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REVIEW ARTICLE

Standard methods for virus research in *Apis mellifera*

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Summary

Honey bee virus research is an enormously broad area, ranging from subcellular molecular biology through physiology and behaviour, to individual and colony-level symptoms, transmission and epidemiology. The research methods used in virology are therefore equally diverse. This article covers those methods that are very particular to virological research in bees, with numerous cross-referrals to other *BEEBOOK* papers on more general methods, used in virology as well as other research. At the root of these methods is the realization that viruses at their most primary level inhabit a molecular, subcellular world, which they manipulate and interact with, to produce all higher order phenomena associated with virus infection and disease. Secondly, that viruses operate in an exponential world, while the host operates in a linear world and that much of the understanding and management of viruses hinges on reconciling these fundamental mathematical differences between virus and host. The article concentrates heavily on virus propagation and methods for detection, with minor excursions into surveying, sampling management and background information on the many viruses found in bees.

Métodos estándar para la investigación de virus en *Apis mellifera*

Resumen

La investigación de los virus de la abeja de la miel es un área sumamente amplia, que abarca desde la biología molecular subcelular hasta la fisiología y el comportamiento, desde síntomas al nivel de individuo hasta al nivel de la colmena, transmisión y epidemiología. Los métodos de investigación en virología son, por tanto, diversos. Este artículo incluye aquellos métodos específicos de la investigación virológica en las abejas, con numerosas referencias cruzadas con otros artículos del BEEBOOK y otros más generales, usados tanto en virología como en otras disciplinas. La base de estos métodos es la comprensión de los virus en su nivel primario de hábitat molecular, ambiente subcelular, que manipulan y con el que interactúan, para producir otros fenómenos de orden superior asociados a la infección del virus y la enfermedad. En segundo lugar, estos virus actúan en un mundo exponencial, mientras que los hospedadores actúan en un mundo lineal y gran parte del entendimiento y manejo de los virus depende de los fundamentos matemáticos de las diferencias entre el virus y el hospedador. El artículo se centra principalmente en la propagación de virus y en los métodos para su detección, con inclusiones menores en su estudio, el manejo del muestreo y la información general sobre los numerosos virus que se encuentran en las abejas.

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西方蜜蜂病毒研究的标准方法

摘要

蜜蜂病毒研究是一个非常广阔的领域，涉及亚细胞分子生物学、生理学和行为学、个体和蜂群症状、传播和流行病学。因此病毒学研究中用到的方法种类繁多。本文涵盖了蜜蜂病毒学研究中特有的一些方法。其中涉及大量在病毒学和其它研究中都有用到的方法，在*BEEBOOK*关于普通方法的章节中已有介绍。这些方法的根源是认识到病毒归根到底生活于一个分子和亚细胞世界，它们操控和作用于这一环境，以产生和病毒感染与疾病相关的更高级别的现象。其次，病毒在指数世界运行，而宿主在线性世界运行，理解和控制病毒很大程度上依赖于协调病毒和宿主之间的这些基本数学差异。本文重点针对病毒复制和病毒检测方法，也提及调查、取样操作及蜜蜂上发现的一些病毒的背景信息。

Keywords: COLOSS *BEEBOOK*, honey bee, virus, sampling, purification, assays, standardisation

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1. Introduction

1.1. Honey bee viruses

There are currently about 24 viruses identified in honey bees, whose physical and biological properties are described in Table 1 and Table 2 respectively. Most of these were discovered by Bill Bailey, Brenda Ball and colleagues at Rothamsted Experimental Station, UK during the 1960s-1980s (Bailey and Ball, 1991; Ribière *et al.,* 2008). More recent additions have come mostly from mass sequencing of RNA and DNA from whole bee extracts (Fujiyuki *et al.,* 2004; Cox-Foster *et al.,* 2007; Cornman *et al.,* 2010; Runckel *et al.,* 2011), and it may well be that there is overlap between the traditionally described viruses and these newly described viral sequences. Several viruses are also closely enough related to be regarded as members of a single species complex (DWV/VDV-1/EBV; ABPV/KBV/IAPV; SBV/TSBV; BVX/BVY and LSV-1/LSV-2), reducing the total to around 16-18 truly unique viruses.

Although some viruses produce recognizable symptoms at sufficiently elevated titres, honey bee viruses generally persist naturally in honey bee populations at low levels, without causing overt symptoms, using a variety of transmission routes (Fig. 1; Table 2). Symptoms are, however, still the principal method by which diseases are diagnosed in the apiary. The advantages of symptom-based diagnosis are that it is robust, simple, fast and cheap and for some diseases accurate. The major disadvantages are that:

- many virus infections do not present visible symptoms at all times
- not all life stages present symptoms
- often different viruses produce similar symptoms (*e.g.* paralysis)
- a single virus may present different symptoms (*e.g.* CBPV)
- symptoms can be confounded if multiple virus infections are present

All viruses are asymptomatic at lower levels of infection and most shorten the life span of bees to varying degrees. The diagnostic symptoms for the major virus diseases have been described in detail by Bailey and Ball (1991) and can be summarized as follows:

1.1.1. Acute bee paralysis virus /Kashmir bee virus /Israeli acute paralysis virus

Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV) are three closely related viruses (de Miranda *et al.,* 2010a) that are largely symptomless, but they can be lethal at individual and colony level (Allen and Ball, 1995; Todd *et al.,* 2007), particularly when transmitted by *Varroa destructor* (Ball, 1985; 1989; Ball and Allen, 1988) which is an active vector of these viruses (Chen *et al.,* 2004a; Shen *et al.,* 2005a; 2005b; DiPrisco *et al.,* 2011). These viruses are characterized by the ability to kill both pupae (after injection; Bailey, 1967; Bailey and Ball, 1991) and adult bees (after injection or feeding: Maori *et al.,* 2007a; 2009; Hunter *et al.,* 2010) very rapidly; 3-5 days after inoculation with sufficient virion loads. This exerts a strong negative selection pressure on the transmission by varroa, since infected pupae fail to complete development, preventing the release of infectious mites from the pupal cells (Sumpter and Martin, 2004). The association of these viruses with varroa infestation is therefore unstable and much influenced by the presence of other viruses that are better adapted to transmission by varroa.

1.1.2. Black queen cell virus

The main symptoms for black queen cell virus (BQCV) consist of blackened cell walls of sealed queen cells, containing dead pro-pupae (Bailey and Ball, 1991; Leat *et al.,* 2000). Diseased larvae have a pale yellow appearance and tough sac-like skin, much like sacbrood. The virus is present in adult bees but without obvious symptoms.

1.1.3. Aphid lethal paralysis virus & Big Sioux River virus

Aphid lethal paralysis virus (ALPV) is a common intestinal dicistrovirus of several major agricultural aphid pests, associated with aphid population declines (van Munster *et al.,* 2002 Laubscher and von Wechmar, 1992; 1993). Big Sioux River virus (BSRV) is closely related to *Rhopalosiphum padi* virus (RhPV; Moon *et al.,* 1998), another common intestinal Dicistrovirus that uses the plant vascular system to transmit horizontally between aphids (Gildow and D'Arcy, 1990). Both can be detected infrequently at very low background levels in adult honey bees throughout the year, with a sharp quantitative increase during late summer (Runckel *et al.,* 2011) when bees often feed on honeydew (aphid excreta) during low nectar flows. It is unclear therefore whether these viruses are incidental or truly infectious in bees. Either of these may be related to Berkeley bee picorna-like virus (BBPV; Lommel *et al.,* 1985), which has not yet been sequenced.

1.1.4. Deformed wing virus /kakugo virus /Varroa destructor virus-1 /Egypt bee virus

The symptoms for deformed wing virus (DWV) consist of bees with crumpled and/or vestigial wings and bloated abdomen and infected bees die soon after emergence. Asymptomatic bees can also be heavily infected, though with lower titres than symptomatic bees (Bowen-Walker *et al.,* 1999; Lanzi *et al.,* 2006; Tentcheva *et al.,* 2006). The virus is detected in all other life stages as well, but without obvious symptoms (Chen *et al.,* 2005a; 2005b; Yue and Genersch 2005; Lanzi *et al.,* 2006; Tentcheva *et al.,* 2006; Fievet *et al.,* 2006; Yue *et al.,* 2006; de Miranda and Genersch, 2010). 'kakugo' virus (KV; Fujiyuki *et al.,* 2004; 2006) and other strains of DWV (Terio *et al.,* 2008) have been associated with elevated aggression in bees, although naturally aggressive bee races are not more infected with DWV than gentle bee races (Rortais *et al.,* 2006). DWV also affects sensory response, learning and memory in adults (Iqbal and Müller, 2007).

Table 1. Summary of the physical properties, such as particle shape, size, capsid protein profile, genome type and length and taxonomy, of the currently known honey bee viruses. Adapted from Bailey and Ball (1991).

		PHYSICAL PROPERTIES					
VIRUS		SHAPE	SIZE	CAPSID PROTEINS	NUCLEIC ACID	GENOME SIZE	TAXONOMY
Acute bee paralysis virus	ABPV	icosahedral	30nm	35-9-33-24kDa	ssRNA	~9.5kb	Dicistroviridae
Kashmir bee virus	KBV	icosahedral	30nm	37-6-34-25kDa	ssRNA	~9.5kb	Dicistroviridae
Israeli acute paralysis virus	IAPV	icosahedral	30nm	35-7-33-26kDa	ssRNA	~9.5kb	Dicistroviridae
Black queen cell virus	BQCV	icosahedral	30nm	31-14-29-30kDa	ssRNA	~9.5kb	Dicistroviridae
Aphid lethal paralysis virus	ALPV	icosahedral	30nm	25-7-32-28kDa*	ssRNA	~10kb	Dicistroviridae
Big Sioux River virus	BSRV	icosahedral	30nm	28-5-29-30kDa	ssRNA	~10kb	Dicistroviridae
Deformed wing virus	DWV	icosahedral	30nm	32-2-44-28kDa	ssRNA	~10kb	Iflaviridae
Varroa destructor virus-1	VDV-1	icosahedral	30nm	32-2-46-28kDa	ssRNA	~10kb	Iflaviridae
Egypt bee virus	EBV	icosahedral	30nm	30-2-41-25kDa	ssRNA	?	Iflaviridae
Sacbrood virus	SBV	icosahedral	30nm	31-2-32-30kDa	ssRNA	~9kb	Iflaviridae
Thai/Chinese sacbrood virus	TSBV	icosahedral	30nm	31-2-32-30kDa	ssRNA	~9kb	Iflaviridae
Slow bee paralysis virus	SBPV	icosahedral	30nm	27-2-46-29kDa	ssRNA	~9.5kb	Iflaviridae
Chronic bee aralysis virus	CBPV	anisometric	30~60nm	23-(30/50/75?)kDa	ssRNA	~2.3kb/~3.7kb	unclassified
Chronic bee paralysis satellite virus	CBPSV	icosahedral	17nm	15kDa	ssRNA	(3x)~1.1kb	satellite
Cloudy wing virus	CWV	icosahedral	17nm	19kDa	ssRNA	~1.4kb	?
Bee virus-X	BVX	icosahedral	35nm	52kDa	ssRNA	?	?
Bee virus-Y	BVY	icosahedral	35nm	50kDa	ssRNA	?	?
Lake Sinai Virus-1	LSV-1	?	?	63kDa*	ssRNA	~5.5kb	unclassified
Lake Sinai Virus-2	LSV-2	?	?	57kDa*	ssRNA	~5.5kb	unclassified
Arkansas bee virus	ABV	icosahedral	30nm	43kDa	ssRNA	~5.6kb	?
Berkeley bee picorna-like virus	BBPV	icosahedral	30nm	37-?-35-32kDa	ssRNA	~9kb	?
Varroa destructor Macula-like virus	VdMLV	icosahedral	30nm	24kDa*	ssRNA	~7kb	Tymoviridae
Apis mellifera filamentous virus	AmFV	rod	150x450nm	12x(13~70kDa)	dsDNA	?	Baculoviridae
Apis iridescent virus	AIV	polyhedral	150nm	?	dsDNA	?	Iridoviridae
		* (genome predicted)		SDS-PAGE	(order in polyprotein)		

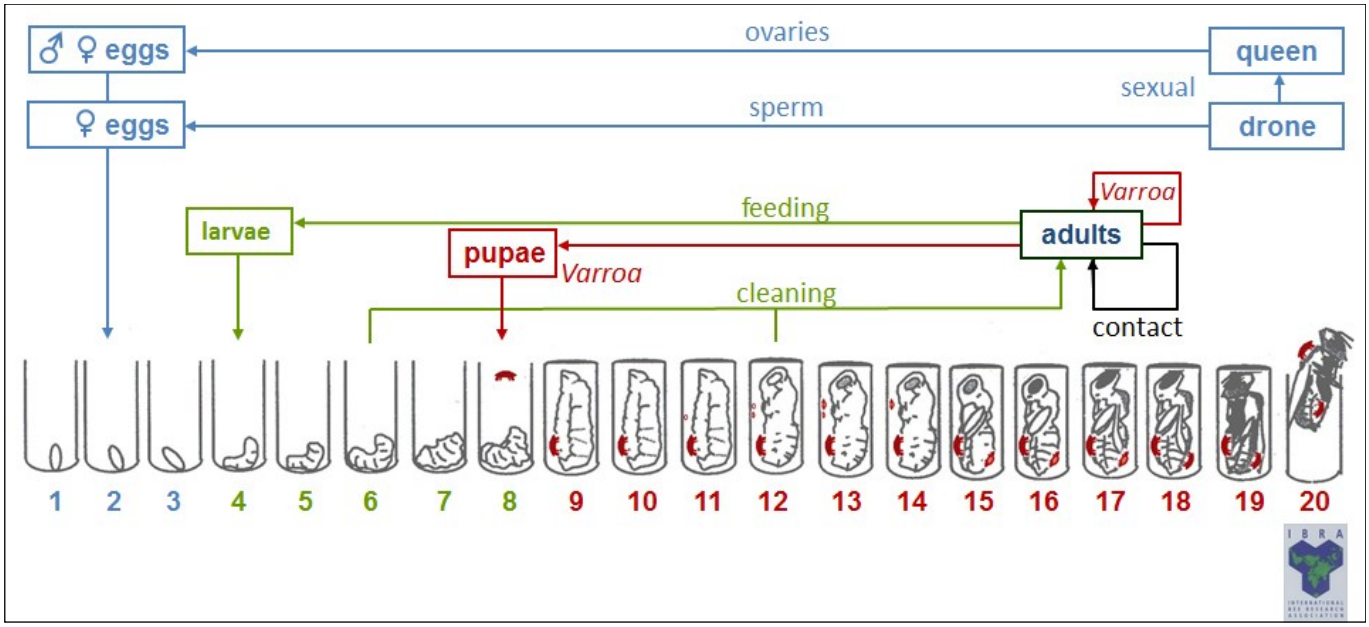

Fig. 1. Diagram describing the different possible transmission routes for honey bee viruses. Adapted from de Miranda *et al.* (2011).

Table 2. Summary of the current state of knowledge concerning biological properties of honey bee viruses, such as transmission routes, associations with other parasites/pathogens, principal life stages affected and seasonal incidences. Adapted from de Miranda *et al.* (2011).

		TRANSMISSION							ASSOCIATION				LIFE STAGE				SEASON		
		HORIZONTAL				VERTICAL							INFECT/SYMPTOMS						
VIRUS		ORAL-FECAL	CONTACT	AIR	VARROA	OVARIES	SEMEN	SPERM	VARROA	ACARAPIS	NOSEMA	MALPIGHAMOEBA	EGGS	LARVAE	PUPAE	ADULTS	SPRING	SUMMER	AUTUMN
Acute bee paralysis virus	ABPV	+	-	?	+	+	+	?	+	?	?	?	+/-	+/-	+/~	+/+	+	+++	++
Kashmir bee virus	KBV	+	-	?	+	+	~	?	+	?	?	?	+/-	+/-	+/+	+/+	+	++	+++
Israeli acute paralysis virus	IAPV	+	-	?	+	+	~	?	+	?	?	?	+/-	+/-	+/~	+/+	+	++	++
Black queen cell virus	BQCV	+	-	?	~	+	?	?	+	?	+	?	+/-	+/-	+/+	+/-	+	+++	+
Aphid lethal paralysis virus	ALPV	?	?	?	?	?	?	?	?	?	?	?	?/?	?/?	-/-	+/?	-	+++	-
Big Sioux River virus	BSRV	?	?	?	?	?	?	?	?	?	?	?	?/?	?/?	-/-	+/?	-	+++	++
Deformed wing virus	DWV	+	-	?	+	+	+	?	+	?	?	?	+/-	+/-	+/+	+/+	+	++	+++
Varroa destructor virus-1	VDV-1	+	-	?	+	+	+	?	+	?	?	?	+/-	+/-	+/+	+/+	+	++	+++
Egypt bee virus	EBV	?	?	?	?	?	?	?	?	?	?	?	?/?	?/?	+/?	+/~	?	?	?
Sacbrood virus	SBV	+	-	?	-	?	?	?	~	?	?	?	?/?	+/+	+/-	+/~	+++	++	+
Thai/Chinese sacbrood virus	TSBV	+	?	?	?	?	?	?	?	?	?	?	?/?	+/+	+/-	+/~	?	?	?
Slow bee paralysis virus	SBPV	+	-	?	+	?	?	?	+	?	?	?	?/?	+/-	+/-	+/+	+	+	+
Chronic bee paralysis virus	CBPV	+	+	?	-	?	?	?	~	~	?	?	~/-	+/-	+/-	+/+	++	++	+
Chronic bee paralysis satellite virus	CBPSV	?	?	?	?	?	?	?	?	?	?	?	?/?	?/?	?/?	+/?	+	+	+
Cloudy wing virus	CWV	?	~	~	-	?	?	?	~	?	?	?	-/-	~/-	~/-	+/+	+	+	+
Bee virus-X	BVX	+	?	?	?	?	?	?	?	?	-	+	-/-	-/-	-/-	+/+	+++	+	+
Bee virus-Y	BVY	+	?	?	?	?	?	?	?	?	+	-	-/-	-/-	-/-	+/+	+	+++	+
Lake Sinai Virus-1	LSV-1	?	?	?	?	?	?	?	?	?	?	?	?/?	?/?	+/?	+/?	++	+++	++
Lake Sinai Virus-2	LSV-2	?	?	?	?	?	?	?	?	?	?	?	?/?	?/?	~/?	+/?	+++	+	+
Arkansas bee virus	ABV	?	?	?	?	?	?	?	?	?	?	?	?/?	?/?	~/?	+/?	?	?	?
Berkeley bee picorna-like virus	BBPV	?	?	?	?	?	?	?	?	?	?	?	?/?	?/?	?/?	+/?	?	?	?
Varroa destructor Macula-like virus	VdMLV	?	?	?	+	?	?	?	+	?	?	?	?/?	?/?	+/?	+/?	+	++	+++
Apis mellifera filamentous virus	AmFV	+	?	?	?	?	?	?	?	?	+	?	-/-	-/-	-/-	+/+	+++	+	+
Apis iridescent virus	AIV	?	?	~	?	?	?	?	?	?	?	?	-/-	-/-	-/-	+/+	+	++	+
		+	(present)									~	(uncertain)			?	(unknown)		

Varroa destructor virus-1 (VDV-1) is genetically closely related to DWV but is reported to be more specific to *Varroa destructor* than to bees (Ongus, 2006). However, both viruses replicate in varroa mites as well as in honey bees (Ongus *et al.,* 2004; Yue and Genersch, 2005; Zioni *et al.,* 2011); both have been detected at high titres in different honey bee tissues (Zioni *et al.,* 2011; Gauthier *et al.,* 2011); both have been found in regions where *V. destructor* is absent (Martin *et al.,* 2012) and natural recombinants between them have been found (Moore *et al.,* 2011). VDV-1 and DWV therefore appear to co-exist in bees and mites as part of the same species-complex (de Miranda and Genersch, 2010; Moore *et al.,* 2011; Gauthier *et al.,* 2011; Martin *et al.,* 2012).

Egypt bee virus (EBV) is serologically related to DWV, but has no known symptoms in adults, pupae or larvae (Bailey *et al.,* 1979).

1.1.5. Sacbrood virus /Thai sacbrood virus

The clearest symptoms of sacbrood virus (SBV) appear a few days after capping, and consist of non-pupated pale yellow larvae, stretched on their backs with heads lifted up towards the cell opening, trapped in the unshed, saclike larval skin containing a clear, yellow-brown liquid. The virus is also present in adult bees, but without symptoms (Lee and Furgula, 1967; Bailey, 1968). Diseased larvae are most commonly seen in spring, but the disease normally clears quickly with rapid expansion. However, the Asian honey bee, *Apis cerana*, frequently suffers from lethal sacbrood epidemics caused by a closely related strain of SBV, variously called Thai sacbrood virus (TSBV), Chinese sacbrood virus (CSBV) or Korean sacbrood. The genetic differences of these strains with the SBV infecting *A. mellifera* are minimal. SBV-infected adults cease to attend brood or eat pollen, start foraging much sooner than normal, and only forage nectar, rarely pollen (Bailey and Fernando, 1972). These may be behavioural adaptations by *A. mellifera* to prevent sacbrood epidemics, since SBV is shed in the hypopharyngeal secretions fed to larvae and combined with pollen to make bee-bread (Bailey and Ball, 1991).

1.1.6. Slow bee paralysis virus

Slow bee paralysis virus (SBPV) is characterised by the paralysis of the front two pairs of legs of adult bees, a few days before dying, after inoculation by injection (Bailey and Woods, 1974). The virus is associated with, and transmitted by, *V. destructor* (Bailey and Ball, 1991; Denholm, 1999). Despite this association, SBPV is rarely detected in bee colonies (Bailey and Ball, 1991; de Miranda *et al.,* 2010b). SBPV can also be detected in larvae and pupae, but produces no symptoms in these.

1.1.7. Chronic bee paralysis virus /satellite virus

Chronic bee paralysis virus (CBPV) manifests itself in adult bees through two distinct set of symptoms. One set consists of trembling of the wings and bodies and a failure to fly, causing them to crawl in front of the hive in large masses. They often have partly spread, dislocated wings and bloated bodies as well. The other set of symptoms consists of hairless, greasy black bees caused by nibbling attacks from healthy bees in the colony. They soon also become flightless, tremble and die (Bailey, 1965; Bailey and Ball, 1991; Ribière *et al.,* 2010). The virus also infects the larval and pupal stages, can be detected in faecal material and is efficiently transmitted through contact and feeding (Bailey *et al.,* 1983b; Ribière *et al.,* 2010). CBPV is sometimes associated with a small "satellite" virus; chronic paralysis satellite virus (CBPSV; originally called chronic bee paralysis virus associate CBPVA), which has a unique genome and capsid protein to CBPV (Ribière *et al.,* 2010) and is of unknown significance to symptomatology (Bailey *et al.,* 1980; Ball *et al.,* 1985).

1.1.8. Cloudy wing virus

The symptoms for cloudy wing virus (CWV) consist of opaque wings of severely infected adult bees, with lower titres resulting in asymptomatic infected bees (Bailey *et al.,* 1980; Bailey and Ball, 1991; Carreck *et al.,* 2010). It cannot be propagated in larvae or pupae. It has an unpredictable incidence, no regular associations with other pathogens or pests. Like chronic bee paralysis satellite virus it has a small particle and very small genome, but they are serologically unrelated and their single capsid proteins are of different size (Table 1; Bailey *et al.,* 1980).

