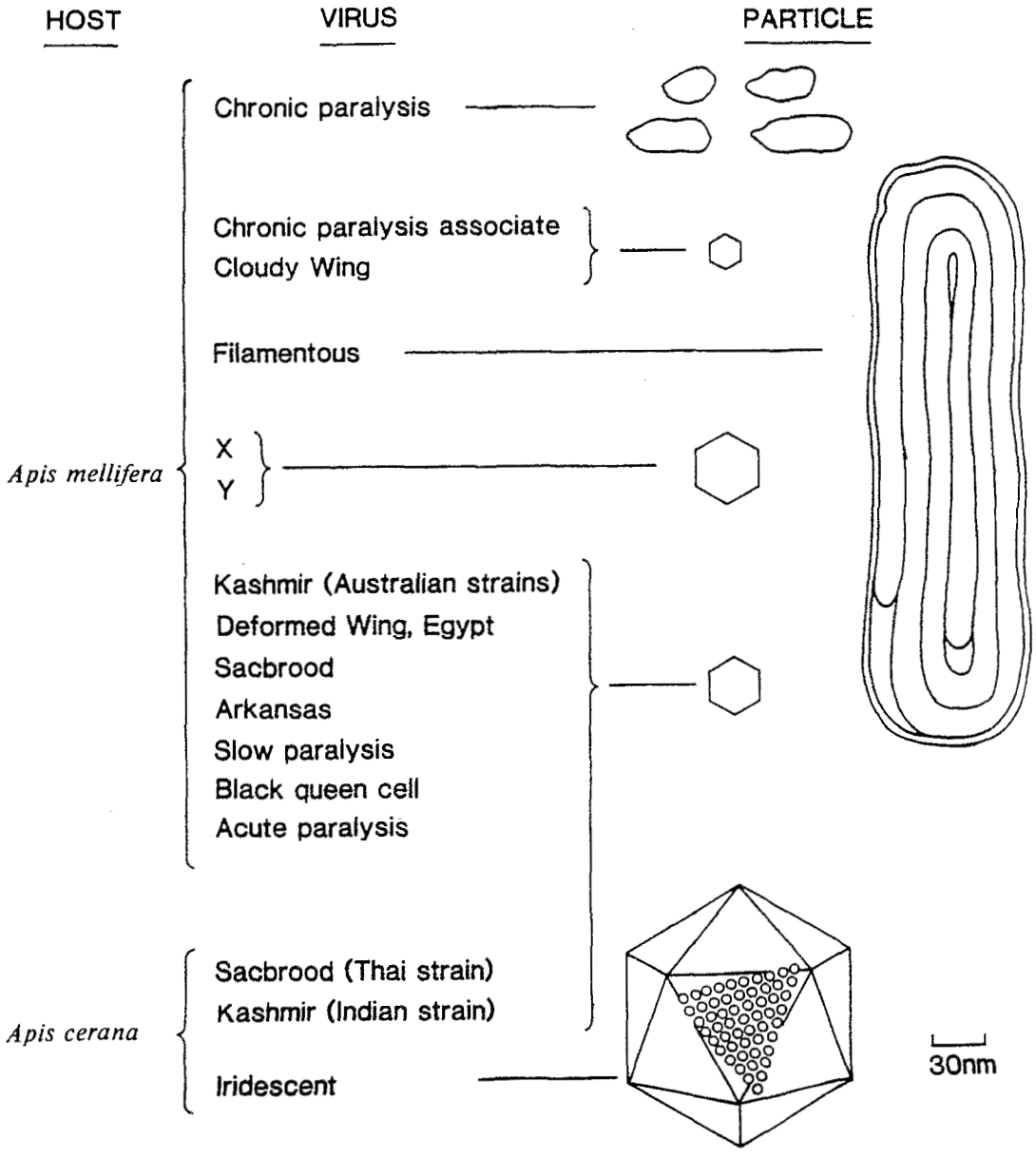


Fig. 2. List and diagrammatic outlines of the viruses of honey bees.