1.1.9. Bee virus X /Bee virus Y

Bee virus X (BVX) is largely symptomless in adult bees and does not multiply in larvae or pupae (Bailey and Ball, 1991). It is associated with the protozoan *Malpighamoeba mellificae* that causes dysentery in winter bees (Bailey *et al.,* 1983a). Bee virus Y (BVY) is serologically related to BVX and is similarly symptomless in adult bees, larvae or pupae. It is associated in adult bees with the dysentery inducing microsporidium *Nosema apis* (Bailey *et al.,* 1983a). Both viruses are common, BVY more so than BVX, with strong peaks in late winter for BVX and early summer for BVY (Table 2; Bailey and Ball, 1991).

1.1.10. Lake Sinai virus-1 /Lake Sinai virus-2

Lake Sinai virus-1 (LSV-1) and Lake Sinai virus-2 (LSV-2) are two closely related viruses that were identified in through a mass metagenomic sequencing survey of honey bee colonies in the USA (Runckel *et al.,* 2011). Their genome organization and sequences place them together with CBPV, in a unique family somewhere between the Nodaviridae and Tombusviridae. Both viruses are common and very abundant at peak incidence. LSV-1 is more common than LSV-2, present throughout the year with a peak in early summer. LSV-2 has a very sharp incidence and abundance peak in late winter with low incidence and abundance the rest of the year. These viruses have also been detected, with similar incidences and titres, in historical European honey bee samples. LSV-1 and LSV-2 have strong similarities in capsid and genome size, seasonal incidence, predominantly adult-based infection and absence of overt symptoms with Bee virus Y and Bee virus X respectively (Table 1 and 2), and may therefore be related.

1.1.11. Arkansas bee virus & Berkeley bee virus

Arkansas bee virus (ABV) and Berkeley bee picorna-like virus (BBPV) are two viruses first identified in the USA (Bailey and Woods, 1974; Lommel *et al.,* 1985; Bailey and Ball, 1991) of which very little is known other than that they often occur together. They have no known symptoms in adult bees or brood. BBPV has typical capsid and genome size characteristics of the Dicistro- and Iflaviruses.

1.1.12. *Apis mellifera* filamentous virus

Apis mellifera filamentous virus (AmFV) is a baculovirus-like DNA virus that has no physical symptoms. It renders the haemolymph of adult bees milky white with rod-shaped viral particles, when examined by electron microscopy (Clark, 1978; Bailey and Ball, 1991).

1.1.13. Apis iridescent virus

The symptoms for Apis iridescent virus (AIV) are similar to the adult flightless clustering symptoms of CBPV (Bailey *et al.,* 1976; Bailey and Ball, 1978). It is only known to occur in adult bees. A partial sequence of AIV has been published (Webby and Kalmakoff, 1999).

1.2. Definitions: pathogenicity *vs* virulence; incidence *vs* prevalence

The terms 'Infectivity', 'Pathogenicity', 'Virulence' and 'Transmissibility' are often used interchangeably, which has led to efforts to tighten and standardize their definition and adapt them to our improved understanding of host-pathogen interactions (Casadevall and Pirofski, 1999; 2001). The same is true for the terms 'Incidence' and 'Prevalence' in surveys and epidemiology. Here are their definitions:

- Prevalence: The proportion of a population that is infected, or diseased, at any one time.
- Incidence: This is the risk of new infection during a specified time. It is globally related to prevalence as a function of time: *prevalence* = *incidence* x *time*
- Infectivity: This refers to the ability of a microorganism to invade and replicate in a host tissue, whether the microbe is pathogenic or not.
- Pathogenicity: This is a qualitative trait, referring to the inherent, genetic capacity of a microorganism to cause disease, mediated by specific virulence factors. Whether or not it does so, is the result of the specific host-pathogen interactions.
- Virulence: This is a quantitative trait, representing the *extent* of the pathology caused by a microorganism. Virulence is therefore a trait expressing the interaction between a pathogen and its host. Its definition has been re-assessed recently (Casadevall and Pirofski, 1999; 2001), in view of the significant influence of the host's immunological condition on the extent of the damage (*i.e.* virulence) caused by a pathogen.

  Virulence is usually correlated to the pathogen's capacity to multiply in the host (Casadevall and Pirofski, 2001) represented, for example, by the virion titre when symptoms appear (Figure 2) or the rate of multiplication. It can also be affected by host and environmental factors, such as the transmission route or type of tissue/life-stage infected. For example, a pathogen may be virulent when infecting one type of tissue and non-virulent when infecting a different tissue (Casadevall and Pirofski, 1999). Virulence is therefore dependent on the nature of the infection.

  Virulence is also a relative trait, referring to the differences in the degree of pathology caused by strains of the same pathogen, or differences in the efficiency with which different strains can cause symptoms (Pirofski and Casadevall, 2012). For example, a pathogen strain that requires few particles to produce disease symptoms (strain-A in Figure 2) would be more virulent than a strain that requires many particles to produce the same symptoms (strain-B in Figure 2).

  Since virulence is a quantitative measure, methods have been developed to quantify the relative contributions of different virulence factors to a phenotype (McClelland *et al.,* 2006).
- Transmissibility: This refers to the efficiency with which a pathogen is transmitted to naïve hosts. There are valid arguments that at epidemiological level, transmissibility could be considered a component of virulence (Figure 2). The relationship between transmission and virulence is a major topic in pathogen-host evolutionary theory (*e.g.* Ebert and Bull, 2003) and has been discussed within the context of honey bee colony structure (Fries and Camazine, 2001) and honey bee virus transmission (de Miranda and Genersch, 2010).

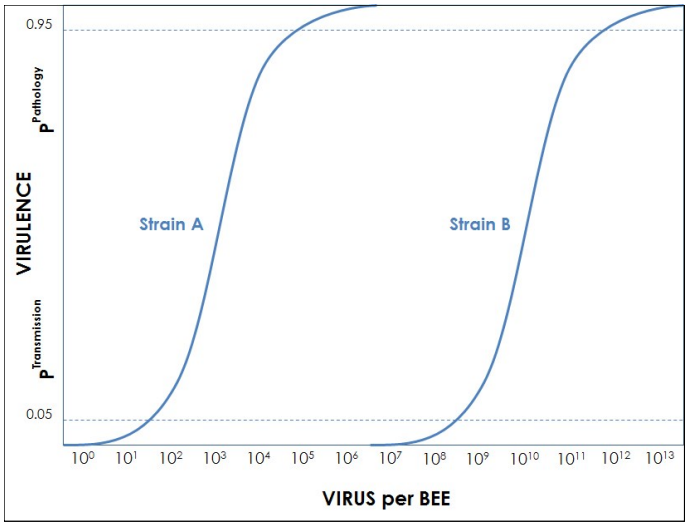

Fig. 2. Diagram describing the Log-linear relationship between virus concentration (X-axis) and virulence (Y-axis), represented by the degree/probability of Pathology ($P^{Pathology}$) or the efficiency/probability of Transmission ($P^{Transmission}$). Other variables can also be plotted on the Y-axis. Image © J R de Miranda.

1.3. Virus replication and variation

Viruses have two main characteristics that are fundamental to the design, analysis and interpretation of virological experiments, surveys and assays. These are:

- The potential for rapid, exponential growth
- The potential for rapid evolution and high levels of molecular variability

Below we briefly discuss these two features and their impact on experimentation and data management.

1.3.1. The mathematics of virus replication and transmission

Viruses are obligatory cell parasites and as such are capable of rapid, exponential growth. This is particularly the case for viral replication within individual organisms. This means that the virus replication dynamics can range from linear (when the virus persists as a covert infection, with minimal replication) all the way to fully logarithmic (when the virus is growing exponentially) and back to linear again when the maximum virus load within diseased or dying organisms is reached, due to exhaustion of the resources for replication (Fig. 2).

The epidemiological spread between organisms is influenced by the transmission medium (air, water, vector), whose rules of dispersion are often not fully exponential. This also applies to other barriers to virus proliferation, such as tissue-specificity, interference, auto-interference, RNA silencing, and immune reactions which all can influence virus multiplication, shedding and dispersal. These restrictions can temper the logarithmic character of the quantitative virus data distribution, at the individual bee, colony or regional level.

What this means is that, from the design of experiments through to the analysis of the data, allowance has to be made for non-linear distributions of the data, ranging from fully logarithmic (pathogenic replication) through semi-exponential (epidemic proliferation) to near-linear (covert replication, dispersal). This can be addressed through transformations, thresholds or non-linear models, but it MUST be dealt with appropriately. Guidelines for this can be found in detail in the *BEEBOOK* paper on statistical methods (Pirk *et al.,* 2013), with aspects specific to virus research also covered in section 3; "Statistical Aspects" of this chapter.

1.3.2. Virus variability and evolution

The second major characteristic of viruses, particularly important when designing molecular assays, is the ease and speed with which they can generate and maintain large amounts of molecular variability. The virus encoded RNA dependant RNA polymerase (RdRp), which facilitates genome replication, lacks proofreading and repair mechanisms causing a high mutation rate in RNA viruses. Therefore a virus is not so much an individual entity with a fixed genome, but rather a large 'swarm' of closely related variants, recombinants and other genetic oddities that are transmitted between individuals as a unit. There are two forces that shape the genetic identity of such a 'mutant swarm' (or 'quasi-species' as it is officially known)

- Fierce *competition* between molecular variants for supremacy in a particular cell, host *etc.*
- Functional *co-operation* between variants, where a temporarily disfavoured variant can remain within the quasi-species by 'borrowing' essential functions such as replication and packaging from the locally dominant variant.

The functional co-operation is an adaptive super-feature of viruses, since it allows a wide range of genetic diversity to persist within a quasi-species across time, hosts and environments. The true adaptive strength of a virus lies therefore more in the diversity within the swarm than in the evolutionary abilities of any one strain.

The importance of this variability for experimentation is in the design of diagnostic assays for virus detection. Serological assays, such as ELISA, are generally not affected by this variability which is mainly expressed at the nucleic acid level. However, nucleic acid assays, especially those based on the Polymerase Chain Reaction (PCR), are often very sensitive to microvariation at the nucleotide level, where even a single base-pair difference can be exploited for specific diagnosis. It is here therefore that supreme care must be taken to ensure that the assays developed for detecting viruses, or virus strains, are designed accurately and conservatively, to avoid non-detection due to assay inadequacy (see also section 12; "Quality Control").

2. Virus surveys

2.1. Introduction

Bee diseases caused by viruses are significant threats to apiculture. Pathogen surveillance is an essential component of a structured (inter)national management strategy to contain or prevent epidemics of viral diseases in honey bee populations. Such surveillance is done both through questionnaires of beekeepers (see the *BEEBOOK* paper on surveys; van der Zee *et al.,* 2013) and through monitoring bee colonies for pathogen prevalence and amount.

Although some honey bee viruses, such as chronic bee paralysis virus (CBPV), deformed wing virus (DWV), black queen cell virus (BQCV), sacbrood bee virus (SBV) and cloudy wing virus (CWV), are capable of causing diseases with recognizable symptoms, most honey bee viruses usually persist and spread between colonies as covert infections without apparent symptoms in bees. Many other bee viruses such as bee virus X and bee virus Y (BVX; BVY) or filamentous virus (AmFV), either do not cause outward symptoms at all, or others, such as slow bee paralysis virus (SBPV) and acute bee paralysis virus (ABPV) only do so under laboratory conditions or produce vague, non-descript symptoms, such as 'early death' by Kashmir bee virus (KBV)

and Israeli acute paralysis virus (IAPV) or 'clustering' associated with Apis iridescent virus (AIV). Many bee virus infections cannot therefore be identified through field observations, because the symptoms are non-existent, inconsistent or absent due to low titres. Until field-ready pathogen ID-kits become available, pathological analysis requires the transport of samples to a laboratory for analysis.

2.2. Types of survey

In epidemiology, there are two broad types of pathogen surveillance systems. Although they are often treated together in summaries and reviews, they are in fact radically different in purpose, strategy, methods and implications. Here we discuss these briefly as they apply to honey bee virus surveillance. For more detail information on how to manage these different approaches, see the *BEEBOOK* papers on epidemiology (vanEngelsdorp *et al.,* 2013) and surveys (van der Zee *et al.,* 2013).

2.2.1. Passive surveillance

This is the type of survey where beekeepers voluntarily send in suspect bee samples to a diagnostic laboratory for analysis. Most countries operate such a service and often the samples are of colonies that died suddenly, usually after winter. The survey is 'passive' since it only analyses material received from the public. It is analogous in human epidemiology to the entry reports of health care facilities for active disease cases. There is no statistically designed sampling strategy, samples will be biased by beekeeper interest, experience and knowledge and proximity to the diagnostic facilities. Samples will arrive in various states of decomposition making definitive diagnosis problematic (see section 4; 'Virus Sample Management'), the data will be heavily biased towards diseased colonies with high pathogen prevalence/titre and the results will be more relevant to the management of epidemics rather than to their prevention.

2.2.2. Active surveillance

Active surveillance schemes fill many of the gaps of passive surveillance. These are usually statistically designed sampling schemes to determine pathogen prevalence within the general bee population, irrespective of symptoms. The bee samples are alive when collected, making molecular detection uniform and reliable, and the data are an accurate representation of the complete pathogen presence within a region. Often samples are taken repeatedly from the same colonies throughout a season, which has to be taken into account during data analysis. A sub-category of active surveillance systems is 'sentinel' surveillance, *i.e.* a series of designated 'monitoring' colonies placed to catch certain pathogens before they reach a particular region.

3. Statistical aspects

3.1. Introduction

Whether experimenting or conducting surveys, data will be generated that will need to be analysed, and this requires the use of statistics. Fortunately, there are excellent statistical tools available right now for helping design experiments, determining the sample size needed to be able to make certain conclusions, for analysing the data and for modelling and prediction. It is highly recommended to include an expert statistician in the project right from the beginning, at the design stage. It will mean that the experiment is set up correctly, that sampling is as efficient as possible and that the data are analysed correctly. The most important statistical concepts and practices relevant to honey bee research and surveying are covered in the *BEEBOOK* paper on statistical methods (Pirk *et al.,* 2013), to which the reader is referred to for guidance.

3.2. Statistical distribution of virus data

The major additional point specifically relevant to honey bee virus research (and probably other pathogens) is that virus titres follow an Exponential (~Logarithmic) distribution, rather than a Normal distribution (Fig. 2; Gauthier *et al.,* 2007; Brunetto *et al.,* 2009; Yañez *et al.,* 2012; Locke *et al.,* 2012). Also, the prevalence of very rare viruses (pathogens, parasites) may follow a Poisson distribution rather than a Binomial distribution. These differences in how the primary virus data is statistically distributed affects the design of an experiment, the determination of sample sizes (Wolfe and Carlin, 1999), surveying strategy, the analysis of pooled samples and the management of the data produced. This subject is treated in more detail in the *BEEBOOK* paper on statistical methods (Pirk *et al.,* 2013).

3.2.1. Log-transformation

Exponentially distributed data (*i.e.* quantitative virus titre data) must be treated with a power transformation (Box and Cox, 1964; Bickle and Doksum, 1981), usually a log-transformation, before they can be used to estimate descriptive statistics (means, medians, variances etc.) or be used in parametric statistical analyses (ANOVAs, correlations, GLMs etc.). This can be done prior to statistical analysis, or can be incorporated as part of the statistical analysis.

3.2.2. Zero values

Since it is not possible to log-transform zero values, instances of non-detection (*i.e.* a 'zero' value) should be replaced by a non-zero constant value appropriate to the variable in question (Cox *et al.,* 2000). A logical constant value to use for replacing zero-values in quantitative virus data sets is one that is set just below the detection threshold for the virus in question (Yañez *et al.,* 2012; Locke *et al.,* 2012). This approach treats zero values as "below detection threshold" rather than as "absence of virus", which is usually also a

more accurate description of the virus status of a sample, especially if the virus is known to be present within the wider bee population.

4. Virus sample management

4.1. Introduction

The aim of a survey or experiment is that the final data should as closely as possible represent the (virus) status of the bee or colony when the sample was taken, since ultimately the data interpretation, conclusions and recommendations will again refer to live bees. Sample management (from collection and field preservation, to transportation, short- and long-term storage, processing and finally analysis) is therefore crucial to the accurate interpretation of survey and experimental data. Honey bee sample management is covered in detail in the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013) with a reduced version given below. Most viruses have an RNA genome and much of the host's molecular response to virus infection is also at the RNA level. However, RNA is highly sensitive to degradation by nucleases and a major criterion for bee sample management is therefore to minimize this degradation (Chen *et al.,* 2007; Dainat *et al.,* 2011). The primary concern is not so much the virus particle (which is usually relatively robust), but rather the viral replicative RNA intermediates and host mRNAs.

4.2. What, where and when to sample

Different viruses have different infection patterns, life-stage/tissue preferences and seasonal prevalences (Table 2), and so the decision as to what bee stages to sample, when/how often to sample and where to sample depends to a large degree on the objective of the experiment/survey and on the virus studied. When the experiments/ surveys are detailed and specific, the sampling regimen should be designed to suit those specific aims. However, often the same samples will be analysed for multiple viruses, or the experiments/ surveys are more global in character, requiring a more consensual approach to sampling. Here are some considerations for making sampling decisions in these situations. See also the *BEEBOOK* paper on statistical methods (Pirk *et al.,* 2013) for decisions on how to determine the optimal sample size and pooled samples.

4.2.1. What to sample?

All viruses described to date can be detected in adult bees (Table 2). This is logical, since adult bees are central to most of the virus transmission pathways (Fig. 1), due to their high mobility, contact rate and diverse contact network. This makes adult bees the most suitable single bee stage for detecting all viruses. Within adult bees, the gut is a major site of accumulation for most viruses (and many other pathogens), and is thus the most suitable single tissue type for sampling.

4.2.2. Where to sample?

The difficulty with adult bees is that the virus titre can be influenced by the age and the tasks of the bee. It is therefore advisable to sample as much as possible the same age/task group throughout the experiment, in order to minimize the influence of such effects on the data (Van der Steen *et al.,* 2012). In practical terms, this means either sampling from the brood chamber (mostly young nurse bees), the honey supers (medium-age bees) or at the entrance (older foraging bees). The choice of age-class for sampling is less important than consistently sampling the same age-class. See also the *BEEBOOK* paper on statistical methods (Pirk *et al.,* 2013).

4.2.3. When to sample?

There are two considerations here. The first is the best time of day to collect a sample and the second how often to sample. The best time to sample is either on sunny days, during the afternoon, when bees are actively foraging and the adult population is most clearly subdivided according to tasks/age, or at the other extreme during cold rainy days, when there is no substructuring of the population and all bees are sampled randomly, irrespective of age class. This choice depends on the design and purpose of the sampling scheme.

The frequency of sampling depends on the type survey/ experiment conducted:

- For single virus geographic prevalence surveys, the best time of year for sample collection would be during the seasonal peak for the virus in question (Table 2).
- For multivirus-pathogen geographic prevalence surveys, and if only a single sample is collected, the best time would be autumn, when most viruses have a seasonal peak.
- For multivirus-pathogen surveys it is advisable to sample at least three times per season; in early spring when the colony is expanding, during peak productivity in summer and during late autumn when the colony is contracting, in order to catch the different pathogens at their peaks, observe seasonal variations in prevalence and identify possible associations between different pathogens-parasites.
- For colony-level experiments with repetitive sampling, it may be useful to take into account the natural turnover of the adult population when considering sampling frequency. During summer, both the brood stage and the adult stage last about three weeks. Sampling every three weeks therefore means that a completely new generation of adult bees is sampled each time, corresponding to the brood generation of the previous sampling point.

4.3. Sample collection

Methods for collecting different sample types (adults *vs* brood; whole bees vs extracted tissues), for sample preservation (chemical and/or temperature) and for transportation (hours *vs* days) are described in

detail in the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013). Faeces may be a useful sample type for the non-destructive or repetitive sampling of the individual bees, such as queens (Hung, 2000). The general rules for sample collection are to get the samples to a freezer as quick as possible, and to keep dead, preserved or processed bee samples as cold as possible during transport.

4.3.1. Adult bees

Adult bees are usually collected from brood frames (young bees); honey frames (older bees); at the hive entrance (foragers) or in dead-bee traps (dead bees). Around 200 bees (about 20g, or a cup-full) are shaken or brushed into either a cage or ventilated box with food (live transport) or in a 1l plastic bag (cold transport). This is enough for both mite and virus analyses.

4.3.2. Pupae

Pupae are usually collected as a 10cm x 10cm section of sealed brood, placed in a suitably sized ventilated box (live transport) or in a plastic bag (cold transport). Individual pupae can be collected in microcentrifuge tubes or on collection cards (see section 4.4.5.).

4.3.3. Larvae

Larvae are usually collected in tubes or on collection cards and transported on ice, since they tend to crawl out of comb sections during live transport.

4.3.4. Eggs

Eggs can be collected as cut comb section, transported in a non-ventilated container to prevent dehydration, or individually in tubes or on collection cards.

4.3.5. Extracted guts

Adult bee guts can be collected by carefully pulling out the stinger plus last integument, and slowly drawing out the hind and midgut. Extracted guts can be transported in tubes or on collection cards, on ice.

4.3.6. Drone endophallus and semen

Drone endophali and/or semen can be collected by squeezing out the endophallus as described in the *BEEBOOK* article on artificial insemination (Cobey *et al.,* 2013). The endophallus or semen can be transported in tubes or on collection cards, on ice.

4.3.7. Faeces

Faeces can be collected destructively by removing the bee gut (see section 4.3.5.) and expelling the faeces, or non-destructively by placing the bee in a petri dish and waiting for defecation.

4.3.8. Dead colonies

Many virus experiments involve bee death as a parameter. Dead bee samples from such experiments should be treated like freshly killed material and frozen as soon as possible to minimize the effects of decay on RNA integrity.

Passive surveys also involve dead bee samples, in this case those sent in by beekeepers for *post-mortem* analysis of the cause of colony death. The RNA from such bees will most likely be degraded, which will affect the reliability of the data, especially of negative results (virus absence). How to manage such samples and data is covered in detail in the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013). Collect the more desiccated dead bees and transport in a ventilated cardboard box at ambient temperature.

4.4. Sample transport

The integrity of samples can stabilized during transport by:

4.4.1. Freezing

Freezing on-site is the gold standard for sample transport, but usually too expensive for mass surveys. There are several alternatives for different purposes and samples sizes (see the *BEEBOOK* paper on molecular techniques; Evans *et al.,* 2013), including the 'dry shipper' which can hold up to 1300 2-ml cryo-tubes below freezing for up to 3 weeks and is approved for international air-shipment.

4.4.2. Ice

Transport on ice is a cheap and practical substitute for freezing, if the samples can be (re)-frozen within 48 hours.

4.4.3. Live transport

Live transport is very practical and cheap, especially if the samples are sent by post. Obviously there is no RNA degradation due to bee death, but live transport may affect the expression of host genes, and possibly virus replication, which should be taken into account when planning experiments.

4.4.4. Chemical stabilizers

There are a number of chemicals that help prevent RNA degradation. For these to work, they have to penetrate the bee exoskeleton and get into the tissues. They are therefore more useful for extracted tissues, eggs and larvae and less useful for adult bees. There are two types of chemical preservation: salts (in solution or impregnated on collection cards) and organic solvents (usually alcohol). Solvents penetrate the exoskeleton better than salt solutions and are more suitable for adult bees. A large excess (>5-fold by weight) should be used to make sure the chemical's concentration in the tissues is high enough to inhibit the nucleases. Various solutions and how to use them are described in the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013).

4.4.5. Sample collection cards

FTA™ collection cards (Whatman) preserve tissues both by desiccation and chemical (salt) preservatives embedded in the filter paper (Becker *et al.,* 2004; Rensen *et al.,* 2005). They are ideal for soft tissues and for remote collecting situations where sample weight and ambient storage are important factors. They are very reliable, but pricey, only suitable for small sample sizes and processing can be messy. See the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013).

4.5. Long-term sample storage

The factors important for long-term storage and preservation are the same ones highlighted for sample transport, which are (in order of effectiveness): temperature, desiccation (lyophilisation) and chemical preservatives. See the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013) for details.

5. Virus propagation

5.1. Introduction

The methods for propagating and purifying honey bee viruses in bees have not changed much from those described by Bailey and Ball (1991). Many viruses, including all of the important 'picorna-like' viruses, can be propagated by injection in either pupae or adult bees. Pupal injections are easier to manage than injection into adult bees, from pretty much every aspect: injection, incubation, homogenization and purification. Some viruses can only be infected orally and/or are only infectious in adult bees. The propagation criteria for each virus are listed in Table 3. Infectivity tests are essentially more precise versions of the propagation protocols. Another way to propagate and purify honey bee viruses is through tissue culture. This removes the potential for contamination and the dependence on the bee season that comes with propagating in bees, and allows for large volume propagation. It also is a highly effective tool for detailed laboratory experimentation at cellular level, without the influence of bee and hive effects. Attempts at virus propagation in bee tissue culture have so far met with limited success. However, significant progress has recently been made with *Nosema* cultivation in commercial (Lepidopteran) insect cell lines (see the *BEEBOOK* paper on cell culture (Genersch *et al.,* 2013).

5.2. Starting material

Often the starting material for propagation is a previous virus preparation that has been checked for the absence of contaminating viruses and retained as a pure isolate. Virus preparations can, however, lose infectivity during prolonged storage. For example deformed wing virus (DWV) and its relatives kakugo virus (KV) and Varroa destructor virus-1 (VDV-1), are particularly sensitive to decay during storage. Since neither serological nor molecular assays can distinguish between degraded or intact virus particles, they are not reliable methods for determining the infectivity of a preparation. Furthermore, such well-characterized and precious reference material is often in limited supply and highly valuable as a historical "reference" isolate for future experiments. Such reference material can be stored long-term either as freeze-dried bees/pupae/larvae, or as a (semi)purified virus solution in an appropriate virus purification buffer (Table 4) and stabilized by 50% glycerol. It is therefore advisable, particularly for infectivity tests but also for propagation, to first prepare a "working" inoculum, by injecting or feeding a small number of bees (larvae, pupae or adults; around 5-10 individuals) with a small amount of the pure reference material. This also serves as an infectivity test for the viability of the stored reference material. After incubating for the appropriate time, a crude extract should be prepared from the bees, the purity and virus concentration of this extract determined, and then this working extract should be used for large-scale propagation or infectivity tests within the next few of weeks.

5.3. Oral propagation

Oral propagation is relatively inefficient for most viruses, requiring high titre inoculums (10^6-10^{11} particles, depending on the virus; Table 3) to establish an infection (Bailey and Gibbs, 1964; Bailey and Ball, 1991).

5.3.1. Larvae

1. Mix purified virus of the appropriate minimum concentration (Table 3) with medium for *in-vitro* larval rearing and allocate this to the wells of a 48-well tissue culture plate (see the *BEEBOOK* paper on *in-vitro* larval rearing (Crailsheim *et al.,* 2013).
2. Transfer two-day old larvae to the wells, making sure there is enough virus so that each larva gets the minimum infectious dose.
3. Follow the procedures for *in-vitro* larval rearing (see the *BEEBOOK* paper on *in-vitro* larval rearing (Crailsheim *et al.,* 2013), transferring the larvae periodically to fresh food, either including or excluding further virus extract.
4. Include a series of control inoculations, using larval food medium without virus.

5.3.2. Adults

1. Mix purified virus with 60% sucrose to the desired concentration for infecting bees individually or in bulk.
2. Feed the virus-sucrose solution individually to newly emerged adult bees in 5-10 µl volumes, using a Pasteur or micro-pipette.
3. Immobilize the bees by either holding their wings or in a suitable restrainer, such as head-first in a 1.5 ml microcentrifuge tube with the bottom cut off (see the section

'standard methods for immobilising, terminating, and storing adult *Apis mellifera*' in the *BEEBOOK* paper on miscellaneous methods; Human *et al.,* 2013).

4. Bulk-feeding of the virus-sucrose solution to adult bees is done either in hoarding cages, using disposable 15 ml plastic feeding tubes (see the *BEEBOOK* paper on maintaining adult *Apis mellifera* workers in cages; Williams *et al.,* 2013) or in whole colonies using internal or top feeders. In both cases it is important to calculate the amount of purified virus needed to ensure that each bee gets the minimum infectious dose (Table 3).
5. Include a series of control inoculations, using sucrose solution without virus.

5.4. Injection propagation

Propagation by injection into pupae or adults is generally very efficient, requiring very low virus doses and concentrations to establish an infection (10^2-10^4 particles, depending on the virus). However, this high efficiency also makes propagation by injection susceptible to the amplification of any contaminating viruses, either those present in the injected inoculum or those present naturally within the bees. Extra care has to be taken therefore to confirm the purity of the propagation, after purification.

5.4.1. Pupae

1. Lay the frame horizontally at a slight angle, bottom-to-top, under good light.
2. Using needle-forceps, remove the wax capping from 10-50 cells containing white-eyed pupae, by cutting along the inside of the cell.

It is easiest to work from the bottom of the frame upwards, clearing room underneath for opening up the cells higher up the frame and picking up the pupae from behind.

3. With blunt, curved forceps remove the top part of each cell, exposing the head of the pupa.
4. Place the curved forceps underneath the head of the pupa, from the back, and carefully lift the pupa out of its cell.

It is critical to remove the white-eye pupae very carefully from the comb, to avoid damage.

5. Collect the pupae in a plastic Petri dish containing a circular filter paper dampened with sterile water.
6. For the purpose of propagation, you need:
   - a 10~50 µl syringe,
   - a thin needle (around 28G~30G)
   - a semi-automatic volume dispenser control unit that can dispense 1~5 µl volumes.

The Hamilton Company produces both dispenser units and 10~50 µl syringes with Luer locks that fit disposable needles. The pupae can be injected by hand, which is faster (but less precise) than doing so under microscope, with moveable trays.

7. Attach the syringe and control unit horizontally to a stand.
8. Close the four fingers of your hand and carefully lay a white-eye pupa on its back in the groove between index and middle finger.

The abdomen of the pupa points towards the tip of the fingers and the head is supported by the tip of the thumb.

9. Move the pupa towards the needle, inserting the needle at the narrowest angle possible under the skin of the pupa, on the lateral side, between the 2nd and 3rd integuments of the abdomen.
10. Inject 1~5 µl of virus suspension using the control unit.
11. Move the pupa backwards off the needle.
12. Move the pupa carefully from the hand to a plastic, disposable tissue culture plate, using forceps to support the pupa underneath.

Use plates with matching lids, both to prevent cross-contamination between wells and to control the humidity.

13. Incubate the plate at 30°C in a humidity-controlled incubator.

If this is not available, place the plates in a closed plastic box containing moistened paper towels.

14. Check the progress of the infection by monitoring the change in eye colour of the pupae.

Those pupae injected with virus that remain alive will change eye colour, but more slowly than those pupae injected with only buffer.

15. Include a control series with buffer-only inoculations, and a control series without inoculation, just incubation of the pupae.

The best propagations are with those inoculum concentrations that keep the pupae alive for as long as possible, generating the highest propagation concentrations. Sometimes parts of the body become necrotic, either only the abdomen or only the thorax/head. This can happen when the virus concentration of the inoculum is too high, killing the tissue too quickly for efficient propagation. Too low a virus inoculums concentration will, however, increase the risk of amplifying unrelated covert infections already present in the pupa.

It is therefore advisable to first propagate a range of log-scale dilutions (1/10, 1/100, 1/1000 *etc.*) of the virus inoculum. Then for large-scale propagation, choose the highest inoculum concentration that does not necrotize the pupae before the 4th day of incubation.

Table 3. Summary of the protocols and conditions for the oral and injection propagation of the different honey bee viruses in larvae, pupae and adult bees. Non-viable propagation routes are marked with an 'x'. Absence of reliable information is marked with '?'. Adapted from Bailey and Ball (1991).

PROPAGATION						
VIRUS	**METHOD**	**STAGE**	**AGE**	**DOSE** (2ng ≈ 10^7 particles)	**INCUBATION***	**NOTES**
Acute bee (ABPV)	ORAL	larvae	48 hour	?	?	
		adult	< 24 hour	> 10^{10} particles/bee	?	
	INJECTION	pupae	white eye	> 10^2 particles/bee	5 days	
		adult	any	> 10^2 particles/bee	5 days	
Kashmir bee virus (KBV)	ORAL	larvae	48 hour	?	?	
		adult	< 24 hour	> 10^7 particles/bee	5 days	
	INJECTION	pupae	white eye	> 10^2 particles/bee	3 days	
		adult	any	> 10^2 particles/bee	3 days	
Israeli acute (IAPV)	ORAL	larvae	48 hour	?	?	
		adult	< 24 hour	> 4ng pure virus/bee	6 days	
	INJECTION	pupae	white eye	> 10^2 particles/bee	3 days	
		adult	any	> 10^2 particles/bee	4 days	
Black queen cell virus (BQCV)	ORAL	larvae	48 hour	?	?	requires N. apis co-infection
		adult	< 24 hour	> 4mg crude extract/bee	40 days	
	INJECTION	pupae	white eye	> 10^3 particles/bee	5 days	
		adult	any			
Deformed wing Varroa destructor virus-1 (DWV & VDV-1)	ORAL	larvae	48 hour	> 10^9 genomes/bee	5 days	
		adult	< 24 hour	> 10^8 genomes/bee	?	
	INJECTION	pupae	white eye	> 10^2 genomes/bee	14 days	
		adult	any	> 10^7 genomes/bee	3 days	
Egypt bee virus (EBV)	ORAL	larvae	48 hour	?	?	
		adult	< 24 hour	?	?	
	INJECTION	pupae	white eye	> 10^2 particles/bee	8 days	
		adult	any	?	?	
Sacbrood virus (SBV)	ORAL	larvae	48 hour		7 days	
		adult	< 24 hour			
	INJECTION	pupae	white eye	> 10^3 particles/bee	5 days	
		adult	any			
Thai/Chinese Sacbrood virus (TSBV)	ORAL	larvae	48 hour		7 days	
		adult	< 24 hour			
	INJECTION	pupae	white eye	> 10^3 particles/bee	5 days	
		adult	any			
Slow bee paralysis virus (SBPV)	ORAL	larvae	48 hour	?	?	
		adult	< 24 hour	?	?	
	INJECTION	pupae	white eye	> 10^3 particles/bee	5 days	
		adult	any	> 10^3 particles/bee	12 days	
Chronic bee paralysis virus (CBPV)	ORAL	larvae	48 hour	> 10^{10} particles/bee	5 days	
		adult	< 24 hour	> 10^{10} particles/bee	? days	
	CONTACT	larvae	x	x	x	
		adult	< 48 hour	> 10^7 genomes/bee		
	INJECTION	pupae	white eye	> 10^2 particles/bee	5 days	
		adult	any	> 10^2 particles/bee	7 days	

Table 3. continued

VIRUS	METHOD	STAGE	AGE	DOSE (2ng ≈ 10^7 particles)	INCUBATION*	NOTES
Cloudy wing virus (CWV)	CONTACT	larvae	?	?	?	
		adult	?	?	?	
	INJECTION	pupae	x	x	x	
		adult	x	x	x	
Bee virus X (BVX)	ORAL	larvae	x	x	x	enhanced by M. mellificae co-infection
		adult	< 24 hour	> 4mg crude extract/bee	30 days	
	INJECTION	pupae	x	x	x	
		adult	x	x	x	
Bee virus Y (BVY)	ORAL	larvae	x	x	x	requires N. apis co-infection
		adult	< 24 hour	> 4mg crude extract/bee	30 days	
	INJECTION	pupae	x	x	x	
		adult	x	x	x	
Arkansas bee virus (ABV)	ORAL	larvae	48 hour	?	?	
		adult	< 24 hour	?	?	
	INJECTION	pupae	white eye	> 10^3 particles/bee	5 days	
		adult	any	> 10^3 particles/bee	21 days	
Filamentous virus (AmFV)	ORAL	larvae	?	?	?	enhanced by N. apis
		adult	< 24 hour	> 4mg crude extract/bee	30 days	
	INJECTION	pupae	x	x	x	
		adult	x	x	x	
Apis iridescent virus (AIV)	ORAL	larvae	48 hour	?	?	
		adult	< 24 hour	?	?	
	INJECTION	pupae	white eye	> 10^3 particles/bee	5 days	
		adult	any			
					* based on minimum	

5.4.2. Adults

Propagation in adult bees is best done with newly emerged bees, whose exoskeleton is still soft, making it easier for controlled injection. Using young bees also avoids any age-related variability in propagation and unintentional propagation of adult-acquired viruses.

1. Collect the bees in lots of 10-20 in queen cages, or similar containers.
2. Anaesthetize the bees for 1 minute with CO_2 from a pressurized cylinder.
3. Make sure to bubble the CO_2 gas through water to melt any CO_2 micro-particles, which can be very injurious to bees. See the section 'Standard methods for immobilising, terminating, and storing adult *Apis mellifera'* in the *BEEBOOK* paper on miscellaneous methods (Human *et al.,* 2013).
4. Proceed quickly to avoid excess anaesthesia for the bees and do not anaesthetize the bees more than once a day. Bees sometimes die from excess anaesthesia. This is especially important for infectivity tests, where the death of a bee is often a recorded experimental variable.
5. Inject 1~5 µl of virus suspension between the 2nd and 3rd integuments of the bee using a similar controlled-volume syringe set-up as for pupal propagation (see section 5.4.1.).
6. Incubate the inoculated bees in a hoarding cage at 30°C in 60 -70% relative humidity with sufficient sterilized food and water (see the *BEEBOOK* paper on maintaining adult *Apis mellifera* workers in cages (Williams *et al.,* 2013) for the appropriate amount of time for each virus (Table 3).
7. Include a control series with buffer-only inoculations, and a control series without inoculation, just incubation of the adult bees.

Table 4. Summary of the protocols for the purification of the different honey bee viruses. Adapted from Bailey and Ball (1991).

PURIFICATION					
VIRUS	**EXTRACTION BUFFER (2 ml/g tissue)**	**LOW-SPEED CENTRIFUGE**	**HIGH-SPEED CENTRIFUGE**	**RESUSPENSION BUFFER (0.1 ml/g tissue)**	**SUCROSE GRADIENTS**
Acute bee paralysis virus (ABPV)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.0) incubate 12-36 hours; 5°C low-speed centrifugation; 5°C	45 000 g 3 hours 4°C collect band
Kashmir bee virus (KBV & IAPV)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.0) incubate 12-36 hours; 5°C low-speed centrifugation; 5°C	45 000 g 3 hours 4°C collect band
Israeli acute paralysis virus (IAPV)	0.01M potassium phosphate (7.6) 0.2% Na-deoxycholate 2% BRIJ-58	10 000 g 20 minutes 15°C supernatant	100 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.6) 0.6 g/ml CsCl * CsCl gradient centrifugation	100 000 g* 24 hours* 20°C collect band
Black queen cell virus (BQCV)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume CCl_4	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.0) incubate 12-36 hours; 5°C low-speed centrifugation; 5°C	45 000 g 3 hours 4°C collect band
Deformed wing virus & Varroa destructor virus-1 (DWV & VDV-1)	0.5M potassium phosphate (8.0) 0.2% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.5M potassium phosphate (8.0) immediately to sucrose gradients	45 000 g 3 hours 4°C collect band
Egypt bee virus (EBV)	0.5M potassium phosphate (8.0) 2% ethylene diamine tetra acetic acid 1% ascorbic acid; 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.5M potassium phosphate (8.0) immediately to sucrose gradients	45 000 g 3 hours 4°C collect band
Sacbrood virus (SBV)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.0) incubate 12-36 hours; 5°C low-speed centrifugation; 5°C	45 000 g 3 hours 4°C collect band
Thai/Chinese Sacbrood virus (TSBV)	0.5M potassium phosphate (8.0) 0.2% diethyldithiocarbamate 0.02M ethylene diamine tetra acetic acid 0.1 volumes ether; carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.5M potassium phosphate (8.0) 0.02M ethylene diamine tetra acetic acid	45 000 g 3 hours 4°C collect band
Slow bee paralysis virus (SBPV)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.0) incubate 12-36 hours; 5°C low-speed centrifugation; 5°C	45 000 g 3 hours 4°C collect band
Chronic bee paralysis virus (CBPV)	0.2M potassium phosphate (7.5) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	3000 g 30 minutes 15°C supernatant	100 000 g 2 hours 15°C retain pellet	0.2M potassium phosphate (7.5) 15°C	45 000 g 4.5 hours 15°C collect band

Table 4. Cont'd.

PURIFICATION					
VIRUS	**EXTRACTION BUFFER (2 ml/g tissue)**	**LOW-SPEED CENTRIFUGE**	**HIGH-SPEED CENTRIFUGE**	**RESUSPENSION BUFFER (0.1 ml/g tissue)**	**SUCROSE GRADIENTS**
Cloudy wing virus (CWV)	0.5M potassium phosphate (8.0) 0.2% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3.5 hours 15°C retain pellet	0.5M potassium phosphate (8.0) immediately to sucrose gradients	45 000 g 4.5 hours 4°C collect band
Bee virus X (BVX)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.0) equal volume 0.2M ammonium acetate (5.0) low-speed & high-speed centrifugation resuspend 0.1M ammonium acetate (7.0)	45 000 g 3 hours 4°C collect band
Bee virus Y (BVY)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.0) equal volume 0.2M ammonium acetate (5.0) low-speed & high-speed centrifugation resuspend 0.1M ammonium acetate (7.0)	45 000 g 3 hours 4°C collect band
Arkansas bee virus (ArkBV)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume ether 0.1 volume carbon tetrachloride	8000 g 10 minutes 15°C supernatant	75 000 g 3 hours 15°C retain pellet	0.01M potassium phosphate (7.0) incubate 12-36 hours; 5°C low-speed centrifugation; 5°C	45 000 g 3 hours 4°C collect band
Filamentous virus (AmFV)	0.01M ammonium acetate (7.0) 0.02% diethyldithiocarbamate	150 g 10 minutes 15°C supernatant	30 000 g 30 minutes 15°C retain pellet	0.1M ammonium acetate (7.0) layer on 50% w/v sucrose centrifuge 75 000g, 3 hours, 15°C resuspend 0.1M ammonium acetate (7.0)	10 000 g 30 minutes 5°C collect band
Apis iridescent virus (AIV)	0.01M potassium phosphate (7.0) 0.02% diethyldithiocarbamate 0.1 volume carbon tetrachloride	150 g 10 minutes 15°C supernatant	30 000 g 30 minutes 15°C retain pellet	0.01M potassium phosphate (7.0) incubate 12-36 hours; 5°C low-speed centrifugation; 5°C	10 000 g 30 minutes 5°C collect band

5.5. Tissue culture

The importance of a viable tissue-culture system for the purification and propagation of honey bee viruses has long been acknowledged. There are two possible approaches to such a system. One is to develop a reliable, immortal honey bee cell line for infection. Only recently has there been any significant progress towards this goal (Bergem *et al.,* 2006; Hunter, 2010; Kitagishi *et al.,* 2011; Gisder *et al.,* 2012). The other approach is to propagate honey bee viruses in existing commercial, heterologous insect cell lines. Many of the honey bee viruses naturally infect other insect hosts, such as other *Apis* spp., varroa and tropilaelaps parasitic mites, bumble bees, wasps, ants and a range of solitary pollinators (Bailey and Gibbs, 1964; de Miranda *et al.,* 2010a; de Miranda and Genersch, 2010; Ribière *et al.,* 2010; Dainat *et al.,* 2009; Singh *et al.,* 2010; Peng *et al.,* 2011; Li *et al.,* 2011; DiPrisco *et al.,* 2011; Zhang *et al.,* 2012; Evison *et al.,* 2012) and the replication-translation control regions of honey bee virus genomes are active in several commercial Lepidopteran and Dipteran cell lines (Ongus *et al.,* 2006). Protocols for the establishment and maintenance of honey bee and commercial insect cell lines can be found in the *BEEBOOK* article on cell cultures (Genersch *et al.,* 2013).

5.5.1. Virus infection

The most common method of infecting tissue culture cells is through passive co-incubation of purified virus particles with the cells, allowing the natural processes of virus entry to establish an infection (Minor, 1985; Gantzer *et al.,* 1998; Rhodes *et al.,* 2011; Amdiouni *et al.,* 2012). However, the virus particles (or the naked viral RNA genome) can also be forced into the cells using electroporation, which involves a short high-voltage pulse of electricity to temporarily open up the cell membrane to allow foreign elements to enter the cell, or chemical-mediated transfection, where a combination of membrane-active ions and concentrating agents interact to encourage the uptake of the virus or nucleic acid into the cell (*e.g.* Boyer and Haenni, 1994;

Benjeddou *et al.,* 2002; Ongus *et al.,* 2006; Yunus *et al.,* 2010). Whichever virus transfection protocol is chosen, it is essential that the virus preparation is free of bacteria or fungi to prevent contamination of the tissue culture (Minor, 1985; Gantzer *et al.,* 1998). Bacteria, fungi and their spores can be effectively removed from a virus preparation using microfilters with appropriate pore size, depending on the size of the virus (Gantzer *et al.,* 1998; Rhodes *et al.,* 2011; Amdiouni *et al.,* 2012), with 0.2 µm suitable for purifying most small, enteric picorna-like viruses of around 30-60nm (Rhodes *et al.,* 2011; Amdiouni *et al.,* 2012), which includes most of the honey bee RNA viruses (Table 2).

5.6. Full-length infectious virus clones

A supremely powerful tool in RNA virus research is cloning full-length genomic sequences of the virus into bacterial vectors. The naked RNA transcribed from such clones is usually infectious when introduced into a suitable host (Yunus *et al.,* 2010), especially for positive-stranded RNA viruses (*i.e.* the majority of known bee viruses). Such clones can be manipulated by site-directed mutagenesis and recombination for functional analysis of different open reading frames or control regions. Reporter genes such as green fluorescent protein can be inserted to make fusion proteins with viral genes or to study promoter function in real-time (Ongus *et al.,* 2006) and of course they can function as a genetically pure source of infectious virus, rather than having to rely on biological propagation with the associated dangers of contamination with other viruses and the changing genetic constitution of the virus, through evolution.

Full-length viral cDNAs are also an important tool in studying the genetic complexity of virus populations, since they make it possible to identify complete sequences of individual viruses within the population, including natural recombinants between major variants (Palacios *et al.,* 2007; Moore *et al.,* 2011).

5.6.1. Full-length viral RNA synthesis strategies

Full-length cloning of viral genomes has been a common tool in virology since the 1980's (Taniguchi *et al.,* 1978; Lowry *et al.,* 1980; Racaniello and Baltimore, 1981), but it is often a long and tedious process, mostly due to the frequent instability of the full-length clones in bacteria (Boyer and Haenni, 1994). It is thought that cryptic bacterial promoters and secondary structures within the viral sequences encourage the bacteria to excise problematic viral regions from the plasmid clone. Full-length clones therefore have to be monitored constantly for possible deletions and re-arrangements as part of their maintenance. The process has been made easier by improvements in cloning techniques and the stability of the cloning vectors with respect to accepting and maintaining long (~10kb) inserts. There are several alternatives to cloning full-length genomes. Sometimes it is easier to clone the genome in several partial clones first, and then recombine these afterwards into a single full-length clone (Rodriguez *et al.,* 2006). Another strategy, used for viruses that prove impossible to clone full-length, is to clone the genome in two halves, which are maintained independently and then recombined *in vitro* prior to RNA transcription using a suitably engineered restriction site (Jakab *et al.,* 1997). This improves the stability of the clones, the engineered restriction site can be used for recombining different virus strains for gene function analysis and also serves as a useful marker for tracing the virus through experiments.