Serology

Polyclonal antiserum production

Electron microscopy is useful for confirming the presence of the 30 nm icosahedral viruses in extracts of diseased bees but they cannot be distinguished morphologically. However, the outer protective shell, or capsid, which encloses the ribonucleic acid (RNA) is made up of proteins which are characteristic of each virus. We can make use of this property by producing specific antisera.

It is essential to use highly purified virus of a single type as an immunogen. If the virus preparation is contaminated with host protein or contains a mixture of different types of particle, antibodies to these proteins will also be produced. This will present problems with the use of the antiserum as non-specific reactions will make interpretation of results difficult.

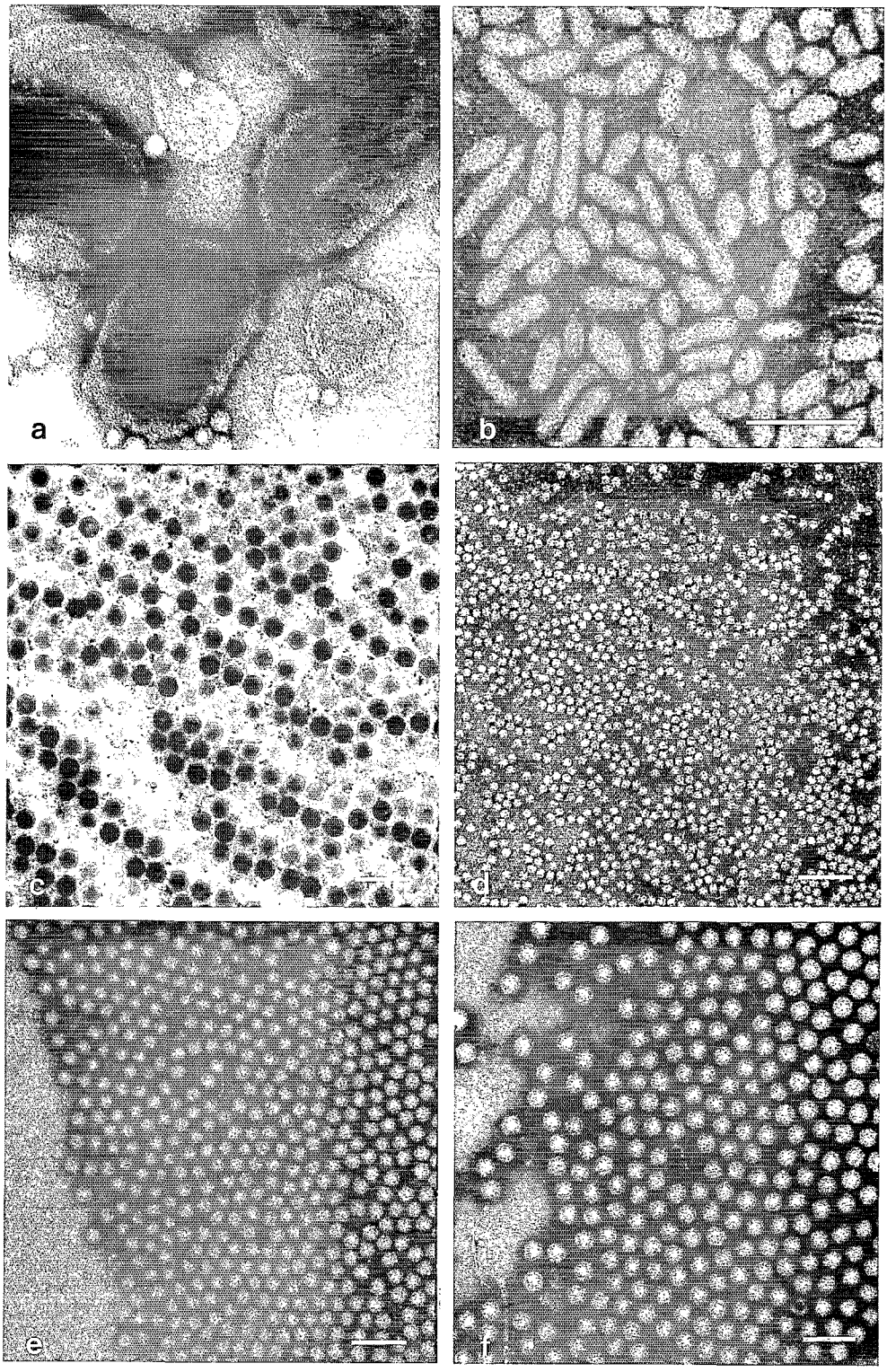


Fig. 3. Electron micrographs of representative types of virus particles from bees. (a) filamentous virus; (b) chronic paralysis virus; (c) *Apis* iridescent virus in ultra thin section of the cytoplasm of adult bee fat body cell; (d) particles 17 nm in diameter (cloudy wing virus, chronic paralysis virus associate); (e) particles 30 nm in diameter (acute paralysis, Arkansas, black queen cell, deformed wing, Egypt bee, Kashmir bee, sacbrood and slow paralysis viruses); (f) particles 35 nm in diameter (bee viruses X and Y). Scale bars = 100 nm except (c) where scale bar = 500 nm.

For most purposes rabbits are convenient laboratory animals for antiserum production and polyclonal antisera (sera that contain antibodies to different virus epitopes) are easier to produce and suitable for diagnostic tests. An outline of a suggested protocol is given in Table 1. A pre-immune bleed from an ear vein is usually made about a month before the rabbit is immunised. About 1 mg or more of antigen (virus preparation) in 0.5 ml of phosphate buffered saline is added to 0.5 ml of an immunostimulant (such as Titermax) and mixed thoroughly to form a good emulsion. 