A third alternative is to dispense with cloning altogether and generate infectious RNA transcripts directly off full-length PCR products that have a suitable recognition site for the T3, T7 or SP6 RNA polymerase incorporated into the full-length amplification primers, for transcript synthesis. This avoids the instability problems of cloned full-length clones but limits the extent to which the genomes can be manipulated genetically. This approach was successful for synthesizing full-length, infectious transcripts of BQCV after it proved impossible to successfully clone full-length BQCV (Benjeddou *et al.,* 2002).

5.6.2. Protocol

Here we describe a method for:

- Producing full-length PCR products of positive-stranded, ssRNA honey bee viruses, with a T7 RNA polymerase promoter site incorporated into the forward primer sequence (sections 5.6.2.1. – 5.6.2.2.)
- Cloning this product into a stable plasmid vector (section 5.6.2.3.)
- Confirming the integrity and character of the full-length clones (section 5.6.2.4.)
- Synthesizing full-length infectious RNA transcripts of the virus (sections 5.6.2.5. – 5.6.2.6.)

5.6.2.1. Full-length reverse transcription

Genomic RNA of most honey bee ssRNA viruses is approximately 10 kb long and contains highly structured 5'-terminal un-translated region with extended hairpin structures. Therefore, the first strand cDNA synthesis should be performed using a reverse transcriptase with a high optimum temperature (*e.g.* InVitrogen's Superscript III), so that RNA secondary structures are also transcribed.

1. Prepare purified virus particles using gradient centrifugation (see section 7; "Virus purification")
2. Extract viral RNA from the purified virus particles (see section 8.3.; "Nucleic acid extraction")
3. Combine in a single 200 µl thin wall tube:
   3.1. 1 µg virus RNA,
   3.2. 1 µl 2 µM "Reverse Primer" 5'-CGGTGTTTAAAC$(T)_{27}(X)_{32}$-3', where $(X)_{32}$ is a sequence complementary to the final 32 nucleotides at the 3' end of the virus genome to be cloned,
   3.3. 1 µl 10 mM dNTPs,
   3.4. Make the total volume 13 µl with RNase-free water.

4. Mix well by pipetting on ice.
5. Incubate at 65°C for 3 minutes in PCR heating block.
6. Transfer to ice and cool down for 1 minute.
7. Add the following:
   7.1. 4 µl 10x First Strand Buffer (supplied with Superscript III),
   7.2. 1 µl 0.1M DTT,
   7.3. 1 µl RNase OUT recombinant RNAse inhibitor (Invitrogen).
8. Mix well and incubate at 52°C for 2 minutes in heating block.
9. Add 2 µl Superscript III reverse transcriptase (Invitrogen) and mix well.
10. Incubate at 52°C for 10 min.
11. Incubate at 55°C for 60 min.
12. Incubate at 70°C for 15 min.
13. Store in freezer as a template for full length cDNA amplification.

5.6.2.2. Amplifying full-length viral RNAs

One critical factor in the successful amplification of viable, full-length cDNAs is the use of a thermostable DNA polymerase with proof-reading capacity. One such high-fidelity, high processivity DNA polymerase is Phusion DNA polymerase (New England Biolabs). If the fragment is to be cloned into a plasmid vector lacking a T7 or T3 RNA polymerase promoter site (required for the synthesis of full-length infectious RNA copies), then such a site should be incorporated into the 5′ amplification primer (*e.g.* Benjedou *et al.,* 2002; Ongus *et al.,* 2006).

1. Combine following in the 200 µl thin wall PCR tube placed on ice:
   1.1. 2 µl of the first strand cDNA reaction,
   1.2. 35 µl sterile nuclease free water,
   1.3. 10 µl of 5x HF Phusion amplification buffer,
   1.4. 1 µl 10mM dNTP mixture,
   1.5. 0.5 µl 2 µM "Reverse primer" (section *5.6.2.1.*; step 3.2.)
   1.6. 0.5 µl 2 µM T7 RNA polymerase promoter-tagged "Forward primer" 5′-GCTATAATACGACTCACTATAGG$(X)_{20}$-3′ where $(X)_{20}$ are the first 20 nucleotides at the 5′ end of the virus genome
2. Mix well by pipetting on ice.
3. Add 2 µl (5U) Phusion DNA polymerase.
4. Mix by pipetting.
5. Place the tube in the thermocycler when the block is 90°C (Hot-start PCR).
6. Amplify with the following cycling programme:
   6.1. 98°C:1 min,
   6.2. 5x [98°C:15 sec – 52°C:60 sec – 72°C:7 min],
   6.3. 25x [98°C:15 sec – 55°C:60 sec – 72°C:7 min],
   6.4. 72°C: 7 min.
7. Purify the reaction products with PCR purification kit (Qiagen), eluting into 50 µl of water.

5.6.2.3. Cloning full-length viral RNAs

Cloning of long (~10 kbp) PCR products into plasmid vectors may be not very efficient. We recommend to using pCR-XL-TOPO cloning, which is vector specifically designed for cloning of large products. This vector requires 3′ terminal A overhangs in the PCR products, therefore the first stage is the addition of 3′ overhangs to the Phusion-generated blunt ends.

1. Mix the following:
   1.1. 50 µl purified PCR fragments,
   1.2. 6 µl 10x Taq polymerase buffer,
   1.3. 3 µl 10mM dNTPs,
   1.4. 1 µl Taq polymerase.
2. Incubate at 72°C for 10 min.
3. Separate the fragments by electrophoresis in 0.8% agarose gel in TAE buffer.
4. Stain the gel with crystal violet (see InVitrogen TOPO XL PCR cloning kit).
5. Excise the 10 kb full-length cDNA fragments.
6. Extract DNA using the Gel Purification reagents included in the TOPO XL PCR Cloning kit (Invitrogen).
7. Ligate fragments into the pCR XL TOPO vector by mixing:
   7.1. 4 µl of the purified product (approximately 10 to 50 ng)
   7.2. 1 µl of pCR XL TOPO vector.
8. Incubate reaction for 5 min at 25°C.
9. Stop reaction by addition of 6x TOPO cloning Stop Solution.
10. Mix for a few seconds at room temperature and place the reaction to ice.
11. Proceed immediately to transformation of the OneShot competent *E. coli* cells (Invitrogen), following the manufacturer's instructions.

5.6.2.4. Confirmation of full-length clones

1. Select the white colonies and transfer to a fresh plate.
2. Amplify insert DNA from all colonies using the PCR primers and protocols from Section 9.3.3.; "RT-(q)PCR – Protocols".
3. Separate PCR products on gel electrophoresis.
4. Isolate clones with inserts > 10kb.
5. Prepare plasmid DNA from full-length clones using a Qiagen plasmid purification kit and corresponding instructions.
6. Sequence the inserts in selected plasmids using a series of oligonucleotide primers that are conserved between all known variants of the virus.
7. Confirm identity of clones through comparing the cloned sequences with the published consensus virus sequences.

5.6.2.5. Synthesizing full-length viral RNA

The plasmid can be used as a template for *in vitro* transcription using T7 RNA polymerase.

1. Linearize plasmid downstream of the 3' poly-A sequence with *Pme*I restriction endonuclease.
2. Purify the linearized templates with a Qiagen plasmid purification kit.
3. Synthesize capped *in vitro* RNA transcripts using "mMESSAGEmMAchine" T7 kit (Ambion).

5.6.2.6. Confirmation of Full-length Viral RNA

The full-length nature and activity of the transcripts is confirmed by *in vitro* translation experiments (Green and Sambrook, 2012), and ultimately by infection of pupae through injection with the synthesized RNA transcripts (see section 5.4). The infectivity of such transcripts is confirmed by comparing virus titres between transcript-inoculated pupae with control-inoculated pupae, and by sequencing the new virus infection (Benjedou *et al.,* 2002).

6. Virus infectivity assays

Infectivity assays were used before sensitive molecular techniques were developed to detect low levels of virus in surveys (Bailey, 1976; Bailey *et al.,* 1981; 1983b). These assays take advantage of the fact that most bee viruses when injected into adult bees or pupae multiply rapidly to high titres that can subsequently be detected by serology (Dall, 1987). Dilution series of the extracts provide a measure of quantitation. Different viruses develop titre and kill pupae at different rates, which can be detected by the 'breaking' of the eye-colour development in white-eyed pupae (Anderson and Gibbs, 1988; 1989). This can provide an early indication of which virus is being multiplied. Although labour intensive, infectivity assays can rival the most sensitive molecular tests available (Denholm, 1999). One serious drawback of honey bee infectivity assays is that often unapparent viruses present at very low levels in the assay pupae can also be amplified, sometimes by the mere injection of buffer (Bailey, 1967; Anderson and Gibbs 1988; 1989). Several important bee viruses (ABPV, KBV and SBPV) were discovered this way, as a by-product of the propagation of CBPV, AIV and BVX respectively (Bailey *et al.,* 1963; Bailey and Milne, 1969; Bailey and Woods, 1974; 1977; Bailey and Ball, 1991), and the technique may yet prove useful for the discovery of other symptomless bee viruses.

In general terms, the procedures for virus infection infectivity assays are the same as for virus propagation. It is especially important is that the larvae and pupae are transferred to the incubation plate as carefully as possible, and that they are checked for vitality and survival before being used for infection experiments. Larvae and pupae should be checked under a stereo microscope for damage and vitality. In both cases it is advisable to incubate them for 12-24 hours prior to conducting the assay, and remove larvae or pupae that show signs of necrosis or low vitality. The infectivity assays should also include a number of additional methodological controls (effects of transfer, incubation, manipulation, feeding etc.), to facilitate the interpretation of the data.

7. Virus purification

7.1. Introduction

The method given below for purifying honey bee viruses is simple but only suitable for non-enveloped viruses, which fortunately covers the vast majority of RNA viruses. However, this method is unsuitable for viruses containing membranes, such as *Apis mellifera* filamentous virus. There are a number of enveloped virus families that have insect -infecting members and there may be more enveloped honey bee viruses to be discovered. The various buffers, solvents and centrifugation conditions for purifying individual viruses are given in Table 4. Most of the buffers shown are the phosphate buffers developed by Bailey and Ball (1991), between 0.01 and 0.5M and of neutral pH (between 7.0 and 8.0). In most cases, TRIS.Cl buffers of similar molarity and pH will perform equally well. Similarly, chloroform can be substituted for the more toxic ether/carbon tetrachloride combination for extract clarification. Nonionic detergents such as Triton X100 (0.05%), BRIJ-58 (2%) and/or sodium deoxycholate (0.2%) are also common agents for lipid solubilisation and extract clarification during virus purification. 0.1 M ascorbic acid is a common alternative to DIECA as antioxidant. Conduct as much of the purification as possible at 4°C (on ice). With each purification step, there is a considerable loss of yield, particularly during the high-speed and gradient centrifugation steps. As much as 80% of the primary extract can be lost during purification. It is therefore important to consider how pure the virus preparation needs to be for your experiments. For infection experiments, purity may be less important while for developing a specific antiserum, high purity is essential. The high-speed and gradient centrifugation steps are excellent for separating the virus from other cellular contents and particles, but are not suitable for separating different virus species: they all have very similar densities.

7.2. Protocols

7.2.1. Primary extract

1. Mix 2 ml of extraction buffer (Table 4) per 1 g of bee tissue.
2. Prepare a primary virus extract by either:
   - Grinding the bee tissues in liquid nitrogen in a mortar-and-pestle.
   - Liquidizing in an automatic blender.
   - Using a large-volume bead mill.

(see also section 8.2.; "Sample Homogenisation")

3. Transfer to a solvent-resistant container.
4. Add 0.5 ml of chloroform or carbon tetra-chloride per 1 g of bee tissue.
5. Shake vigorously by hand.
6. Centrifuge at 8,000 *g* and 4°C for 15 minutes.
7. Carefully collect the supernatant.
8. Discard the organic phase.
9. Remove 10 µl for virus analysis by RT-qPCR or ELISA (see section 9.3. and the *BEEBOOK* article on molecular methods (Evans *et al.,* 2013) to determine the viral purity of the extract.
10. The crude extract at this stage is appropriate for long-term storage. Add glycerol to a final concentration of 50%, aliquot and store at -80°C.

7.2.2. High speed centrifugation

1. Centrifuge the supernatant at 75,000 *g* and 4°C for 3 hours.
2. Discard the supernatant.
3. Re-suspend the pellet 5 ml extraction buffer.

This is best achieved by storing the pellet with buffer overnight at 4°C, to loosen the pellet.

4. Next day vortex the pellet lightly.
5. Centrifuge at 8,000 *g* and 4°C for 15 minutes.
6. Retain the supernatant for gradient centrifugation.

7.2.3. Gradient Centrifugation

The purpose of gradient centrifugation is to concentrate the virus particles according to their specific density and thereby separate them from other cellular material with different density. For high purity requirements, where the virus needs to be separated from other particles with similar density (e.g. ribosomes), 'continuous' gradients are used. These have a gradual transition from high to low density so that each particle-type can band at its own specific density. For lower purity requirements 'discontinuous' gradients can be used. These have low density solution layered on top of high density solution, with a sharp interface between them where all material with a specific density between the high and low solutions concentrates. Discontinuous gradients are slightly easier to prepare and to fractionate, but continuous gradients are cleaner and more secure if the specific density of a virus is not known. Many different substances can be used for creating the density differential (sugars, salts, polyethylene glycol, synthetic polymers), each with their (dis) advantages, but for most virus purification purposes sucrose gradients are adequate. The most common alternative is CsCl (caesium chloride) gradients. These are easier to prepare and generally leave cleaner virus preparations, but require longer centrifugation. CsCl is also chaotropic, stripping virus particles from other cellular constituents, and is therefore not suitable for purifying enveloped viruses. For these, sucrose gradients should be used. Gradient centrifugation is best done in a high-speed, swing-out rotor. These usually have 6 buckets, which should all be used during each centrifugation run, since the rotor is only fully balanced when each bucket is present in its correct position. This means that for each run, six centrifugation tubes should be prepared and balanced.

7.2.3.1. Sucrose gradients

1. Prepare four solutions, containing the appropriate virus extraction buffer (Table 4) and either 10%, 20%, 30% or 40% sucrose.

This is best done by mixing the appropriate amounts of 10x buffer, 60% sucrose solution and water.

2. Divide the total volume of a centrifuge tube by 5.
3. Add 1/5th volume of the 10% sucrose-buffer solution to every centrifugation tube.

Be accurate with the volumes, to avoid problems with balancing the tubes later-on.

4. Using a syringe with a long needle, layer 1/5th volume of the 20% sucrose-buffer solution underneath the 10% solution.
5. Repeat with 1/5th volumes of the 30% and 40% sucrose-buffer solutions.
6. Place the tubes in a freezer with minimum disturbance,.
7. Once completely frozen, take the tubes out and thaw completely.

The higher concentration solution will thaw earlier than the lower density solutions, causing the boundaries between the concentration layers to blur.

8. Repeat the freeze-thaw process twice more to extend this process, generating a continuous density gradient.

Do not freeze-thaw too often, or you will end up with a single-density solution.

9. For discontinuous gradients, layer 1/5th volume of the 40% sucrose-buffer solution underneath 3/5th volume of the 10% sucrose-buffer solution.
10. Layer 1/5th volume of virus extract carefully on top of the gradient.
11. Balance the tubes by weight to within 1 mg, using buffer solution.
12. Insert tubes carefully in the buckets and hang the buckets in the correct orientation in their appropriate place on the rotor.
13. Centrifuge in a swing-out rotor at the appropriate speed, time and temperature for the virus in question (see Table 4).

7.2.3.2. Caesium chloride gradients

CsCl gradients can be used either instead of sucrose gradients, or as an additional purification step after sucrose gradients. CsCl gradients are formed automatically during centrifugation, from a single-density solution (isopycnic, or self-forming, gradients). This is a property of the heavy salt.

1. Resuspend the virus in its buffer (Table 4).

2. Make the solution 1.37 g/ml CsCl; final concentration.

This is the density where most picorna-like viruses will band.

3. Centrifuge 28,000 rpm for 16-24 hours at 15°C-20°C.

Higher centrifugation speeds will create steeper gradients.

7.2.3.3. Fractionation

1. After centrifugation, carefully remove the centrifuge tubes from each bucket.

If there is a lot of virus then the virus particles can be seen as an iridescent band underneath a top-light (Figure 3).

Often two bands can be seen; a lighter band higher in the gradient and a more intense band lower in the gradient. These correspond to 'empty' and 'filled' (with RNA) particles respectively.

2. Remove the band(s) with a disposable syringe and needle.

This is best done using a needle with a 'flat' end, rather than the 'angled' end. Slide the needle along the centrifuge wall to just below the band and draw up the band slowly into the syringe.

3. If no bands can be seen, either because of low virus amounts or because the centrifuge tube is opaque, then the gradient needs to be fractionated by removing 0.5 ml volumes at a time.

The best way is to use an automated fractionator, which removes the fractions from the bottom of the gradient. The manual alternative is to remove 0.5 ml fractions from the top of the gradient.

4. Analyse 10 µl of each fraction for the presence of virus, using either ELISA (see section 9.2.) or RT-(q)PCR (see section 9.3. and the *BEEBOOK* article on molecular methods; Evans *et al.,* 2013).

Since there will be some virus contaminating every fraction, qualitative RT-PCR will not be able to distinguish very well between high and low virus fractions.

5. Pool the 3-4 fractions containing the highest virus concentrations, giving a final volume of approximately 2 ml.
6. If necessary, the virus can be concentrated further by another high-speed centrifugation (Table 4), although this will reduce the yield.

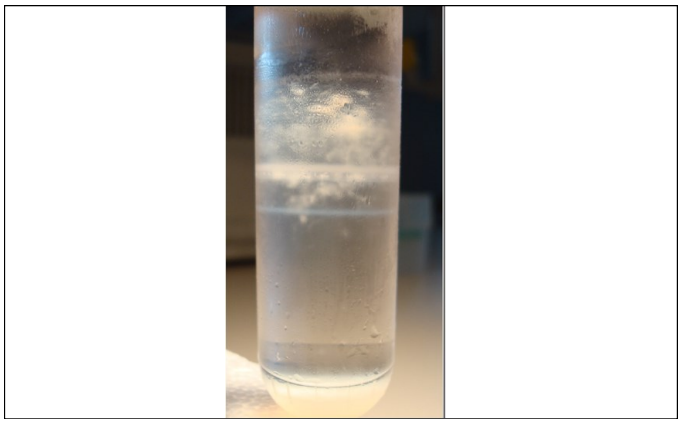

Fig. 3. White translucent band containing DWV particles after CsCl density gradient centrifugation. Image © E Ryabov.

8. Virus sample processing

8.1 Introduction

The primary processing of a sample is crucial for the uniformity of a diagnostic method and should be optimized for high yield and low variability (Bustin, 2000). There are two main stages: sample homogenization and nucleic acid extraction. This section is covered in detail in the *BEEBOOK* article on molecular methods (Evans *et al.,* 2013), with a reduced version given below.

8.2. Sample homogenisation

The most variable step in sample processing is sample homogenization. Different sample types require different homogenization methods (see below), but all should be optimized experimentally to ensure minimal variability between replicate samples.

8.2.1. Bead-mill homogenizers

These are mechanical shakers that homogenize samples with glass, ceramic or steel beads and give excellent and highly uniform homogenization of small samples (1-10 bees).

8.2.2. Blender

Similar homogenization uniformity to the beadmills, but for large samples (50-1000 bees). There is a risk of cross-contamination between samples, through re-use of the blender, which should be assessed.

8.2.3. Mortar and pestle

This method is suitable for medium-size samples (10-50 bees) but is much less uniform than beadmills or blenders, and also has a cross-contamination risk due to re-use of the equipment.

8.2.4. Mesh bags

Mesh bags are a disposable alternative to mortars, also for medium-size samples (10-50 bees). Homogenization uniformity is also moderate, but without the cross-contamination risk.

8.2.5. Micropestle

These are disposable pestles for manually grinding individual bee samples in microcentrifuge tubes. They are much inferior to bead-mills in terms of homogenization uniformity, for the same samples size. At best they should only be used for soft (brood) stages.

8.2.6. Robotic extraction

Robotic extraction stations are very reliable and consistent, but generally only suitable for small sample sizes and soft tissues. They are best used after bead-mill homogenization, as part of a semi-automated homogenization-extraction chain.

8.3. Nucleic acid extraction

A denaturing buffer should be used during homogenization to protect the nucleic acids from degradation. The most common denaturants used are: high concentrations of chaotropic (guanidine) salts, strong antioxidants (β-mercaptoethanol), detergents and/or organic solvents. The nucleic acid is purified from the buffer using either cheap, disposable affinity purification columns or even cheaper precipitation with ethanol, isopropanol or lithium chloride (RNA only). Both methods are reliable, though not particularly uniform (Tentcheva *et al.,* 2006). Affinity columns generally produce cleaner nucleic acid samples, due to the column washing steps. Precipitation can produce higher yields, since columns have a limited binding capacity, or extract volume, equivalent to ~¼ bee.

8.3.1. Protocol 1 – affinity column purification

The processing consists of making a primary homogenate from 1-30 bees and purifying RNA from 100 µl aliquots of the homogenate (equivalent to 20 mg bee tissue: the maximum loading capacity of one affinity column). The protocol is based on the Qiagen RNA purification columns. β-mercaptoethanol is toxic.

1. Prepare fresh GITC buffer:
   1.1. 5.25 M guanidinium thiocyanate (guanidine isothiocyanate),
   1.2. 50 mM TRIS.Cl (pH 6.4),
   1.3. 20 mM EDTA,
   1.4. 1.3% Triton X-100,
   1.5. 1% β-mercaptoethanol.
2. Place frozen bees in the homogenizer of choice.
3. Per bee add the following amount of GITC buffer:

Bee	Weight	Buffer	Total volume
Worker bee	120 mg	500 µl	600 µl
Drone	180 mg	700 µl	900 µl
Worker pupa	160 mg	650 µl	800 µl
Drone pupa	240 mg	1000 µl	1200 µl

4. Proceed according to the Qiagen Plant RNA extraction protocol using 100 µl extract as sample. The Qia-shredder option significantly increases yield and purity of nucleic acid (see Qiagen instructions booklet).
5. Elute in 100 µl nuclease-free water.
6. Determine nucleic acid concentration and purity (see section 8.4.; "Nucleic acid quality assessment").
7. Store as two separate 50 µl aliquots at -80°C, one for working with and one for storage.
8. Include a 'blank' extraction (*i.e.* an extraction of purified water) after every 24 bee samples, to make sure none of the extraction reagents have become contaminated.

8.3.2. Protocol 2 – TRIzol extraction and isopropanol precipitation

This protocol uses TRIzol®; a proprietary mixture of phenol and high concentration salt solution (InVitrogen). The RNA is recovered through isopropanol precipitation.

1. Homogenize bees directly at 4°C in TRIzol® reagent in a glass -walled blender or mortar and pestle. Use 1 ml reagent per bee (~120 mg tissue).
2. Add 0.5 ml chloroform per bee.
3. Shake hard for 1 minute.
4. Centrifuge 8,000 g; 15 minutes; 4°C.
5. Recover the upper (aqueous) layer containing the nucleic acids, discarding the lower red (organic) phase and the semi-solid, white interphase (containing proteins and lipids).
6. Add an equal volume of iso-propanol, mix and precipitate at -20°C for at least 15 minutes.
7. Centrifuge 8,000 g; 15 minutes; 4°C.
8. Remove iso-propanol supernatant.
9. Resuspend nucleic acid pellets in 100 µl RNase-free water.
10. Determine nucleic acid concentration and purity (see section 8.4.; "Nucleic acid quality assessment").
11. Store as two separate 50 µl aliquots at -80°C, one for working with and one for storage.

8.4. Nucleic acid quality assessment

A number of sophisticated methods are available to determine the quantity, quality and integrity of an RNA sample, as described in the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013). The minimum requirements are to determine the yield of the RNA, and its purity with respect to protein and phenolic metabolite contaminants. This can be determined by UV spectrophotometry (Green and Sambrook, 2012), through comparing peak absorbance at 260 nm (nucleic acids), 280 nm (proteins) and 230 nm (phenolic metabolites):

- A^{260} of 1.0 = 40 ng/µl ssRNA
  = 37 ng/µl ssDNA
  = 50 ng/µl dsDNA
- A^{260}/A^{280} < 2.0 indicates contamination with proteins.
- A^{260}/A^{230} < 2.0 indicates contamination with phenolics.

9. Virus detection

9.1. Introduction

There are numerous techniques available for detecting and quantifying viruses (de Miranda, 2008; see also the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013). Most of these detect only a small portion of the viral genome or the capsid proteins, and almost all require some sort of amplification, either of the target (most of the

nucleic acid-based detection technologies) or the detection signal (most of the protein-based detection technologies). Both are important considerations to bear in mind when interpreting virus diagnostic data. Here we will only cover the most commonly used methods.

Secondly, despite the popular classification of molecular assays as either 'qualitative' (presence/absence) or 'quantitative' (concentration), ultimately all assays are quantitative: qualitative assays are simply quantitative assays with a detection threshold (a visible colour; a band on a gel; a fluorescence level; a C_q value; a statistical index). This is an important consideration, since there are many factors besides the initial virus amount that can influence whether or not an assay reaches a detection threshold, such as degradation of the sample, changes to storage-extraction procedures, assay deterioration *etc.*. Furthermore, the molecular and mathematical rules underpinning any assay are the same whether this assay is 'qualitative' or 'quantitative'. The only difference is that in 'quantitative' assays these rules are specifically acknowledged and accounted for, whereas in 'qualitative' assays they are often ignored. It is therefore advisable to approach any experiment or assay from a quantitative perspective first, and include the appropriate controls for threshold-conversion to 'qualitative' data, if this is desired.

9.2. Enzyme-Linked ImmunoSorbent Assay (ELISA)

There are many versions of the ELISA, using different blocking agents, primary/secondary antibodies, reporter enzymes and their specific colorimetric substrate solutions for detection and quantification (Harlow and Lane, 1988). They generally fall into one of two major categories:

9.2.1. Normal ELISA

In conventional ELISA, the sample is adsorbed directly into the wells, to be detected by the specific antibody. This antibody is either conjugated directly to an enzyme (Fig. 4A), usually either horse radish peroxidase or alkaline phosphatase, or more commonly is detected in a subsequent incubation by a commercial enzyme-conjugated protein that recognizes antibodies in general (Fig. 4B).

9.2.2. Sandwich ELISA

In "sandwich" ELISA, a modified version of the primary antibody is adsorbed to the well first, in order to 'capture' the virus particles after the sample is added. The captured virus particles are then detected as before, either with the reporting enzyme directly conjugated to the detecting antibody (Fig. 4C) or with an extra incubation using an antibody-detecting protein conjugated to the reporter enzyme (Fig. 4D). The sandwich ELISA is cleaner and much more sensitive than conventional ELISA, but has a less predictable relationship between virus concentration and signal (depending on which component in the assay is limiting).

The most common reporter enzyme systems are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Both are relatively robust enzymes that can be conjugated to the primary or secondary antibody. They convert a colourless substrate into a coloured reaction product, such that the absorbance at a wavelength appropriate for the specific colour produced is proportional to the amount of enzyme activity present in the sample, which in turn is proportional to the amount of antibody captured by the sample, and thus also the amount of virus in the sample. The protocols below are generic ones for conventional ELISA and sandwich ELISA, based on the methods of Allen *et al. (*1986), using horseradish peroxidase as the reporter enzyme. See also Harlow and Lane (1988) for alternatives and more extensive laboratory protocols involving antibodies.

9.2.3. Protocols

9.2.3.1. Sample preparation

1. Mix phosphate-buffered saline (PBS):
   1.1. 0.8% NaCl,
   1.2. 0.14% $Na_2HPO_4.2H_2O$,
   1.3. 0.02% KH_2PO_4,
   1.4. 0.02% KCl,
   1.5. Adjust to pH 7.4.
2. Grind each bee in 1 ml PBS.
3. Add 300 µl chloroform.
4. Mix on a vortex.
5. Centrifuge for 3 minutes to clarify.

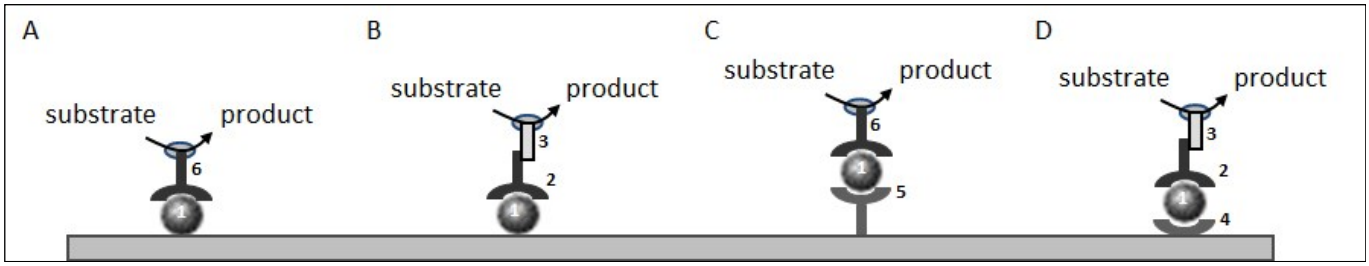

Fig. 4. Different types of ELISA, depending on whether the antigen[1] is adsorbed directly onto the assay well (A & B) or is captured by the F_{ab} fragment of a specific antibody[4] or the full antibody[5] (sandwich ELISA; C & D) and whether the detection system involves an enzyme reporter conjugated directly to the detecting antibody[6] (A & C) or a reporter conjugated to a generic antibody-recognizing protein[3] recognizing the detecting antibody[2] (B & D). Adapted from de Miranda (2008).

9.2.3.2. ELISA

1. Mix coating buffer (CB):
   1.1. 0.159% Na_2CO_3,
   1.2. 0.293% $NaHCO_3$,
   1.3. Adjust to pH 9.6.
2. Seed each well with 180 µl of CB.
3. Add 5-20 µl sample.
4. Incubate 3 hrs at ambient temperature, or overnight at 4°C.
5. Tip out and wash the wells 3X with PBS-T (= PBS containing 0.05% Tween20 detergent), shaking the ELISA plate dry each time.
6. Prepare a 1/2,000 - 1/5,000 dilution of the primary antibody in PBS-TPO:
   6.1. 2% Polyvynylpyrrolidone (PVP) mw 440000,
   6.2. 0.2% Bovine serum albumin (BSA),
   6.3. in PBS-T (fresh daily).
7. Add 200 µl of antibody/PBS-TPO to each well.
8. Incubate 3 hrs at ambient temperature, or overnight at 4°C.
9. Tip out fluid and wash the wells 3X with PBS-T, shaking the ELISA plate dry each time.
10. Prepare a ProteinA-HorseRadishPeroxidase (PrA-HRP) conjugate stock solution at 100 µg/ml.
11. Make a 1/2,000 – 1/5,000 dilution of PrA-HRP stock solution in PBS-TPO.
12. Add 200 µl to each well.
13. Incubate 3hrs at ambient temperature, or overnight at 4°C.
14. Tip out fluid and wash the wells 3X with PBS-T, shaking the ELISA plate dry each time.

9.2.3.3. Sandwich ELISA

1. Seed each well with 200 µl of a 1/2,000-1/5,000 dilution of the F_{ab} fragment (Harlow and Lane, 1988) of the primary antibody in coating buffer (CB, see section 9.2.3.2.).
2. Incubate 3hrs at ambient temperature, or overnight at 4°C.
3. Tip out and wash the wells 3X with PBS-T, shaking the ELISA plate dry each time.
4. Add 180 µl PBS-TPO to each well.
5. Add 20 µl sample to each well.
6. Incubate 3hrs at ambient temperature, or overnight at 4°C.
7. Tip out fluid and wash the wells 3X with PBS-T, shaking the ELISA plate dry each time.
8. Add 200 µl 1/2,000 – 1/5,000 dilution of PrA-HRP stock solution in PBS-TPO to each well.
9. Incubate 3hrs at ambient temperature, or overnight at 4°C.
10. Tip out fluid and wash the wells 3X with PBS-T, shaking the ELISA plate dry each time.

9.2.3.4. Development

The development of the enzymatic reaction is the same for conventional and sandwich ELISA. This method is appropriate for horseradish peroxidase as a reporter enzyme. A different substrate is required if alkaline phosphatase is used as the reporter enzyme (Harlow and Lane, 1988), together with a different wavelength for determining the absorbance, but the overall procedure is the same.

1. Prepare TMB substrate solution:
   1.1. 100 ml water,
   1.2. 1 ml 10 mg/ml TMB (33'55'TetraMethylBenzidine) in DMSO (DiMethylSulfOxide),
   1.3. 10 ml 1M Na Acetate (pH 5.8 with 1.0M citric acid),
   1.4. 20 µl 30% H_2O_2.
2. Add 200 µl substrate solution to each well.
3. Let colour develop for 10-15 minutes .
4. Add 50 µl 3M H_2SO_4 to terminate the reaction.
5. **Immediately** read the absorbance at 450 nm (the termination reaction will continue to develop colour).

9.2.3.5. Controls

ELISA is a complex, multistep assay involving sensitive enzymatic reporters, which means that there are many opportunities for assay failure, either through false-positive or false-negative results. Enzymatic reporter systems, such as used by ELISA, are sensitive to any native enzymatic activity present in the sample (peroxidases, phosphatases). The initial coating step in a highly alkaline buffer abolishes most of such background activity, as does the specific capture of virus particles in sandwich ELISA and the washes with PBS. However, the user should be aware of the possibility of residual enzymatic activity in the samples, particularly if the substrate incubation step is extended to allow more colour to develop (for instance, when trying to detect very low amounts of virus). Secondly, either the enzyme or the substrate may be faulty, preventing colour development even though there has been antibody recognition of the sample. Alternatively, the primary or secondary antibody may fail, for a number of reasons. All ELISA assays should therefore have a number of controls to establish the correct functioning of the assay itself, thus validating the results from the samples.

- Reporter-free negative control (quantification of background substrate absorbance).
- Sample-free negative control (Absence of non-specific binding of antibodies/reporters to wells; test of blocking and washing efficacy).
- Primary antibody-free negative control (Absence of non-specific binding of secondary antibody and/or reporters).
- Secondary antibody/reporter-free negative control (quantification of background enzymatic activity in samples).

- Substrate-free negative control (quantification of background absorbance in the system).
- Primary antibody positive control (direct adsorption of antibody in CB: test for recognition of antibody by secondary antibody-reporter).
- Secondary antibody positive control (direct adsorption of secondary antibody-reporter in CB: test for functioning reporter enzyme).
- Purified virus positive control (correct recognition of virus by primary antibody; calibration standards).

9.3. RT-(q)PCR

The most common current methods for honey bee virus detection are based on Reverse Transcription Polymerase Chain reaction (RT-PCR), essentially the PCR amplification of cDNA. A detailed coverage of the principles and practices of PCR is found in Yuryev (2007) and the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013). A reduced version, including those elements specifically relevant to virus detection, is presented here.

9.3.1. Primer design

Designing RT-(q)PCR assays for detecting (honey bee) RNA viruses poses some unique challenges. The most critical components of a PCR assay are the two amplification primers. RNA viruses are genetically highly variable while PCR is very sensitive to nucleotide mismatches between primer and target, particularly at the 3′ primer termini where extension occurs (Onodera, 2007). A mismatch at the 3′ terminus of just one of the primers will result in non-amplification. Mismatches further away from the 3′ terminus have increasingly less influence on the success of amplification and generally only the last two 3′ nucleotides are critical for amplification specificity. The 3′ mismatch issue is therefore crucial to the specificity, accuracy, reliability and sensitivity of a PCR-based virus assay. Here we outline how to use this to our benefit, and how to avoid it when needed.

9.3.1.1. What do we want to detect?

The first decision is to establish precisely what the assay should detect and what it should not detect:

- For distinguishing closely related strains, locate the 3′ terminus of one primer at a position where the strains differ consistently. The other primer can be common for all strains (de Miranda *et al.,* 2010b).
- For detecting all potential variants within a virus species or complex, the primer sequences should be conserved between all known variants, so as to be able to detect both known and as-yet-unknown variants in the complex. Locate the primers at least 200 nucleotides apart, so that new variants can be identified by sequence analyses of the intervening region.
- Avoid locating the 3′ terminus of a primer on the 3rd base of a codon in the coding region of a virus genome, since these are by far the most variable nucleotides in any virus genome (Grabensteiner *et al.,* 2001; Bakonyi *et al.,* 2002b; de Miranda *et al.,* 2004; Lanzi *et al.,* 2006; Olivier *et al.,* 2008; de Miranda *et al.,* 2010b).
- Use deoxyinosine as the 3′ nucleotide, which can pair with all nucleotides (Benjeddou *et al.,* 2001; Topley *et al.,* 2005), thus avoiding the 3′ mismatch problem altogether.

9.3.1.2. Where in the genome?

The genome of positive-strand RNA viruses is usually compact and efficiently coded, and there is normally no duplication of sequences within the genome. This facilitates the assay design enormously, since one can choose between many alternative assays on virological and assay performance-quantitation criteria, no matter where in the genome they are located, since they should all only amplify a single region of the genome.

9.3.1.3. Primer annealing temperature, length and composition

Both amplification primers should have similar melting temperature (T^m), length and composition. It is useful to design all assays and primers around the same annealing temperature, so that a single cycling program can be used for all assays, and that different assays can be run concurrently with the same program, on the same plate. 56°C is a good, standard, robust target for the *in silico* estimated T^m for primers.

9.3.1.4. Primer-dimer and other PCR artefacts

PCR is susceptible to qualitative and quantitative errors caused by the accidental, and highly efficient, amplification of short non-target PCR templates formed by fleeting complementarity of the primers with non-target templates, or among the primers themselves (SantaLucia, 2007; see the *BEEBOOK* paper on molecular methods; Evans *et al.,* 2013). Such artefacts can be identified by gel electrophoresis during assay optimization. The easiest solution to persistent PCR artefacts is to design new primers and test these experimentally (SantaLucia, 2007).

9.3.1.5. Fragment length

PCR assay design software packages usually design very short amplicons (< 100 nucleotides), with high amplification efficiency and short cycling times. However, amplicons up to 300 nucleotides amplify equally efficiently but are easier to separate from PCR artefacts, provide more room for designing probes and can be used to characterize new variants, through sequence analyses.

9.3.2. Detection and analysis of PCR products

9.3.2.1. "End-point" vs "real-time" detection

The PCR products can be detected after the PCR is completed, usually for "qualitative" analysis (presence/absence of product), either by (gel) electrophoresis or Melting Curve analysis. Detection can also be done after each cycle, as PCR proceeds, using laser optics (*i.e.* in 'real

-time'). The amount of initial target cDNA in a reaction can then be very accurately related to how many amplification cycles are required for a product to appear. This is the basis for "quantitative" PCR (qPCR), which is extremely accurate over a wide range of target concentrations (see the *BEEBOOK* paper on molecular methods; Evans *et al.,* 2013).

9.3.2.2. Cycles and thresholds

Continuous real-time detection also allows multiple detection thresholds (*i.e.* the cycle at which product appears) to be set for the same reaction, which can be related to different levels of risk for disease. For most practical (or even experimental) purposes, 35 cycles of amplification is sufficient. Beyond 35 cycles, the rapidly increasing risk of both false-positive and false-negative detection errors outweighs the marginal gains in sensitivity (see the *BEEBOOK* paper on molecular methods; Evans *et al.,* 2013).

9.3.2.3. Detection chemistry

There are many different detection chemistries available for following qPCR in real-time (de Miranda, 2008). The two most common are SYBR-green and similar DNA-binding dyes, and fluorophore-labelled hydrolysis (TaqMan™) probes. The merits of both systems are discussed in the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013). TaqMan™ assays are best suited for well-optimized, stable assays for widespread, routine diagnosis. SYBR-green assays are better suited for discovery, characterization of new strains and analysis of strain mixtures (Papin *et al.,* 2004). They are also much cheaper to design, adapt and optimize (Bustin and Nolan, 2004).

9.3.3. Assay optimization

Once a PCR assay has been designed, it should be optimized experimentally for annealing temperature (using annealing temperature gradients), primer concentration and cycling times (see the *BEEBOOK* paper on molecular techniques; Evans *et al.,* 2013). Optimization usually identifies the highest annealing temperature, the lowest primer concentrations and the shortest incubation time that consistently generates the right product, without artefacts, at a consistent amplification cycle (see section 12: "Quality control").

9.3.3.1. Reverse transcription

Reverse transcription is the most variable step in RT-PCR, whose efficiency is easily affected by inhibitors, reaction conditions (including primers) and even nucleic acid concentration (Ståhlberg *et al.,* 2004a; 2004b). To minimize this variability, the nucleic acid should be optimally prepared and a constant amount used in every reaction. Since PCR does not require large amounts of initial target, the RNA can be diluted to minimise the effects of any inhibitors. cDNA is best prepared with random hexamer primers that generate a bias-free cDNA copy of the entire RNA population, suitable for a multitude of analyses.

9.3.3.2. One-Step/Two-Step RT-PCR

Reverse transcription and PCR can be conducted in a single buffer, PCR following reverse transcription (One-Step RT-PCR) or in two separate reactions (Two-Step RT-PCR). The advantages of One-Step RT-PCR are speed and reduced contamination risk; the disadvantages are wasteful use of precious RNA and inability to control for differences in cDNA synthesis efficiency between reactions (Bustin, 2000; Bustin *et al.,* 2009). These (dis)advantages are reversed for Two-Step RT-PCR, with the additional advantage that the cDNA produced can be used for many other purposes as well. Two-Step RT-PCR also tends to be considerably more sensitive and more prone to artefacts, unless steps are taken to avoid this (see the *BEEBOOK* paper on molecular methods; Evans *et al.,* 2013).

9.3.4. Protocols

Numerous qualitative and quantitative RT-(q)PCR protocols have been published for honey bee viruses (Annex 1: http://www.ibra.org.uk/downloads/20130805/download), although few have been optimized experimentally. The European Reference Laboratory for bee diseases at ANSES (France) is in the process of designing fully optimized, validated RT-qPCR protocols for all bee viruses (see Blanchard *et al.,* 2012) for routine, standardised diagnostic use by accredited laboratories. For experimental purposes, existing published protocols can be used and optimized, many of which can be easily adapted to qPCR using SYBR-green dye detection. Alternatively, new protocols can be designed based on the following practical, robust protocols for Reverse Transcription, One-Step RT-qPCR and Two-Step RT-qPCR, suitable for either quantitative or qualitative analyses. These provide a useful basis for individual adaptation and optimization, using the guidelines given above and in the *BEEBOOK* paper on molecular techniques (Evans *et al.,* 2013).

9.3.4.1. Reverse transcription

The following is a robust, standard reverse transcription protocol for generating cDNA that is fully representative of the original RNA population:

1. Mix:
   1.1. 0.5 µg sample RNA template,
   1.2. 1 ng exogenous reference RNA (*e.g.* Ambion RNA250),
   1.3. 1 µl 50 ng/µl random hexamers,
   1.4. 1 µl 10mM dNTP,
   1.5. up to 12 µl RNAse free water.
2. Heat the mixture to 65°C for 5 min and chill quickly on ice.
3. Add:
   3.1. 4 µl 5X First-Strand Buffer,
   3.2. 2 µl 0.1 M DTT,
   3.3. 1 µl (200 units) of M-MLV RT.
4. Mix by pipetting gently up and down.
5. Centrifuge briefly to collect the contents at the bottom of the tube.

6. Incubate 10 min at 25°C.
7. Incubate 50 min at 37°C.
8. Inactivate the reaction by heating 15 min at 70°C.
9. Dilute the cDNA solution tenfold with nuclease-free water before using in PCR assays, to reduce the risk of PCR artefacts.

9.3.4.2. Two-Step RT-qPCR

The following is a robust, standard qPCR protocol for amplifying and quantifying cDNA templates < 300bp in length. The protocol is based on SYBR-green detection chemistry, with modifications for probe-based detection and qualitative PCR indicated:

1. Mix:
   1.1. 3 µl cDNA (pre-diluted 1/10, in nuclease-free water),
   1.2. 0.6 µl 10 µM Forward primer (0.3 µM final concentration),
   1.3. 0.6 µl 10 µM Reverse primer (0.3 µM final concentration),
   *[1.4. 0.4 µl*10 µM TaqMan™ probe* (0.2 µM final concentration*)],*
   1.5. x µl *TwoStep Buffer* + dNTP(0.2 mM final dNTP),
   1.6. y µl nuclease-free water,
   1.7. z µl Thermostable DNA polymerase mix,
   1.8. 20 µl total volume.

* Use appropriate buffer for either SYBR-green or TaqMan™ probe assays. dNTP is usually included in pre-optimized buffers. If not, add separately to 0.2 mM final concentration.

2. Incubate in real-time thermocycler:
   2.1. 5 min:95°C,
   2.2. 35 cycles [10 sec:95°C - 30 sec:58°C - read].
3. For SYBR-green assays, follow with Melting Curve analysis:
   3.1. 1 min:95°C,
   3.2. 1 min:55°C,
   3.3. 5 sec:0.5°C:read from 55°C to 95°C.
4. For qualitative PCR, a conventional thermocycler can be used and the products can be analysed by gel, capillary or chip-based electrophoresis.

9.3.4.3. One-Step RT-qPCR

The following is a robust, standard One-Step RT-qPCR protocol for amplifying and quantifying targets < 300bp in length, using SYBR-green detection chemistry, and starting with an RNA template:

1. Mix:
   1.1. 3 µl 5 ng/ µl RNA,
   1.2. 0.6 µl 10 µM Forward primer (0.3 µM final concentration),
   1.3. 0.6 µl 10 µM Reverse primer (0.3 µM final concentration),
   *[1.4. 0.4 µl*10 µM TaqMan™ probe* (0.2 µM final concentration*)],*
   1.5. x µl *OneStep Buffer* + dNTP(0.2 mM final dNTP),
   1.6. y µl nuclease-free water,
   1.7. z µl Reverse Transcriptase/Thermostable DNA polymerase mix,
   1.8. 20 µl total volume.

* Use appropriate buffer for either SYBR-green or TaqMan™ probe assays. dNTP is usually included in pre-optimized buffers. If not, add separately to 0.2 mM final concentration.

2. Incubate in real-time thermocycler:
   2.1. 15 min:50°C,
   2.2. 5 min:95°C,
   2.3. 35 cycles [10 sec:95°C - 30 sec:58°C - read].
3. For SYBR-green assays, follow with Melting Curve analysis:
   3.1. 1 min:95°C,
   3.2. 1 min:55°C,
   3.3. 5 sec:0.5°C:read from 55°C to 95°C.
4. For qualitative PCR, a conventional thermocycler can be used and the products can be analysed by gel, capillary or chip-based electrophoresis.