0.5 ml of the mixture is injected into each thigh muscle of the rabbit. This method of inoculation gives a better antigenic response as the antigen is released slowly and produces a prolonged challenge; intravenous injection often requires several immunisations. The first bleed is taken from the rabbit four weeks after immunisation and subsequent bleeds are at three week intervals. Approximately 25 ml of blood are collected at each bleed and this is maintained at room temperature for about four hours to clot. The blood is then centrifuged for 15 min at 2000 g and the clear, straw-coloured serum collected. If the titre of the antiserum from initial bleeds is low, a booster injection of a further 0.5 ml of virus (approx. 0.5 mg) with adjuvant is given. The antiserum is best stored frozen at -20°C and the bleed selected for use may be divided into small aliquots or stored at appropriate dilutions for use so that repeated thawing and freezing does not occur. Each batch of antiserum should be titrated against the virus preparation used for immunisation, to obtain the maximum dilution at which a visible line of reaction is produced by immunodiffusion. Working strength antiserum is usually about two dilution steps below the maximum titre. The antiserum should also be checked for cross reactivity against other virus isolates if these are available, and for non-specific reactivity to both adult bee and pupal protein.

Table 1. Production of polyclonal antibodies†

1. Bleed the rabbit, via the lateral ear vein, prior to immunisation. Up to 30 ml can be collected in a centrifuge tube. This pre-bleed is used as a control to ensure any antibody activity is due to the immunisation.
2. Immunise the rabbit intramuscularly, with up to 2 mg of antigen in 0.5 ml PBS emulsified in 0.5 ml Freunds complete adjuvant. The injection is given in two sites in the thigh muscle.
3. Wait for 4 weeks and immunise as above, but with the antigen emulsified in Freunds incomplete adjuvant.
4. After a 2 week rest, bleed the rabbit as for the pre-bleed.
5. Allow the blood to clot at room temperature (up to 4 h) and then spin at 2000 g for 15 min.
6. Carefully decant the serum fraction (supernatant) from the tube.
7. Test the antibody titre by immunodiffusion or ELISA. If the titre is sufficiently high (1:1000) continue bleeding at 3 week intervals. If not, repeat step 3.
8. Continue to take up to 10 bleeds, or until the titre drops to below 1:1000.
9. Store the collected serum in aliquots at -20°C, or with glycerol added to a final concentration of 50% at 4°C.

Note: Antisera should not be frozen and thawed repeatedly, as this can lead to loss of activity due to antibody aggregation which blocks the binding sites.

†Most polyclonal antibodies are produced in rabbits, as they are large enough to give a useful amount of sera, but not so large as to be difficult to handle.

The immunodiffusion test

For diagnostic purposes the simplest, quickest and most inexpensive serological test is immunodiffusion (Fig. 4). The agar for the test contains buffer of suitable molarity and pH to maintain the virus of interest dispersed. Large aggregates of virus particles formed under unsuitable conditions will not diffuse through the agar. A template is used to ensure that the wells cut in the agar are of the same size and equidistant and the test extracts and antisera are added in an appropriate arrangement. Several extracts of adult bees, parts of bees or individual larvae may be tested against one antiserum or one extract may be tested against several different antisera. A virus and its homologous antiserum diffuse through the agar and where they meet in optimum concentration an insoluble complex is formed which produces a visible line (Fig. 5a). The antiserum will only bind to the protein against which it was produced; non related viruses will not react. Although the immunodiffusion test is very specific (provided that the antiserum is monospecific) it is relatively insensitive; about 10^9 particles are required to give a clear positive reaction. However, single bees or even parts of a bee that have been killed by virus replication will contain sufficient virus to be readily detected in this way. The results of tests can be read the following

day and the immunodiffusion plates only require a moist environment and a stable temperature for best results. The test is also useful for determining relationships between viruses where reactions of partial identity can provide information on differences in antigenic sites (epitopes) between virus isolates.

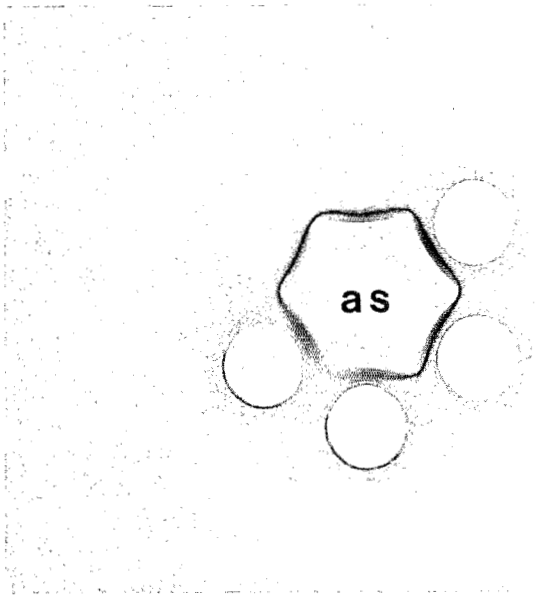


Fig. 4. Immunodiffusion test for the diagnosis of honey bee viruses. Extracts of bees infected with acute paralysis virus in the outer six wells have reacted with acute paralysis virus antiserum (as) in the central well. The confluent lines of reaction have been stained to improve visualisation.

Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay is a much more sensitive means of virus detection and it has the added advantage of providing a quantitative estimate of virus present in a sample, when used in conjunction with virus standards of known concentration. The specificity of the antiserum is very important since even low cross-reactivity to contaminating bee protein can be amplified to obscure the results. There are many different types of ELISA now in general use but the indirect method described here has the advantage that the protein A-enzyme conjugate can be used with any antiserum and the only pre-treatment that is required is the production of virus specific $F(ab')_2$ fragments from the IgG (Fig. 5b).