9.3.5. Quantitation controls

A number of controls are required for quantifying the amount of virus in a sample. These can be broadly divided into "external reference standards", which are used to quantify the absolute amount of target in each reaction, and "internal reference standards", which are used to correct the quantitative data for unique differences between individual samples. The *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013) describes in detail the function, preparation and application of these standards.

9.3.5.1. External reference standards

These consist of dilution series of known concentrations of (cloned) target DNA or RNA, which is used to establish a calibration curve for converting RT-qPCR data to absolute amounts of target (genome copies) in a reaction (Pfaffl and Hageleit, 2001; Bustin *et al.,* 2009). External reference standards should be prepared for every target assayed, including all internal reference standards.

9.3.5.2. Internal reference standards

Unfortunately, external standards cannot correct for factors unique to each sample that affect the RT and/or PCR reactions, such as RNA quality and quantity, enzyme inhibitors, sample degradation, internal fluorescence *etc.* To correct for these factors, internal reference standards are used. These come in two forms:

"*Exogenous internal reference standard"*, which is a pure, unrelated RNA of known concentration that is added to the RT mastermix prior to RT-qPCR (Tentcheva *et al.,* 2006). The amount used should be < 1% of the amount sample RNA, so as not to affect

the RT-qPCR reaction efficiencies. These are used to calculate cDNA reaction efficiencies of individual samples (correcting for RT inhibitors).

"Endogenous internal reference standards" (commonly called 'housekeeping genes'), are relatively invariant host mRNAs present in every sample. These can be used to normalize quantitative data for differences between samples in RNA degradation or the presence of inhibitors (Bustin *et al.,* 2009; Radonić *et al.,* 2004) and to guard against 'false-negative' data (due to RNA degradation).

There are a couple of practical difficulties with endogenous internal reference standards. First, one can never be certain that they are truly invariant (Radonić *et al.,* 2004). The current recommendations are therefore to use an index of 3 or 4 endogenous reference standards for data correction (Bustin, 2000). Second, contaminating genomic DNA in an RNA sample can interfere with accurate quantification of the endogenous gene mRNA. This can be avoided by digesting the RNA sample with DNAse prior to RT-PCR, or more elegantly by designing intron-spanning primers for the endogenous reference gene (Bustin, 2000; Yañez *et al.,* 2012; Locke *et al.,* 2012), such that only cDNA to the mRNA can be amplified.

Internal reference standards are costly, since they are run for all samples. Their inclusion should therefore be evaluated in relation to their importance to the project. There are probably more relevant for fully-quantitative experiments and less for semi-quantitative surveys.

9.3.6. Multiple assays

With careful primer design (see section 9.3.1.) it should be possible to approach 100% correct detection (no false positive or false negative results) for most viruses with a single primer pair. This is, however, very much conditional on the natural variation and variability (*i.e.* the capacity to generate new variants) for each virus. There are valid arguments that PCR is perhaps too specific for the reliable detection of highly variable entities such as RNA viruses, even when employing several different primer sets (Gardner *et al.,* 2003). When the reliability of a primer set with respect to virus variability is in doubt, the best resolution is to employ several primer sets in parallel so that the failure of one set does not necessarily result in misdiagnosis. Multiple primer sets also allows one to estimate the rate of misdiagnosis by different primer sets due to virus variability (Chui *et al.,* 2005). Within the honey bee viruses, multiple primer sets may be needed for reliable diagnosis within the highly variable ABPV complex (de Miranda *et al.,* 2010a) and the slightly less variable DWV-VDV-1 complex (de Miranda and Genersch, 2010). Multiple assays are available for most honey bee viruses, and comparisons of multiple assays have been made for SBV (Grabensteiner *et al.,* 2001), ABPV (Bakonyi *et al., 2002a;* 2002b) and DWV (Genersch, 2005).

9.3.7. Multiplex RT-(q)PCR

Multiplex RT-PCR refers to the simultaneous amplification of several targets in the same reaction. The different end-products are usually identified by size, through (gel) electrophoresis. Several such qualitative multiplex protocols have been designed for honey bee viruses (Chen *et al.,* 2004b; Topley *et al.,* 2005; Grabensteiner *et al.,* 2007; Weinstein-Texiera *et al.,* 2008; Meeus *et al.,* 2010). Real-time qPCR can also be multiplexed, usually for the simultaneous amplification of a target and internal reference standards, by using TaqMan™ probes with different fluorophores.

The main reason for multiplexing is to save cost and time. However, multiplex PCR is less sensitive than uniplex PCR, more complex to optimize, more prone to artefacts and requires post-PCR fragment analysis, nullifying any gains in time and cost. Most importantly, the late amplification of low-abundance targets is strongly affected by the prior amplification of high-abundance targets, through the auto-inhibition of the PCR by the DNA it produces (SantaLucia, 2007). For these reasons, it is often more effective to use uniplex RT-PCR, even for large projects.

9.4. Microarrays

Multiplexing is far more effective through a microarray, which is an ordered array of hundreds of molecular probes specific for different target RNAs bound to a solid support, usually a slide. Most microarray technology has been developed for nucleic acid probes, although protein-based arrays are also being developed (Sage, 2004). The hybridization of RNA target sequences to these probes to these probes can be detected by a variety of methods (de Miranda, 2008), including PCR and sequencing. Numerous honey bee microarrays have been designed, including honey bee immune gene-pathogen arrays (Evans, 2006; Runckel *et al.,* 2011) and a honey bee virus array (Glover *et al.,* 2011). Microarrays are being superseded for research purposes by high-throughput sequencing technologies, but retain a future in routine screening applications, due to their adaptability and high multiplexing capacity (Glover *et al.,* 2011). See also the microarray section in the *BEEBOOK* paper on molecular methods (Evans *et al.,* 2013).

10. Virus replication

10.1. Introduction

The detection of viral replication is crucial for differentiating between an active infection and just the presence of virus particles in a host, or between a mechanical (virus non-replicating) and a biological (virus replicating) vector of a virus. Evidence that a virus is actively infecting a host includes the presence of viral particles and structures within host cells, revealed by electron microscopy, preferably including a specific nucleic acid or serological probe to positively identify the virus. Another approach is to detect the non-structural proteins involved in virus replication, which for most positive-stranded RNA viruses are only produced after invasion and mark the start of a

replication cycle. Negative-stranded RNA viruses often carry their replicative proteins within the particle. A related philosophy, which is more sensitive and accessible, is to specifically detect the replicative strand RNA of a virus. Most of the described bee viruses are single- and plus-strand RNA viruses which replicate through a negative-strand RNA intermediate serving as template for the generation of new viral plus-strand RNA genomes. The specific detection of viral negative strand RNAs can therefore serve as a marker of active replication of these RNA viruses in a certain host, tissue or cell type. Below are outlined two methods for strand-specific detection of RNA virus sequences.

10.2. Strand-specific RT-qPCR

One of the most popular methods in bee virology for detecting virus replication is the specific detection of negative strand viral RNA, using strand-specific Reverse Transcriptase-PCR (Peng *et al.,* 2012; DiPrisco *et al.,* 2011; Boncristiani *et al.,* 2009; Dainat *et al.,* 2009; Eyer *et al.,* 2009; Gisder *et al.,* 2009; Celle *et al.,* 2008), following its first application in bee virology by Yue and Genersch (2005). The procedure is illustrated in Fig. 5, including the most common cause of false-positive results (non-specific cDNA synthesis) and how best to avoid this (tagged-cDNA primer followed by tag-specific PCR). Theoretically, strand-specificity can be achieved by performing the reverse transcription reaction in the presence of only one primer specifically annealing with a unique region of the viral negative strand before amplifying the obtained cDNA by adding the second primer or a specific primer pair for PCR amplification. Unfortunately, strand-specific RT-PCR is highly susceptible to false positive results (Gunji *et al.,* 1994; Lanford *et al.,* 1995; Lanford *et al.,* 1994; McGuiness *et al.,* 1994; Craggs *et al.,* 2001; Peyrefitte *et al.,* 2003; Boncristiani *et al.,* 2009) due to:

- False-priming of the incorrect strand by the cDNA primer.
- Self-priming of positive-strand RNA in areas of complex secondary structures.
- Random priming by contaminating cellular nucleic acids.
- Incomplete inactivation of the reverse transcriptase, leaving residual activity during PCR amplification (which contains both negative and positive strand primers).

To overcome these drawbacks and improve the specificity of the assays, certain effective techniques have been developed that enhance strand-specificity. These include:

- Thermostable reverse transcriptases.
- Tagged-cDNA primers.
- Inactivation/removal of residual tagged-cDNA primers prior to PCR.
- Chemical blocking of free 3' ends before or after reverse transcription.

10.2.1. Thermostable reverse transcriptases

Thermostable reverse transcriptases, operating at temperatures up to 50-70°C, avoid much non-specific priming of the RNA through elevated reaction temperatures (Lanford *et al.,* 1995; Laskus *et al.,* 1998; Craggs *et al.,* 2001; Horsington and Zhang, 2007; Carrière *et al.,* 2007; Celle *et al.,* 2008). Thermostable reverse transcriptases need to be inactivated thoroughly prior to the PCR step, otherwise the reverse transcriptase has access to primers for both strands (thus nullifying the strand-specificity). Another strategy is to inactivate the (viral) RNA by alkaline treatment or digestion with RNase H (McGuiness *et al.,* 1994), thereby removing any target for reverse transcription during PCR.

When using thermostable reverse transcriptase, make sure that the virus-specific portion of the tagged cDNA primer has a theoretical T^m ~60°C, to ensure adequate priming at elevated temperatures.

10.2.2. Chemical blocking of RNA 3' ends

The free 3' ends of the RNA can be blocked with borohydride, so that the RNA cannot serve as a primer for cDNA synthesis by self-priming or random priming with small cellular RNAs. This means that only RNA primed with the strand-specific cDNA primers can be elongated by the reverse transcriptase (Gunji *et al.,* 1994). The protocol involves oxidation of the RNA free ends with sodium periodate ($NaIO_4$) followed by reduction with sodium tetrahydroborate ($NaBH_4$).

10.2.3. Tagged cDNA primers

To further improve strand-specific detection of viral RNA, tagged RT-PCR can be used (Craggs *et al.,* 2001). This method relies on a primer for cDNA synthesis which contains a tag sequence at the 5'-end that is unrelated to either virus or host. PCR amplification is then carried out with a primer consisting of only the tag sequence, together with a virus-specific upstream primer. This ensures that only cDNA's derived from the tagged cDNA primer are amplified, and not cDNAs from false-, self- or mis-priming events. It is therefore important to ensure that the chosen tag sequence does not show any homology with a known bee pathogen or invertebrate sequence, by checking the tag sequences against the nucleotide sequence databases available on the NCBI website, using BLAST (Altschul *et al.,* 1990). Tag sequences screened for use with honey bee viruses are shown in Table 5.

10.2.4. Removal of tagged-cDNA primers from the cDNA reaction

Since the purpose of using tagged-cDNA primers is to amplify only with the tag sequence, it is important to either remove or inactivate the original tagged-cDNA primers after the cDNA reaction, and prior to PCR. If not, then these tagged-cDNA primers (which contain virus-specific sequences) can participate in the PCR reaction, just like a

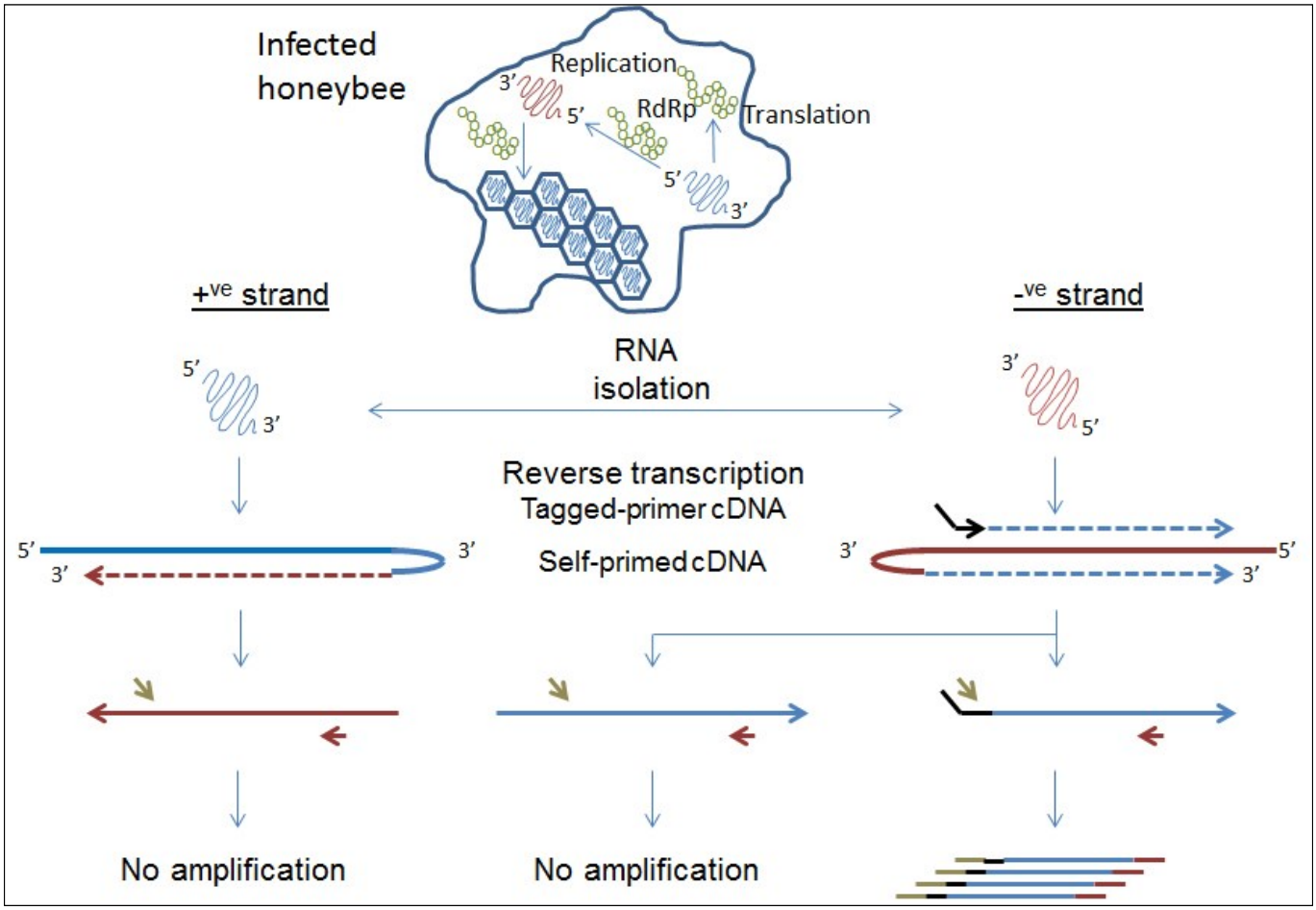

Fig. 5. Outline of the procedure for strand-specific RT-PCR amplification of negative-strand viral RNA, using tagged-cDNA primers to avoid amplification of non-target cDNAs. Only cDNA produced with tagged-cDNA primers, and amplified with the tag and a virus-specific primer will be amplified. RdRp refers to the viral RNA-dependent RNA polymerase. Image © JR de Miranda.

non-tagged virus-specific cDNA primer would. The presence of tagged -cDNA primers in the PCR reaction therefore permits the amplification of false-, self- or misprimed cDNAs of the 'wrong' strand, leading to an incorrect conclusion of strand-specificity (Craggs *et al.,* 2001; Peyrefitte *et al.,* 2003; Plaskon *et al.,* 2009; Boncristiani *et al.,* 2009).

Tagged-cDNA primers can be most easily removed from the cDNA reaction using commercial PCR/cDNA purification columns (Peyrefitte *et al.,* 2003). An alternative is to use biotinylated tagged-cDNA primers and then capture the tagged cDNA with Streptavidin-conjugated magnetic beads (Boncristiani *et al.,* 2009). Although highly effective at removing primers, the disadvantage of cDNA purification is that its DNA recovery efficiency of individual columns can be highly variable (Tentcheva *et al.,* 2006), leading to different types of error in (quantitative) detection and interpretation. This can be managed by adding a passive 'reference' DNA prior to cDNA purification (similar in concept to the "exogenous internal reference standards" used in RT-qPCR quantification, discussed in the *BEEBOOK* paper on molecular techniques; Evans *et al.,* 2013), which can be used to normalize the data again afterwards.

10.2.5. Inactivating tagged-cDNA primers in the cDNA reaction

It is also possible to inactivate the tagged-cDNA primer in the cDNA reaction, and so prevent it from participating in the PCR reaction. This can be done using exonuclease-I, which specifically digests only single-stranded DNA, *i.e.* the tagged-cDNA primer (Craggs *et al.,* 2001; Purcell *et al.,* 2006; Plaskon *et al.,* 2009; Lin *et al.,* 2009) or by phosphorylating the 3' end of the tagged-cDNA primer (making it impossible for the polymerases in PCR to synthesize DNA from this primer). These enzymatic reactions can be done right after the cDNA reaction, in the same reaction tube, after which the enzymes can be heat-inactivated prior to the PCR reaction. The exonuclease-I digestion has become the method of choice for quantitative strand-specific RT-qPCR (*e.g.* Purcell *et al.,* 2006; Plaskon *et al.,* 2009; Lin *et al.,* 2009; Runckel *et al.,* 2011). The main advantages of enzymatic inactivation of the tagged-cDNA primers, compared to the primer removal methods (see section 10.2.4.), is that enzymatic inactivation is much faster and cheaper, with fewer handling and contamination errors (no tube changes), and that it avoids the possible quantitation errors of the primer removal methods.

Table 5. Primers and tags used for detection of positive and negative strand honey bee virus RNAs by strand-specific RT-PCR.

TAG	VIRUS	STRAND	PRIMER	SEQUENCE	FUNCTION	REFERENCES
agcctgcgcaccgtgg	DWV	+ (pos)	tag-B23 F15	agcctgcgcaccgtggCCACCCAAATGCTAACTCTAAGCG TCCATCAGGTTCTCCAATAACGGA	tagged-cDNA virus-sense	Yue and Genersch, 2005 Gisder *et al.*, 2009 Dainat *et al.*, 2009
		- (neg)	tag-F15 B23	agcctgcgcaccgtggTCCATCAGGTTCTCCAATAACGGA CCACCCAAATGCTAACTCTAAGCG	tagged-cDNA virus-antisense	
	DWV	+ (pos)	tag-DWVas DWV-s	agcctgcgcaccgtggTCGACAATTTTCGGACATCA ATCAGCGCTTAGTGGAGGAA	tagged-cDNA virus-sense	Boncristiani *et al.*, 2009
		- (neg)	tag-DWVs DWV-as	agcctgcgcaccgtggATCAGCGCTTAGTGGAGGAA TCGACAATTTTCGGACATCA	tagged-cDNA virus-antisense	
	IAPV	+ (pos)	tag-IAPVas IAPV-s	agcctgcgcaccgtggCTTGCAAGATAAGAAAGGGGG GCGGAGAATATAAGGCTCAG	tagged-cDNA virus-sense	DiPrisco *et al.*, 2011
		- (neg)	tag-IAPVs IAPV-as	agcctgcgcaccgtggGCGGAGAATATAAGGCTCAG CTTGCAAGATAAGAAAGGGGG	tagged-cDNA virus-antisense	
	BQCV	+ (pos)	tag-BQCVas BQCV-s	agcctgcgcaccgtggGCAACAAGAAGAAACGTAAACCAC TCAGGTCGGAATAATCTCGA	tagged-cDNA virus-sense	Peng *et al.*, 2011
		- (neg)	tag-BQCVs BQCV-as	agcctgcgcaccgtggTCAGGTCGGAATAATCTCGA GCAACAAGAAGAAACGTAAACCAC	tagged-cDNA virus-antisense	
atcggaatcgcctagctt	CBPV	+ (pos)	tag-R23 R20	atcggaatcgcctagcttCCCAATGTCCAAGATGGAGT GCTTGATCTCCTCCTGCTTG	tagged-cDNA virus-sense	Celle *et al.*, 2008
		- (neg)	tag-R20 R23	atcggaatcgcctagcttGCTTGATCTCCTCCTGCTTG CCCAATGTCCAAGATGGAGT	tagged-cDNA virus-antisense	
ggccgtcatggtggcgaataa (Plaskon *et al.*, 2009)	LSV-1	+ (pos)	tag-LSVU-R1717 LSV1-F1434	ggccgtcatggtggcgaataaCCATATCATAAGTTGGCAAGTG CAGGTGCAGAGCAATTGGATTCA	tagged-cDNA virus-sense	Runckel *et al.*, 2011
		- (neg)	tag-LSV1-F1434 LSVU-R1717	ggccgtcatggtggcgaataaCAGGTGCAGAGCAATTGGATTCA CCATATCATAAGTTGGCAAGTG	tagged-cDNA virus-antisense	
	LSV-2	+ (pos)	tag-LSVU-R1717 LSV2-F1434	ggccgtcatggtggcgaataaCCATATCATAAGTTGGCAAGTG TAGGTGTCGGGCCATAGGGTTTG	tagged-cDNA virus-sense	
		- (neg)	tag-LSV2-F1434 LSVU-R1717	ggccgtcatggtggcgaataaTAGGTGTCGGGCCATAGGGTTTG CCATATCATAAGTTGGCAAGTG	tagged-cDNA virus-antisense	

10.2.6. Dilution

Another strategy to minimize the chance of illegitimate amplification of non-strand-specific cDNA molecules through the involvement of residual tagged-cDNA primer, is to dilute the cDNA reaction mixture 10-fold prior to PCR (Craggs *et al.,* 2001).

10.2.7. Strand-specific real-time RT-qPCR

Finally, a very effective way to manage the consequences of illegitimate priming events during cDNA synthesis is to use real-time qPCR for strand-specific detection (Purcell *et al.,* 2006; Gisder *et al.,* 2009; Boncristiani *et al.,* 2009; Plaskon *et al.,* 2009; Lin *et al.,* 2009; Zioni *et al.,* 2011). This allows all the PCR products arising from rare cDNAs generated by false-, self- or mis-priming events to be excluded from the data on quantitative grounds.

10.2.8. Protocols

All these conditions can easily be incorporated into a one-tube protocol combining the benefits of an RT-reaction at higher temperature, tagged primers, exonuclease-I digestion of the tag-cDNA primer and dilution of the cDNA, prior to real-time qPCR with a primer complementary to the tag and a virus-specific forward primer.

10.2.8.1. High temperature reverse transcription

Using an elevated temperature for the cDNA reaction significantly reduces mis-priming events, and thus the risk of falsely detecting the incorrect strand. Common alternatives are: SuperScript-III (50°C: Peyrefitte *et al.,* 2003; Purcell *et al.,* 2006; Plaskon *et al.,* 2009; Lin *et al.,* 2009; Runckel *et al.,* 2011), OmniScript/SensiScript (55°C: Yue and Genersch, 2005; Gisder *et al.,* 2009), Thermoscript (60°C: Carrière *et al.,* 2006; Horsington and Zhang, 2007) and r*Tth* reverse transcriptase (70°C: Lanford *et al.,* 1995; Laskus *et al.,* 1998; Craggs *et al.,* 2001; Celle *et al.,* 2009). The method below is a generic one, with individual adaptations for the different reverse transcription options:

1. Mix:
   1.1. 5 µl 50 ng/µl RNA,
   1.2. 1 µl 10 µM tagged-cDNA primer (0.5 µM final concentration),
   1.3. 2 µl nuclease-free water.
2. Heat 70°C for 5 min. Cool on ice for 2 min .

3.a. For SuperScript-III reactions, add:
   3.a.1. 10 µl 2x SuperScript-III buffer (containing 1 mM dNTP),
   3.a.2. 2 µl SuperScript-III/RNAseOUT mixture,
   3.a.3. Incubate 30 min at 50°C,

3.a.4. Inactivate 15 min at 95°C,
3.a.5. Cool reaction to room temperature, store on ice.
3.b. For OmniScript/SensiScript reactions, add:
3.b.1. 4 µl 5x Qiagen OneStep RT-PCR buffer,
3.b.2. 0.8 µl 10 mM dNTP (400 µM final concentration),
3.b.3. 0.8 µl Qiagen OneStep enzyme mix,
3.b.4. 5.4 µl nuclease-free water,
3.b.5. Incubate 30 min at 50°C. Go to section ***10.2.8.4***.
3.c. For ThermoScript reactions, add:
3.c.1. 4 µl 5x ThermoScript buffer,
3.c.2. 2 µl 10 mM dNTP (1 mM final concentration),
3.c.3. 1 µl 0.1 M DTT (5 mM final concentration),
3.c.4. 1 µl 40 u/µl RNAseOut,
3.c.5. 1 µl 15 u/µl ThermoScript,
3.c.6. 4 µl nuclease-free water,
3.c.7. Incubate 30 min at 60°C,
3.c.8. Inactivate 15 min at 95°C,
3.c.9. Cool reaction to room temperature, store on ice.
3.d. For r*Tth* reactions, add:
3.d.1. 2 µl 10x r*Tth* buffer,
3.d.2. 0.4 µl 10 mM dNTP (200 µM final concentration),
3.d.3. 1 µl 10 mM $MnCl_2$ (1 mM final concentration),
3.d.4. 2 µl 2.5 u/µl r*Tth* reverse transcriptase,
3.d.5. 6.6 µl nuclease-free water,
3.d.6. Incubate 30 min at 70°C,
3.d.7. Add 2 µl 10x chelating buffer (to chelate the Mn^{+2}),
3.d.8. Inactivate 15 min at 98°C,
3.d.9. Cool reaction to room temperature, store on ice.

10.2.8.2. Exonuclease-I digestion of tagged primer

Exonuclease-I specifically digests only single-stranded DNA, in a 3'-5' direction, and thus inactivates unincorporated tagged-cDNA primer prior to PCR. This reduces ten-fold the chance of falsely detecting the incorrect strand (Craggs *et al.,* 2001) and is a common step in strand-specific RT-PCR (Purcell *et al.,* 2006; Lin *et al.,* 2009; Plaskon *et al.,* 2009; Runckel *et al.,* 2011).

1. Add to the cDNA reaction:
   10 u Exonuclease-I.
2. Incubate 30 min at 37°C; inactivate 15 min at 70°C.
3. Dilute cDNA reaction ten-fold, to 200 µl.

10.2.8.3. Column purification of cDNA

The cDNA can also be purified to remove unincorporated tagged-cDNA primer, using Qiagen affinity purification columns, and thus significantly reduce the chance of falsely detecting the incorrect strand through participation of residual tagged-cDNA primer in the early PCR reactions. This procedure is a common alternative to Exonuclease-I digestion (Peyrefitte *et al.,* 2003; Carrière *et al.,* 2007) and used in strand-specific detection of several honey bee viruses (Boncristiani *et al.,* 2009; DiPrisco *et al.,* 2011; Peng *et al.,* 2012).

1. Follow Qiagen DNA affinity column purification protocol.
2. Elute the purified cDNA in 100 µl nuclease-free water.

10.2.8.4. OneStep PCR

Yue and Genersch (2005) developed a modified OneStep protocol for strand-specific RT-PCR that does not include specific steps to remove the tagged cDNA primer prior to PCR. Occasionally, weak bands are produced derived from non-strand-specific cDNA priming events (Gisder *et al.,* 2009).

1. For the OmniScript/SensiScript OneStep RT-PCR reactions, add:
   1.1. 0.5 µl 10 µM tag primer (0.25 µM final concentration),
   1.2. 0.5 µl 10 µM virus-specific primer (0.25 µM final concentration).
2. Incubate
   2.1. 15 min at 95°C,
   2.2. 35 cycles of [94°C:30 sec – 54.5°C:60 sec – 72°C:30 sec],
   2.3. 72°C:10 min.

10.2.8.5. Real-time qPCR

The SuperScript-III, Thermoscript and r*Tth* cDNA reactions all enter a separate (TwoStep) PCR protocol, which can be conveniently adapted to real-time qPCR, using a real-time qPCR kit containing SYBR-green:

1. Mix:
   1.1. 3 µl cDNA (column purified, or diluted 1/10),
   1.2. 0.4 µl 10 µM tag primer (0.2 µM final concentration),
   1.3. 0.4 µl 10 µM virus-specific primer (0.2 µM final concentration),
   1.4. 0.4 µl* 10 mM dNTP* (0.2 mM final concentration*),
   1.5. x µl Buffer + SYBR-green (as per manufacturer),
   1.6. y µl nuclease-free water,
   1.7. z µl Taq polymerase (as per manufacturer),
   1.8. 20 µl total volume.

(* dNTPs are often included in the optimized buffer)

2. Incubate in real-time thermocycler:
   2.1. 5 min:95°C,
   2.2. 35 cycles [10 sec:95°C - 30 sec:58°C - read].
3. For Melting Curve analysis of the products, incubate:
   3.1. 1 min:95°C,
   3.2. 1 min:55°C,
   3.3. 5 sec:0.5°C:read from 55°C to 95°C.

10.2.9. Controls

By now it should be evident that strand-specific RT-PCR should include a large number of controls, to account for the many ways by which an incorrect result can be generated. Most of these involve the reverse transcription reaction, since this is where most of the errors

come from. The one essential control that should be run for every individual sample is:

- A primer-free cDNA reaction (proof that self-primed cDNA is not amplified).

Other controls that should be included at least once for the experiment are:

- A template-free cDNA reaction (absence of contamination of reagents/pipettes with target DNA).
- A reverse-transcriptase-free cDNA reaction (absence of reverse-transcriptase activity during PCR).
- An exonuclease-I-free cDNA reaction (disappearance of signal from mis-primed cDNA reactions).

The PCR step for all these controls should also include tagged-cDNA primer, equivalent to the estimated carry-over from the cDNA reaction, in addition to the regular concentrations of tag primer and virus-specific primer necessary for the PCR. Through this, the controls will contain the complete primer composition of the experimental reactions, which (as explained above) is an essential condition for excluding possible false positives.

Whether or not false-positive results during strand-specific RT-PCR presents a major problem also depends on the question to be answered. If the virus replication in a certain host, tissue or cell type is expected, then false-positive results are not a major factor. In contrast, if the absolute presence or absence of replication needs to be proven, then extreme care must be taken when conducting and interpreting the experiments.

10.3. Multiplex Ligation-dependent Probe Amplification (MLPA)

10.3.1. Introduction

Multiplex Ligation-dependent Probe Amplification (MLPA) technology is an amplification technique that allows simultaneous detection of up to 40 targets with the use of a single PCR primer pair. The procedure uses a series of paired oligonucleotides (half-probes), each pair specific for one target. The two half-probes; the Left Probe Oligo (LPO) and the Right Probe Oligo (RPO) lie adjacent to each other on the target genome so that they can be joined together by a ligation reaction, to produce an amplification probe (Fig. 6). In addition to a target-specific sequence, each of the half-probes contains one of two sequences recognized by a universal PCR primer, for probe amplification. Since these PCR primer sequences are common to all half-probe pairs, a single pair of PCR primers can amplify all target probes in a multiplex reaction. The half-probe pairs also contain a 'stuffer' fragment of variable length, allowing each amplified probe to be identified by its size, using (capillary) electrophoresis (Fig. 6). This technique was recently adopted to detect the most common honey bee viruses including CBPV, DWV (KV & VDV-1), ABPV (IAPV & KBV), BQCV, SBPV, SBV (De Smet *et al.,* 2012). Because these are all RNA viruses, the MLPA is preceded by a reverse transcription of RNA into cDNA. Since the probes are strand-specific, this technique is highly suitable for the selective detection of either the positive-strand genomic viral RNA or the negative-strand virus replicative intermediate RNA, which is a marker for virus replication.

Since several targets are amplified at the same time, there will be competition between different targets for the amplification resources (primers, nucleotides, enzyme). This 'competitive' PCR allows for a measure of relative quantification between the targets, in the sense that the relative proportion of the targets after amplification should, if all targets amplify equally efficiently, reflect their initial proportions in the sample. By including one or more internal reference genes or exogenously added absolute quantification standards among the targets, the procedure can be made (semi-) quantitative.

10.3.2. Protocol

The reactions are performed in a thermocycler with heated lid (105°C) in 0.2 ml thin-walled PCR tubes. The specific MLPA reagents can be obtained from MRC-Holland. The various probes and oligonucleotides used in the honey bee virus MLPA are given in Table 6. It is recommended to use the wildtype MuMLV Reverse Transcriptase from Promega (M1701). The right probe oligos (RPO) are phosphorylated and should be synthesized as 'ultramer' grade.

10.3.2.1. Primer and probe mixtures

1. Prepare RT-primer mix:
   1.1. 5 mM each dNTP,
   1.2. 5 µM each RT primer (Table 6).
2. Prepare probe mix:
   1.33 nM of each half-probe (Table 6) in TE(8.0) buffer.

10.3.2.2. Reverse transcription

1. Mix on ice:
   1.1. 10~500 ng RNA,
   1.2. 1 µl SALSA RT buffer,
   1.3. 0.5 µl RT-primer mix,
   1.4. Sterile water to 4.5 µl total volume.
2. Incubate 1 min at 80°C.
3. Incubate 5 min at 45°C.
4. Add 1.5 µl 20 u/µL MuMLV Reverse Transcriptase.

If necessary, dilute in 1:1 water: SALSA enzyme dilution buffer.

5. Mix.
6. Incubate:
   6.1. 15 min at 37°C,
   6.2. 98°C for 2 min (reverse transcriptase inactivation),
   6.3. Cool to 25°C.

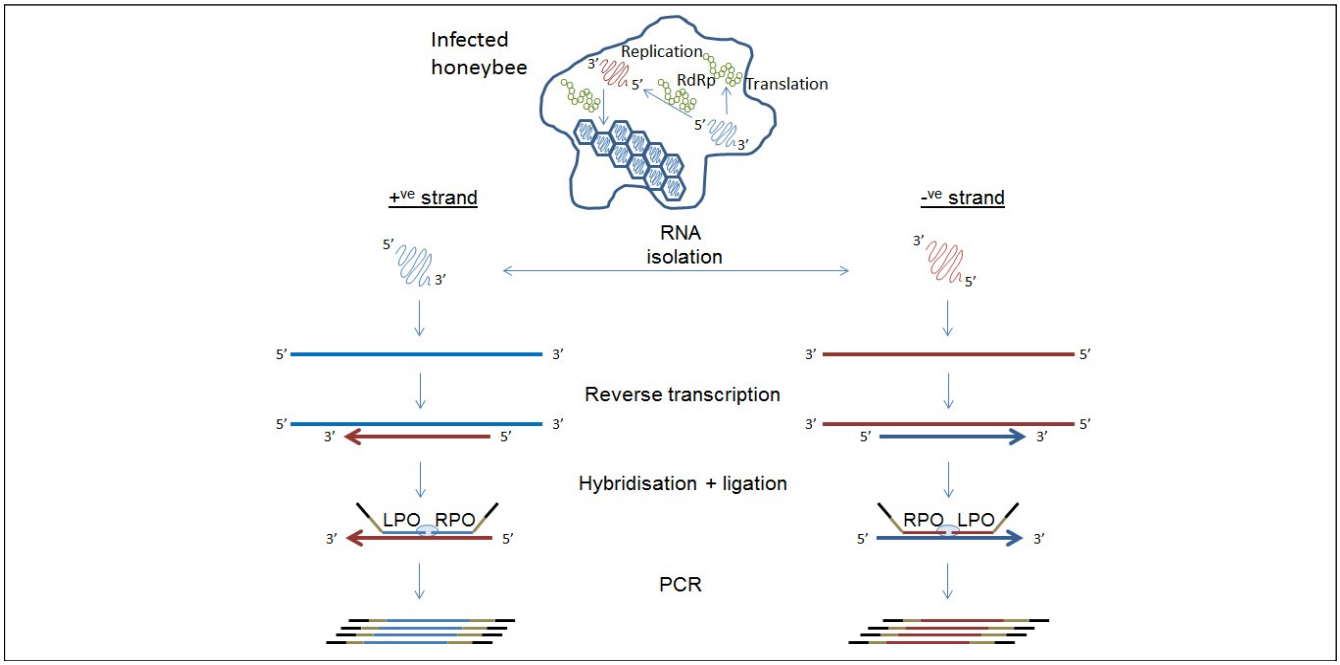

Fig. 6. Outline of the MLPA procedure for amplifying strand-specific ligated probes. LPO and RPO refer to the Left Probe Oligo and Right Probe Oligo respectively. RdRp refers to the viral RNA-dependent RNA polymerase. Image © L De Smet.

Table 6. Primers and probes used for detection of positive and negative strand honey bee virus RNAs by MLPA. Adapted from De Smet *et al.* (2012).

VIRUS	STRAND	PRIMER	FUNCTION	SEQUENCE (5'-3')	SIZE (bp)
CBPV	+ (pos)	LDS22	(-)cDNA	GCCCCGATCATATAAGCAAA	88
		LDS23	(+)MLPA-LPO	gggttccctaagggttggaCCGTAGCTGTTTCTGCTGCGGT	
		LDS24	(+)MLPA-RPO	P-ACTCAGCTCAGCTCGACGCTCAGAtctagattggatcttgctggcac	
	- (neg)	LDS59	(+)cDNA	GAACATCCGGAACAGACGAT	88
		LDS60	(-)MLPA-LPO	gggttccctaagggttggaTCTGAGCGTCGAGCTGAGCTGAGT	
		LDS61	(-)MLPA-RPO	P-ACCGCAGCAGAAACAGCTACGGtctagattggatcttgctggcac	
DWV/KV VDV-1	+ (pos)	LDS8	(-)cDNA	TCACATTGATCCCAATAATCAGA	95
		LDS9	(+)MLPA-LPO	gggttccctaagggttggaTGACCGATTCTTTATGCAGCGAGCTCT	
		LDS10	(+)MLPA-RPO	P-TACGTGCGAGTCGTACTCCTGTGACAtctagattggatcttgctggcac	
	- (neg)	LDS31	(+)cDNA	GTGTGGTGCATCTGGAATTG	95
		LDS32	(-)MLPA-LPO	gggttccctaagggttggaGTTGTCACAGGAGTACGACTCGCA	
		LDS33	(-)MLPA-RPO	P-CGTAAGAGCTCGCTGCATAAAGAATCGGTtctagattggatcttgctggcac	
ABPV KBV IAPV	+ (pos)	LDS1	(-)cDNA (ABPV)	CAATGTGGTCAATGAGTACGG	104
		LDS2	(-)cDNA (KBV&IAPV)	TCAATGTTGTCAATGAGAACGG	
		LDS19	(+)MLPA-LPO	gggttccctaagggttggaCTCACTTCATCGGCTCGGAGCATGGATGAT	
		LDS4	(+)MLPA-RPO	P-ACGCACAGTATTATTCAGTTTTTACAACGCCCtctagattggatcttgctggcac	
	- (neg)	LDS62	(+)cDNA	TGAAACGGAACAAATCACCA	104
		LDS63	(-)MLPA-LPO	gggttccctaagggttggaCGAGCCGATGAAGTGTCTTGAGCCATGG	
		LDS64	(-)MLPA-RPO	P-GGGTATTGATCCTATTTGGAGTTTCCACATCATGtctagattggatcttgctggcac	
BQCV	+ (pos)	LDS16	(-)cDNA	CGGGCCTCGGATAATTAGA	122
		LDS21	(+)MLPA-LPO	gggttccctaagggttggaCTTCATGTTGGAGACCAGGTTTGTTTGCCGACTTACGGAA	
		LDS18	(+)MLPA-RPO	P-TGTCGTTAAACTCTAGGCTTTCCGGATGGCTTCTTCATGGtctagattggatcttgctggcac	
	- (neg)	LDS65	(+)cDNA	TTAAAAGCCCCGTATGCTTG	122
		LDS66	(-)MLPA-LPO	gggttccctaagggttggaTCAGCGCAACAGAAGCCATCCGGAAAGCCTAGAGTTTAACG	
		LDS67	(-)MLPA-RPO	P-ACATTCCGTAAGTCGGCAAACAAACCTGCCTTATCTGGTtctagattggatcttgctggcac	
SBPV	+ (pos)	LDS25	(-)cDNA	CGCAAACACGACGAATTTTA	131
		LDS26	(+)MLPA-LPO	gggttccctaagggttggaCGTTCAATGGTCGAGATAGAAGCCACAGTAGAAGTATTACGCGCT	
		LDS27	(+)MLPA-RPO	P-TCTTGTGTTTTGGCTTATGGGCGTGGGCCTGATCTTCATTCAGCtctagattggatcttgctggcac	
	- (neg)	LDS68	(+)cDNA	GGTGTCATAAACAGAATGACGAG	131
		LDS69	(-)MLPA-LPO	gggttccctaagggttggaTCAGCGCAACACTCAGGCCCACGCCCATAAGCCAAAACACAAGAA	
		LDS70	(-)MLPA-RPO	P-GCGCGTAATACTTCTACTGTGGCTTCTATCTCGCCTTATCTGGTtctagattggatcttgctggcac	
SBV	+ (pos)	LDS28	(-)cDNA	TGGACATTTCGGTGTAGTGG	140
		LDS29	(+)MLPA-LPO	gggttccctaagggttggaCGTTGATCCAATGGTCAGTGGACTCTTATACCGATTTGTTTAATGGTTGG	
		LDS30	(+)MLPA-RPO	P-GTTTCTGGTATGTTTGTTGACAAGAACGTCCACCTTCAGCCATTCAGCtctagattggatcttgctggcac	
	- (neg)	LDS71	(+)cDNA	CCTTACCTCTAGTAAGAAGACATTTGA	140
		LDS72	(-)MLPA-LPO	gggttccctaagggttggaTAAAAAACTACCGTGTAGTGGACGTTCTTGTCAACAAACATACCAGAAA	
		LDS73	(-)MLPA-RPO	P-CCCAACCATTAAACAAATCGGTATAAGAGTCCACTGAAAAGTCGGTGGAtctagattggatcttgctggcac	
β-Actin	+ (pos)	LDS58	(-)cDNA	TTTCATGGTGGATGGTGCTA	182
		LDS56	(+)MLPA-LPO	gggttccctaagggttggaGCAGGAAGTCGTTACCACCTGGCCCAC-GGAGCCAATTTCTCATGCTTGCCAACACTGTCCTTTCTGGAGGT	
		LDS57	(+)MLPA-RPO	P- ACCACCATGTATCCTGGAATCGCGAAAACGTGGTGTACCGGCTGTCTGGTATGTATGAG-TTTGTGGTGAtctagattggatcttgctggcac	
PCR		LDS11	PCR-Forward	gggttccctaagggttgga	n.a.
		LDS12	PCR-Reverse	gtgccagcaagatccaatctaga	

10.3.2.3. Hybridisation of MLPA half-probes

1. Add to the reverse transcription reaction and mix with care:
   1.1. 1.5 µl Probe-mix,
   1.2. 1.5 µl MLPA buffer.
2. Incubate:
   2.1. 1 min at 95°C,
   2.2. 16 h at 60°C in a PCR ThermoCycler.

10.3.2.4. Ligation of MLPA half-probes

1. Reduce the temperature of the thermal cycler to 54°C.
2. While at 54°C, add to each sample:
   2.1. 3 µl Ligase-65 buffer A,
   2.2. 3 µl Ligase-65 buffer B,
   2.3. 25 µl sterile water,
   2.4. 1 µl Ligase-65.
3. Mix well.
4. Incubate:
   4.1. 10-15 min at 54°C,
   4.2. 5 min at 98°C (inactivation of Ligase-65).
5. Cool on ice.

10.3.2.5. PCR amplification of MLPA probes

1. Mix in new tubes:
   1.1. 10 µl MLPA ligation reaction,
   1.2. 4 µl SALSA PCR buffer,
   1.3. 26 µl sterile water.
2. While the tubes are in the thermal cycler at 60°C, add to each tube:
   2.1. 2 µl SALSA PCR primers,
   2.2. 2 µl SALSA enzyme dilution buffer,
   2.3. 5.5 µl sterile water,
   2.4. 0.5 µl SALSA polymerase.
3. Incubate:
   3.1. 35 cycles [30 sec:95°C - 30 sec:60°C - 60 sec:72°C],
   3.2. 20 min:72°C.

10.3.3. Fragment analysis

The MLPA reaction products can be analysed on conventional slab electrophoresis, using a 4% agarose-TBE gel (De Smet *et al.,* 2012; Green and Sambrook, 2012), or using a high-resolution, semi-automatic electrophoresis system such as the BioAnalyzer (Aligent), Experion (Biorad), Qiaxcel (Qiagen) or MultiNA (Shimadzu), which are designed for separating short fragments. In all cases, interpretation of the results is simplified by loading a specific MLPA ladder, generated amplifying each of the MLPA targets individually from cloned controls and pooling these into a single ladder.

Agarose gel electrophoresis:

1. Prepare a 4% high resolution agarose gel in 1x TRIS-Borate-EDTA buffer (Green and Sambrook, 2012).
2. Mix:
   2.1. 10 µl aliquot of the MLPA reaction,
   2.2. 5 µl 4x Sample Buffer.
3. Load gel.
4. Run for 45-60 minutes at 75-90 volts.

Semi-automatic high-resolution gel electrophoresis:

1. Use gel system appropriate for 25-500 bp DNA fragments.
2. Follow manufacturers' instructions for sample preparation, loading, running and data analysis.

10.3.4. Controls

As for strand-specific RT-PCR, MLPA requires a large number of controls to rule out the possibility of artefactual results due to the methods used.

- A nucleic acid-free control (the two half-probes should not be able to ligate without a template).
- A reverse transcriptase-free control (the two half-probes should not be able to ligate on an RNA template).
- A cDNA primer-free control (identifies possible self-priming of RNA for cDNA synthesis).
- A half-probes-free control (only the two half-probes should generate an amplifiable template for PCR).
- A ligase-free control (the two half-probes should not be able to ligate without ligase0.
- A PCR primer-free control (the two half-probes should not be able to function as PCR primers either with each other or with the cDNA primer).

11. Virus variation

11.1. Introduction

Due to the importance of genetic variability to virus virulence and evolution, the detection and quantification of virus genetic variability as a trait in itself has been an interest throughout the history of virus research. Throughout the history of molecular diagnostics, new technologies have been adapted for the detection and quantification of polymorphisms or genetic variation, reviewed by Ahmadian and Lundeberg (2002). Below are a few of the more current methods.

11.2. Protocols

11.2.1. Nuclease protection assays (RPAs and SNPAs)

Nuclease protection assays are an efficient way to analyse the genetic complexities of natural populations of organisms (Kurath *et al.,* 1993; Arens, 1999; Wang and Chao, 2005). A labelled probe is hybridised to the nucleic acid sample of a population of organisms (usually viruses or other pathogens, sometimes related mRNA species) and then digested with RNAse (RNA probe) or S1-nuclease (DNA probe) which will cut the probe wherever there is a mismatch between probe and target. The resulting pattern of digested probe fragments, revealed by gel electrophoresis, is qualitatively and quantitatively indicative of the mismatch polymorphisms present in the nucleic sample. These procedures are called RNAse Protection Assay (RPA) and S1-Nuclease Protection Assay (SNPA).