An outline of a generalised method for an indirect ELISA is given in Fig. 6. The $F(ab')_2$ fragments of the IgG are used at a pre-determined dilution to coat the wells of a plastic microtitre plate. After incubation at 30°C for four hours the contents of the wells are discarded and the plates are thoroughly washed. The samples to be assayed are then added, the plates are sealed with plastic film and incubated in the fridge overnight. Any virus in the test samples which is homologous to the antibody coating the wells, will be bound. The plates are washed again the following morning and a suitable dilution of intact IgG (with the Fc' portion still present) is added. This second portion of the same antiserum binds to virus that has been trapped on the surface of the well by the coating antibody. The plates are incubated at 30°C for 3 h, washed and the protein A-enzyme conjugate added. The protein A binds to the Fc' portion of the IgG so that it may be used for an antiserum produced against any honeybee virus. Several enzymes can be conjugated to protein A but the ones that are most commonly available from commercial companies are alkaline phosphatase and horseradish peroxidase. Excess protein A-enzyme conjugate is removed by washing after incubation at 30°C for three hours and the enzyme substrate added. The reaction is stopped after 20 min by the addition of the appropriate inhibitor and the optical density of each well is read in a spectrophotometer or microelisa reader. The intensity of the colour (or absorbance reading) is proportional to the amount of enzyme and therefore the amount of virus, in the original test sample. If a dilution series of purified virus of known concentration is included on

the plate a standard curve may be plotted of absorbance against virus concentration. From this, an estimate of the amount of virus in test extracts may be determined.

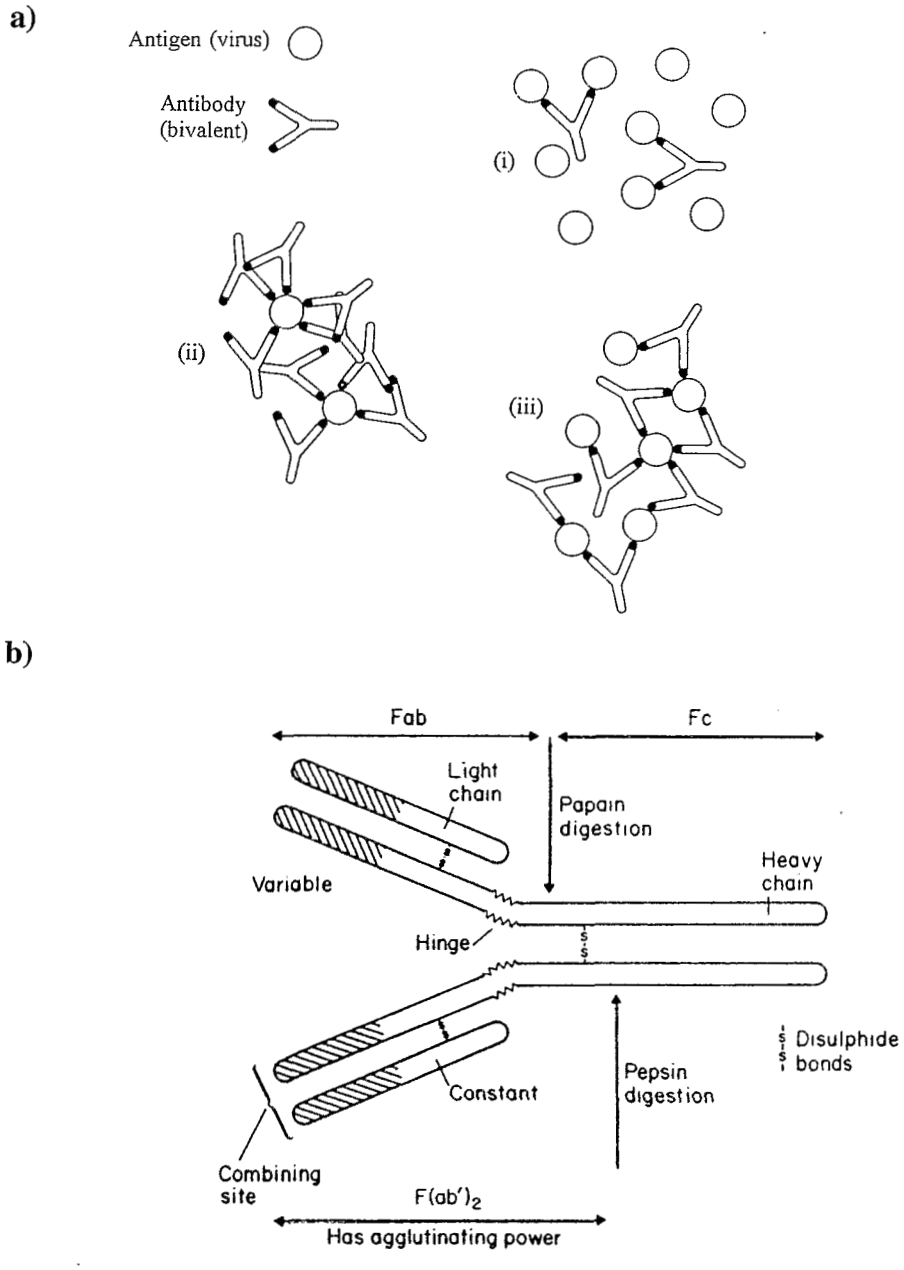
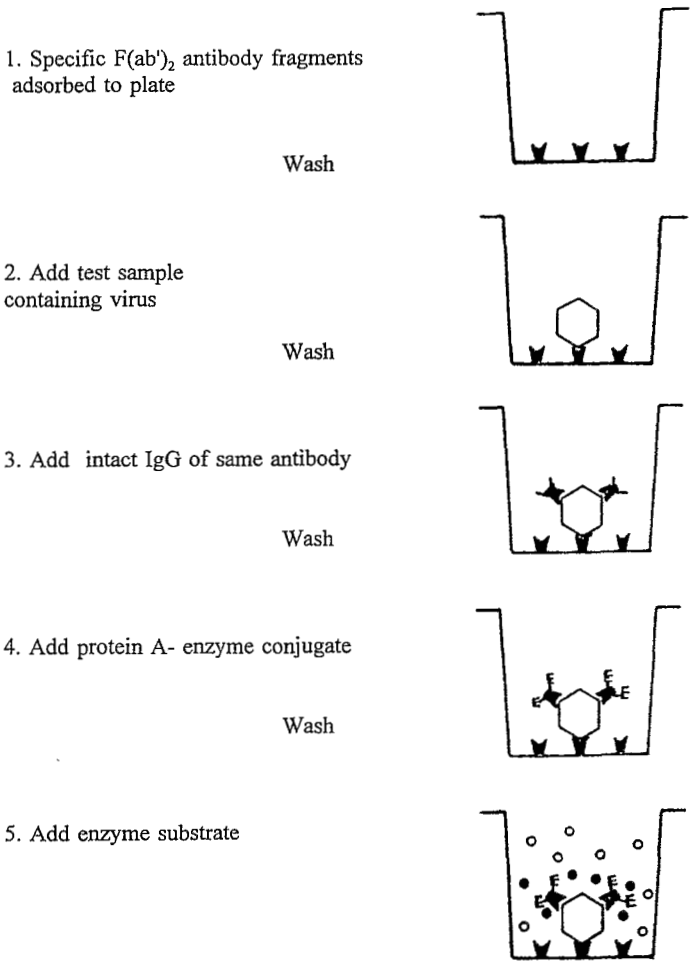


Fig. 5. (a) The antigen/antibody precipitation process. Antigen excess (i) and antibody excess (ii) form soluble invisible precipitates. Antigen and antibody in optimum proportions (iii) give a visible lattice. (b) Diagrammatic structure of the IgG molecule.