Pros: Entire populations can be screened for genetic complexity within the target sequence in a single reaction. The polymorphic sites can be mapped on the genome, through the sizes of the fragments produced.

Cons: Assay is limited to about 300 bases, requiring many assays to cover a genome. Protocols are complex and subject to errors. The nature of the polymorphs requires further analysis.

11.2.2. Gel retardation assays (SSCP and DGGE)

Single Strand Conformation Polymorphism (SSCP) and Denaturing Gradient Gel Electrophoresis (DGGE) are two techniques that use electrophoresis to differentiate directly between variants in a population of sequences (Hauser *et al.,* 2000; Stach *et al.,* 2001). In SSCP the nucleic acids are made single-stranded, to fold into a preferred secondary structure. In DGGE, the nucleic acids are separated in an electrophoretic gel containing a salt gradient that will progressively denature the nucleic acids. In both methods, minor nucleotide differences between polymorphs in the population affect the migration of the DNA. Another technique with a similar philosophy is the heteroduplex mobility shift assay, where single nucleotide mismatches between a probe and target affect the mobility of the hybridised complex during electrophoresis, (Arens, 1999).

Pros: Entire populations can be screened for genetic complexity within the target sequence in a single reaction.

Cons: Assay is limited to about 300 bases, requiring many assays to cover a genome. Protocols are complex, sensitive to procedural accuracy and subject to errors. The nature of the polymorphs requires further analysis.

11.2.3. High Resolution Melting (HRM) analysis

Double stranded DNA can disassociate (or melt) into two single strands upon heating, and can re-associate (or hybridize) upon cooling, in a highly predictable fashion. This fundamental property of nucleic acids underpins all nucleic acid technologies. The principal parameters governing disassociation/hybridization are the length and composition of the DNA, the temperature and the salt concentration of the solution. Work in the 1950s demonstrated that the G-C pairing, with three hydrogen bonds, gave higher thermal stability than the A-T pairing, which has only two such bonds (Marmur and Doty, 1959). This made it possible to predict the temperature at which a DNA molecule would melt (T^m) from its length and base pair composition (Marmur and Doty, 1962). The discovery of DNA binding dyes such as SYBR-green, that fluoresce only when intercalated with double stranded but not single stranded molecules, provided a practical method to quantify the melting process based on a reduction in fluorescence during gradual heating, as the two DNA strands separated. This fluorescence-based detection was integrated with real -time PCR thermocyclers that can very precisely control the temperature of a DNA sample and collect fluorescence data between 10 and 200 times per °C, providing high-resolution melting curves that can distinguish single base pair differences between two PCR products (Wittwer *et al.,* 2003). This makes it possible to use High Resolution Melting (HRM) analysis to analyse the composition of mixed samples, *i.e.* samples containing two or more genetic variants of the same region, by comparing the melting curve of the mixed sample with those of the individual variants.

HRM analysis is a versatile method that can be applied to any sample that contains double stranded DNA, including cDNA or PCR products. The flexibility of HRM analysis has led to a diverse array of applications including pre-sequence screening, Single Nucleotide Polymorphism (SNP) typing, methylation analysis, microsatelite or Simple Sequence Repeat (SSR) marker screening (Arthofer *et al.,* 2011) and copy number quantification. Several of these techniques are covered in the *BEEBOOK* paper on molecular techniques (Evans *et al.*, 2013). Such applications of HRM are also relevant to virology, and the first record of a virological use of HRM analysis was to strain type West Nile virus (Papin *et al.,* 2004). Recently, HRM analysis has been used to monitor the relationship between varroa infestation and virus diversity (Martin *et al.,* 2012).

Many standard real-time PCR machines can be used for HRM analyses. Often an upgrade of the software package and the running of a calibration plate is all that is required to enable a real-time PCR machine to run HRM analyses. Since HRM is a highly technical and sensitive procedure that integrates reaction biochemistry with machinery and analysis software, the best advice is to follow the protocol, reagents and incubation profile recommended by manufacturer. The basic procedure is as follows:

1. Amplification: Amplify your chosen fragment from your experimental samples and cloned controls, using specific HRM reagents containing a saturating DNA DNA intercalating dye and the recommended incubation profile.

2. Replicates: Use a minimum of three technical replicates for each sample. The replicate melting profiles will be averaged and used to assess whether the sample is distinct from other samples/controls.
3. HRM: Immediately after amplification the PCR products are subjected to a high-resolution melting step, within the same tube, during which the decrease in fluorescence due to the transition of the DNA from double- to single-stranded shape is monitored.
4. Analysis: The melting curve of the experimental sample, containing a mixture of different variants, is compared to the melting curves of pure, cloned versions of each of the individual variants.

Pros: Simple; fast; flexible; cheap; sensitive; specific; low contamination risk.
Cons: Requires individual melting curves of (cloned) variants. Cannot identify nature of novel variants. Limited quantification of variants. Limited capacity to resolve complex mixture. Limited to very short genome fragments.

11.2.4. Sequencing

The most powerful means for detecting variation is sequencing, since every possible variant is identified and precisely mapped on the genome. There are several approaches that can be used. The purest and most expensive approach is to clone PCR products of the target(s) and sequence batches of individual clones. This also allows the relative frequencies of individual variants to be determined, even those variants occurring at very low frequencies. A second and cheaper approach is to sequence the PCR products directly and identify the polymorphisms at sites of ambiguity in the sequence (Forsgren *et al.,* 2009; Fig. 7). Since such double peaks can also be the result of sequencing artefacts, each polymorphic site has to be confirmed by a matching pattern when sequencing the complementary strand. Only major polymorphisms can be identified and quantitation is moderate, similar to HRM. The new, high volume automated sequencing methods (Next Generation Sequencing, or NGS) have the capacity to directly analyse complex DNA and RNA mixtures through sequencing followed by automated similarity searches. These methods are rapidly becoming cheaper and more accurate, mostly through massive multiplexing of reactions and samples. They are increasingly being used as a one-step diagnostic method capturing millions of different targets, thus benefiting also from economy of scale in the data generated. They have recently also been used in honey bee pathology studies (Cox-Foster *et al.,* 2007; Runckel *et al.,* 2011) and are covered in detail in the *BEEBOOK* paper on molecular techniques (Evans *et al.*, 2013).

Pros: Comprehensive; fast; flexible; accurate; sensitive; specific; low contamination risk; approximate quantification (NSG).
Cons: Expensive - precise - limited quantification (Sanger sequencing); Very expensive - approximate quantification (NGS).

12. Quality control

12.1 Introduction

Standardization of the diagnostic methods for detecting and quantifying bee viruses and of the interpretation of the results is the first requirement for improved harmonization of the data collected by different laboratories. The protocols and assays for different animal diseases registered with the World Organization for Animal Health (OIE; previously the Office International des Epizooties) ensure the global harmonization and standardization of detection methodologies. Moreover, the OIE provides criteria for the technical requirements and quality management in veterinary testing laboratories, in the form of a series of standards and recommendations that each laboratory should address in the design and maintenance of its quality management program. Valid laboratory results are essential for diagnosis, obtained by the use of good management practices, validated protocols and calibration methods as described in the ISO/IEC 17025 International Standard. By following these standards, a laboratory is able to obtain accreditation, linked to the international certification standard ISO 9001. OIE guidelines provide an interpretation of the ISO/IEC 17025 guidelines in the context of veterinary laboratories working with infectious diseases, including the validation of diagnostic assays, the production of international reference standards and laboratory competence testing. The European reference laboratory for honey bee diseases at ANSES in France is developing a set of standard diagnostic procedures for honey bee viruses, following these procedures and criteria.

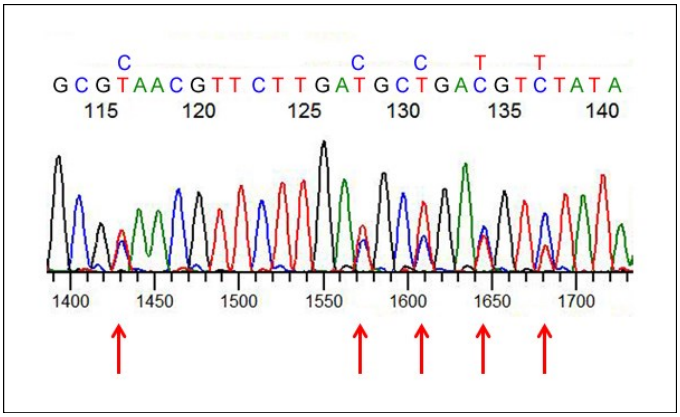

Fig. 7. Mixed virus sequences as revealed in sequence electropherogram. The mixed sequence can be resolved into component sequences using specifically designed software. Adapted from Forsgren *et al.,* 2009.

12.2. Assay selection and validation

Full validation of molecular techniques, as per guidelines issued by the World Organization for Animal Health (OIE) is a relatively new concept in the field of honey bee virus diagnosis. In general terms, a diagnostic protocol is designed in response to a particular diagnostic need and this is then developed into an optimized, documented and fixed procedure, using a series of intra-laboratory validation steps that demonstrate the reliability of results and the performance of the method. Recently, a new standard (XP U47-600) was developed by the French Standards Institute (AFNOR) concerning the minimum requirements for the development, validation and implementation of veterinary PCR-based diagnostic methods in animal health, based on the recommendations by the OIE and following the ISO/IEC 17025 criteria (NF, 2005; OIE, 2010). The validation procedure establishes the performance characteristics for each test method, such as sensitivity, specificity, detection and/or quantification limits.

The initial validation of a RT-qPCR assay involves two steps. The first concerns the validation of the qPCR assay itself, in terms of:

1. Analytical specificity.
2. The PCR detection limit (DL_{PCR}).
3. The PCR quantification limit (QL_{PCR}).
4. The linearity and efficiency of the qPCR assay.

The second step concerns the evaluation of the entire diagnostic protocol in terms of:

1. The method's detection limit (DL_{method}).
2. The diagnostic specificity and sensitivity on samples of known status.
3. The method's quantitation limit (QL_{method}) based on a validation range and accuracy profile.

In each of the two steps, various performance parameters are calculated, including measurement uncertainty (MU), deviations of repeatability and intermediate reliability.

The following is a step-by-step outline of how to develop an accredited RT-qPCR assay for the detection and quantitation of honey bee viruses, based on the successful development of such an assay for CBPV (Blanchard *et al.,* 2012).

12.2.1. Analytical specificity

12.2.1.1. Analysis in silico

Multiple nucleic acid sequences of the virus, obtained from public sequence databases and from a diverse range of biological and geographic sources, are compared *in silico* with each other and unrelated viruses using BLAST (Basic Local Alignment Search Tool; Altschul *et al.,* 1990), to identify regions of variability and conservation. See also the *BEEBOOK* paper on molecular techniques (Evans *et al.,* 2013).

A series of possible diagnostic assays are designed using bioinformatics tools, based on the particular diagnostic requirements for the method.

12.2.1.2. Experimental specificity

The specificity of the PCR assay is then tested experimentally, using **inclusivity** and **exclusivity** tests.

- **Inclusivity tests**
  Inclusivity tests assess the robustness of an assay, *i.e.* its ability to detect genetically diverse isolates. The PCR assay is evaluated against a panel of samples representing the full range of genetic diversity of the virus in question. This diversity is determined beforehand through phylogenetic analysis of bio-geographic isolates (see section 12.2.1.1.).
- **Exclusivity tests**
  Exclusivity tests assess the specificity of an assay, *i.e.* its ability to detect only the virus in question, and not any other viruses. The PCR assay is evaluated against a panel of viruses unrelated to the virus being tested, but which are found in the same environment or ecological area as the virus being tested.

12.2.2. PCR detection limit

The detection limit of a qPCR assay is the lowest number of nucleic acid targets in a given template volume that can be detected in at least 95% of replicate assays. The detection limit is established by performing at least three independent trials, each with trial consisting of three independent two-fold serial dilutions of a template of known concentration. At each dilution level in each serial dilution series, eight replicate qPCR assays are run, *i.e.* a total of 24 replicate assays at each dilution level. The detection limit is the highest dilution level (*i.e.* lowest amount of target nucleic acid template) giving at least 23 positive results from the 24 assays (95% of the replicates).

12.2.3. qPCR dynamic range and quantitation limit

PCR is an exponential (*i.e.* logarithmic) amplification process that is extremely consistent (*i.e.* predictable) over the entire reaction (35~40 cycles) and over a large range of initial target concentrations (at least 10^6-fold). This dynamic range and the quantitation limits of qPCR are determined using a 10-fold serial dilution series of known concentrations of (cloned) target DNA. A standard calibration curve is generated by linear regression of the quantification cycle (C_q) at which the PCR product is detected *vs.* the $\log_{10}$[target copy number]. The resulting algebraic equation:

$$C_q = a * \log_{10}[\text{target}] + b$$

(where '*a*' is the slope and '*b*' the intercept)

is then used to estimate the amount of target in a sample, given the C_q value (Bustin *et al.,* 2009). For accurate calibration of the curve and determining the error associated with data conversion, at least three independent trials of three independent 10-fold serial dilutions

should be run. For each series and trial, the known amounts of target in each dilution are compared to the theoretical amounts estimated from the calibration curve, to obtain the individual bias, which is the averaged for all series and trials to obtain the mean bias (*mb*) at each dilution (an example is shown in Table 7). These values are then used to calculate the standard deviation of the obtained values (*SD*), and the uncertainty of the linearity is obtained by the formula $U_{LINi} = 2[\sqrt{SD^2 + mb^2}]$. The combined linearity uncertainly is defined for the entire calibration range by the formula $U_{LIN} = |\sqrt{\Sigma U_{LINi}^2 / k}|$ where k is the number of dilution levels. The quantitation limit of the assay is then set at the target concentration of the calibration range.

12.3. Method validation

12.3.1. Method detection limit

The method detection limit (DL_{method}) is the lowest amount of biological target in a sample that can be detected by the entire method (from processing through RT-qPCR). The DL_{method} is evaluated with biological reference samples obtained by spiking virus-free bee homogenates with known amounts of purified virus. At least two independent trials must be performed on two independent two-fold serial dilutions, with four replicate RNA extractions at each dilution level. The DL_{method} is the last dilution at which viral RNA can be detected in all replicates (100% frequency).

12.3.2. Method diagnostic sensitivity and diagnostic specificity

The diagnostic specificity and sensitivity is assessed on complete method analysis (processing through assay) of biological samples of known virus status (positive or negative). Diagnostic sensitivity is determined by the percentage of positive results among the known positive samples. Diagnostic specificity is determined by the percentage of negative results among the known negative samples.

12.3.3. Method quantitation limit and accuracy profile

The assessment of a method's quantitation limit is based on the construction and interpretation of an accuracy profile to estimate the precision and reliability of the values. Three independent trials must be performed on three independent 10-fold serial dilutions, including two replicate RNA extractions for each level of dilution. For each dilution series and each target amount, various parameters are determined from estimated target amounts, such as the inter-series variance and the repeatability variance, the sum of both giving the reliability variance. The standard deviation of the reliability (SDrl) is then obtained by the square root of the reliability variance. The mean bias is determined (difference between the theoretical value and the mean of the observed values). To construct the accuracy profile, the lower and upper tolerance interval limits of the quantitation method are determined using the following formula:

mean bias +/- 2 × SDrl

and compared to the acceptability limits defined by the laboratory, *e.g.* +/- 0.5 $\log_{10}$ (Blanchard *et al.,* 2012). The tolerance interval limits of the accuracy profile have to be within the acceptability limits, validating the method for the thus defined calibration range. The quantitation limit of the method is then determined by the first level load of the validated calibration range. An example of the confidence and acceptability limits of an RT-qPCR calibration curve is given Fig. 8, where the evaluated method is validated for a calibration range between 10^3 and 10^6 copies, with a quantitation limit of 10^3 copies.

12.4. Laboratory Validation

The final validation of a diagnostic method is through inter-laboratory proficiency tests, to evaluate the reproducibility and the overall uncertainty of the method, and to assess performance of other laboratories to conduct specifically this method (Birch *et al.,* 2004; Valentine-Thon *et al.,* 2001; Verkooyen *et al.,* 2003). To achieve this, candidate laboratories must submit to a training and accreditation programme.

12.4.1. Training and accreditation

The International Laboratory Accreditation Cooperation (ILAC) is an international cooperation of laboratory and inspection accreditation bodies created more than 30 years ago. It has published specific requirements and guides for laboratories and accreditation bodies. Under the ILAC system, ISO/IEC 17025 is to be used for accreditation. This procedure attests for the laboratory's technical competence and the reliability of its results. In each country, a sole national accreditation body is designated, as the French Accreditation Committee (COFRAC) in France, the Deutsche Akkreditierungsstelle GmbH (DAssK) in Germany or the Swedish Board for Accreditation and Conformity Assessment (SWEDAC) in Sweden. Performance of the method must thus be validated according to the OIE or AFNOR standards and approved by the national accreditation committee of each country. Furthermore, inter-laboratory proficiency tests should be carried out to evaluate the reproducibility of the method.

12.4.2. Inter-laboratory proficiency testing

The basic purpose of proficiency testing is to assess performance of laboratories in conducting specific method. Proficiency testing provides an opportunity to have an independent assessment of each laboratory's data compared to reference values or to the performance of other laboratories (*e.g.* Apfalter *et al.,* 2002). The participation of the laboratory to proficiency testing programs assesses if the laboratory's performances is satisfactory. In case of any potential problems within the laboratory, investigations to detect the difficulties are required. In order to successfully run proficiency test programs, the production and the distribution of reference materials (positive control, extraction control) are key points, as well as technical trainings of laboratories if necessary. In this framework, data harmonization could contribute to a better understanding of honey bee diseases and to a better diagnosis of pathological issues.

Table 7. Worked example of the estimation of primary and secondary statistics relating to the accuracy and confidence limits of a qPCR calibration curve. After Blanchard *et al.,* (2012).

Target amount (copies/reaction)	**30**	**300**	**3000**	**30000**
Theoretical value Log_{10} (Tv)	1.477	2.477	3.477	4.477
Measured value Log_{10} (Mv)	1.426	2.524	3.539	4.420
	1.490	2.443	3.507	4.469
	1.462	2.475	3.528	4.444
	1.492	2.439	3.509	4.468
	1.494	2.435	3.511	4.468
Bias (Mv - Tv)	-0.052	0.046	0.062	-0.057
	0.013	-0.034	0.030	-0.009
	-0.016	-0.002	0.051	-0.033
	0.014	-0.038	0.032	-0.009
	0.017	-0.043	0.034	-0.009
Sum of Mv	7.363	12.316	17.594	22.270
Mean Mv	1.473	2.463	3.519	4.454
Mean bias	-0.005	-0.014	0.042	-0.023
Standard deviation of Mv	0.029	0.037	0.014	0.022
U_{LINi}	0.060	0.080	0.088	0.063
U_{LINi^2}	0.004	0.006	0.008	0.004
U_{LIN}	0.074			

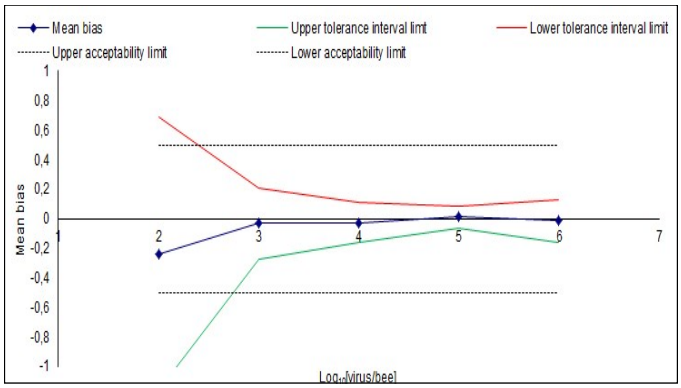

Fig. 8. Example of the mean bias, confidence interval, acceptability limits and quantitation limit for a RT-qPCR calibration curve. After Blanchard *et al.,* 2012.

13. Future perspectives

The future is bright for disease diagnosis and pathogen detection. The molecular biology revolution of the past quarter century has matured through the experimental, labour driven phase to high volume automated systems delivering reliable, high quality information. The revolution is likely to continue, with new methods being developed annually, increasing the options available to the diagnostic virologist. In the 1960's, 70's and 80's the development of semi-automated, sensitive serological assays precipitated a similar revolution in pathogen detection that made more insightful research into disease and epidemiology possible. The most pioneering honey bee virology revelations were made during this time, in particular the discovery and serological characterisation of most of the honey bee viruses that we know today. Several of these remain to be characterised at the nucleic acid level. The development of cheap, high throughput mass sequencing of genomes and transcriptomes has overtaken these efforts somewhat, leading to the identification of novel viral nucleic acid sequences in bee and mite samples that may very well represent the genomes of viruses that had already been discovered previously. Matching these historical virus discoveries and their serological data to these nucleic acid genomes is therefore an important and urgent task, to avoid confusion in bee virus classification and to make sure that the historical literature on these viruses remains relevant in the current molecular age.

The principal criteria for an ideal diagnostic system are sensitivity, accuracy, reliability, universality, simplicity, speed and cost. Most modern detection technologies are now sensitive enough to detect down to a single target molecule. This means that any future development will increasingly focus on quantitative detection (depending on the diagnostic requirements), with a concomitant change to a more integrated, quantitative disease management style. Accuracy of detection at the molecular level (and virus detection is largely molecular) depends essentially on the nature of the primary molecular recognition event, *i.e.* the interaction between target and probe. In this regard, nucleic acid-based detection has a considerable advantage over serological detection, since the kinetics of nucleic acid hybridisation is much more predictable and reliable than that of protein interactions. This also makes nucleic acid-based detection much more adaptable to changing requirements due to the discovery or emergence of new virus variants. The principal area of concern for molecular virus detection is reliability, *i.e.* avoiding misdiagnosis due to false-positive or false-negative results. The nucleic acid genomes of viruses are naturally highly variable and can evolve very quickly, while current molecular diagnostic methods are highly sensitive to minor variations in the nucleic acid target, making it prone to possible false-negative errors. This sensitivity is largely linked to the enzymes used for molecular detection and future developments in molecular virus diagnostics may therefore increasingly feature enzyme-free technologies (Liepold *et al.,* 2005).

The variability of virus genomes is an important component of a virus' adaptive response. It is in many ways a defining and unique characteristic for individual viruses. Other areas of virology now distinguish which viral forms offer increased pathogenicity, or which spread more easily. New methods that can directly describe and quantify this variability, such as HRMC, may become increasingly important in honey bee virology to clarify how the interactions between host factors, individual variants, combinations of variants or the variability as a whole, can induce a diseased state.

Disease is the result of a breakdown in a host's normal physiological state due to the presence or proliferation of a pathogenic agent. The simpler component of this interaction is the pathogen, and its detection. Future developments however, will increasingly focus on the host component of disease and the interplay between pathogen and host. This means that future technological direction in disease diagnosis will emphasise multiplexing, miniaturisation (Fiorini and Chui, 2005) and automation (Service, 2006; Belák *et al.,* 2009), to provide epigenetic data to better understand how the breakdown in the homeostasis between host and pathogen results in disease. Such information is important, since it can inform disease prevention, treatment and potential cures.

Finally, automation and increased demand for simpler, faster and cheaper technologies for routine diagnosis with wide applicability in low-tech settings (Higgins *et al.,* 2003; Schaad *et al.,* 2003) will ultimately drive the costs down to where disease surveillance and routine monitoring becomes cost-effective (Service, 2006), even in low priority areas like honey bee pathology.

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