This type of indirect ELISA has been shown to be able to detect as little as 3 ng ml^{-1} of virus and it is a useful technique for studies of virus epidemiology or for detecting virus in individual *Varroa jacobsoni* mites that are acting as virus vectors. However, most virus infections of bees persist in the population as inapparent infections which are at concentrations below the threshold of detection by ELISA. The technique is also too time consuming and costly to be used for routine diagnosis where a number of different antisera may need to be used. For determining the cause of mortality in natural disease outbreaks the immunodiffusion test is still the most appropriate means of diagnosis.



After 20 mins stop reaction and read absorbance. Colour intensity in the final evaluation is proportional to virus concentration.

Fig. 6. Outline method for an indirect ELISA.

Western blotting

This technique uses antibodies to probe the virus proteins after they have been denatured and separated by polyacrylamide gel electrophoresis (PAGE). Although the reactivity of antisera to denatured protein is usually weaker than that to native protein the technique can be useful to compare the reactivity of capsid proteins of similar molecular weight from related virus strains.

The first stage of the process requires the denaturation of the virus coat proteins by heating a purified virus preparation to 100°C for 2-5 min in the presence of sodium dodecyl sulphate (SDS) and reducing agents. The proteins unfold and bind to the SDS which imparts a very strong negative charge to the protein, dominating its native charge. Thus the charge:mass ratio becomes constant for virtually all proteins. Under these conditions the electrophoretic mobility in acrylamide gels is inversely proportional to the logarithm of the molecular weight. A range of standard proteins of known molecular weight are included on the same gel and their relative mobility plotted against the logarithm of their molecular weight. The distance migrated by viral proteins can therefore be used to estimate their molecular weight. This technique alone is useful for the characterisation of different viruses since the number and size of their constituent capsid proteins differ and characteristic banding patterns are produced.

For Western blotting, the proteins are eluted electrophoretically from the PAGE gel onto a membrane which binds protein tightly. The membrane is then probed with homologous antiserum (produced in a rabbit) and finally incubated in anti-rabbit IgG conjugated to the enzyme alkaline phosphatase. The protein bands which have bound the antibody are visualised on addition of the enzyme substrate. The technique can be useful in differentiating virus strains and clarifying relationships. However, virus structural proteins may not all be exposed or highly antigenic and because secondary and tertiary protein structure may be destroyed some antigenic sites may also be lost.

Cultivation and Purification of bee viruses

Many of the viruses of bees can be readily cultivated in the laboratory, either in adult bees or in bee pupae. Experimental infection can provide information on the incubation period, the mode of infection and the minimum infective dose. Inoculation by injection into the haemocoel through the abdominal intersegmental membrane is a very sensitive means of detection of certain viruses and the technique is useful for propagating virus for antiserum production. Suitable life stages and methods of inoculation for the cultivation of honeybee viruses are shown in Table 2.

Table 2. Cultivation of honey bee viruses

Virus	Instar†	Method of infection††	Incubation period (days) †††
Chronic paralysis virus	A	I	7
	P	I	5
Chronic paralysis virus associate	A queens	I	7
	A queens	I	5
Cloudy wing virus	A	?	?
	P	I	?
Acute paralysis virus	A	I	5
	P	I	5
Arkansas bee virus	A	I	21
	P	I	5
Black queen cell virus	P	I	5
Deformed wing virus	P	I	14
Egypt bee virus	P	I	8
Kashmir bee virus	A	I	3
	P	I	3
Sacbrood virus	L	F	7
	P	I	5
Sacbrood virus (Thai strain)	P	I	5
Slow paralysis virus	A	I	12
Apis iridescent virus	P	I	5
Bee virus X	NA	F	30
Bee virus Y	NA	F	30
Filamentous virus	A	?	?

†A = adults in cages at 35°C; P = pupae in Petri dishes at 96% R.H. and 35°C; L = larvae two days old, kept in bee colonies until cells sealed, then incubated without adult bees at 35°C; NA = newly emerged adults in cages at 35°C supplied with pollen.

†† I = by injection into haemocoele through abdominal intersegmental membrane; F = in food.

††† Based on infection with minimum infective doses.

Adult bees are best collected by placing a comb taken from the outer edges of the brood nest into a suitable box that can be filled with carbon dioxide. The gas is taken from a pressurised cylinder and passed through water, or a large empty vessel, to remove or melt frozen particles of solid carbon dioxide which would otherwise damage the bees. The bees quickly become anaesthetised and can be placed in suitable cages to recover at room temperature, before transferring them to incubators at 30°C or 35°C. The cages should be supplied with 65% sucrose solution and water. The bees are anaesthetised again

when they are injected with virus preparations by micro-syringe, but this is best done the next day because they sometimes die after they have been anaesthetised more than once in 24 hours.

Some of the viruses of bees are only infective by feeding, and incubation periods are protracted. Newly emerged individuals are the most convenient life stage for propagating these particles. Combs containing sealed brood of the correct age can be cleaned of adult bees and maintained in an incubator until the brood emerges. Bees less than 24 hours old can be fed individually with measured amounts of the virus inoculum mixed with a little dilute honey. The bees are then maintained in cages, as above, except they must be supplied with fresh pollen for their first week of life.

Injection of bee pupae at the white-eyed stage of development is an excellent means of propagating most of the viruses. Pupae can be carefully removed from their cells, inoculated and incubated in Petri dishes at 96% relative humidity and 30°C or 35°C. Pupae inoculated with sterile water or buffer and similarly maintained provide an indication of the practical skills of the experimenter since most, if not all, should develop to emergence and they are a useful guide to the arrestment of development of virus inoculated individuals.

When propagating viruses by injection it is always best to work at the highest dilution of inoculum that produces infection. This will tend to dilute out any other undesirable virus particles that may be present in inocula prepared from natural infections and also usually increases virus yields. Serially passaging inocula should be avoided as this increases the risk of contaminating preparations with viruses that normally persist in bees and brood as inapparent infections; these may multiply at a faster rate than the virus being propagated.

A generalised method for the extraction and purification of bee viruses is given in Table 3 and more detailed information on the conditions found suitable for the purification of individual viruses are given in Bailey and Ball (1991). However, these are not inflexible and may be adapted or improved upon.

Table 3. Outline method for the extraction and purification of bee viruses

1. Grind insects in appropriate buffer and solvents.
2. Filter through cotton or cheesecloth.
3. Clarify by slow-speed centrifugation.
4. Sediment virus from supernatant fluid by high-speed centrifugation.
5. Resuspend pellet in buffer.
6. Clarify by slow-speed centrifugation.
7. Repeat 4, 5 and 6.
8. Centrifuge down 10-40% (W/V) sucrose gradients.
9. Locate and remove virus fraction.
10. Dialyse fractions against buffer.
11. Sediment virus by high speed centrifugation.
12. Resuspend pellet in buffer.
Note: Virus to be used for antiserum production should be purified by two cycles of centrifugation down sucrose gradients followed by centrifugation in caesium chloride solutions, adjusted to the density of the particle.

References

Bailey, L. and Ball, B.V. (1991). *Honey Bee Pathology.* 2nd edn. Academic Press, Harcourt Brace Jovanovich, Publishers, London.

Further reading

Hampton, R., Ball, E. and De Boer, S. (eds) (1990). *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens: A Laboratory Manual.* American Phytopathological Society, USA.

Johnstone, A. and Thorpe, R. (1987). *Immunochemistry in Practice.* 2nd edn. Blackwell Scientific Publications, London.

Jones, R.A.C. and Torrance, L. (eds) (1986). *Developments and Applications in Virus Testing.* Proceedings of a Conference at the University of Cambridge, 1985. Association of Applied Biologists, Wellesbourne, UK.

Maizel, J.V. Jr. (1971). Gel electrophoresis of proteins. In *Methods in Virology,* Vol. 5, Maramorosch, K. and Koprowski, H. (eds). Academic Press, New York, pp. 179-246.