



Viruses that affect Argentinian honey bees (*Apis mellifera*)

Marcos D. Salina^{1,2} · María L. Genchi Garcia^{1,4} · Bárbara Bais^{1,4} · Maria E. Bravi^{1,3} · Constanza Brasesco^{3,5} · Matías Maggi^{3,5} · Marcelo Pecoraro¹ · Alejandra Larsen⁶ · Hernan G. Sguazza¹ · Francisco J. Reynaldi^{1,3}

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Abstract

Beekeeping is a widespread activity in Argentina, mainly producing honey that has gained both national and international recognition. There are more than 3,000,000 hives in the country, mainly concentrated in Buenos Aires Province (approximately 1,000,000 hives). In recent decades, worrying rates of hive loss have been observed in many countries around the world. In Latin America, the estimated loss of hives is between 13% (Peru and Ecuador) and 53% (Chile). Argentina had annual losses of 34% for the period of October 1, 2016 to October 1, 2017. The causes of these losses are not clear but probably involve multiple stressors that can act simultaneously. One of the main causes of loss of bee colonies worldwide is infestation by the ectoparasitic mite *Varroa destructor* in combination with viral infections. To date, 10 viruses have been detected that affect honey bees (*Apis mellifera*) in Argentina. Of these, deformed wing virus, sacbrood virus, acute bee paralysis virus, chronic bee paralysis virus, and Israeli acute bee paralysis can be transmitted by mites. Deformed wing virus and the AIK complex are the viruses most often associated with loss of hives worldwide. Considering that bee viruses have been detected in Argentina in several hymenopteran and non-hymenopteran insects, these hosts could act as important natural reservoirs for viruses and play an important role in their dispersal in the environment. Further studies to investigate the different mechanisms by which viruses spread in the environment will enable us to develop various strategies for the control of infected colonies and the spread of viruses in the habitat where they are found.

Introduction

Beekeeping is a widespread activity in Argentina, mainly producing honey that has gained both national and international recognition [90]. In recent years, the average annual honey production has been 76,000 tons [59], of which 92% was exported in bulk with an approximate value of 182 million dollars [6]. Argentina has a wide range of conditions for honey production, so beekeeping can be divided into four main production zones (Buenos Aires zone, Central zone, Littoral zone and Patagonian zone). There are more than 3,000,000 hives in the country, mainly concentrated in Buenos Aires Province (approximately 1,000,000 hives), followed by Entre Ríos (~ 680,000 hives) and Santa Fe (~ 312,000 hives). These provinces account for 68% of the total inventory and have the largest numbers of extraction rooms and processing plants [108].

Native to Africa, Europe, and East Asia, *Apis mellifera* is currently the species of honey bee that is most widely distributed throughout the world by human action and was first introduced into Latin America during the seventeenth century [109]. In addition to producing honey, *A. mellifera* is

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✉ Marcos D. Salina
marcosdsalina@gmail.com

¹ Laboratorio de Virología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (UNLP), La Plata, Argentina

² Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Buenos Aires, Argentina

³ Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

⁴ Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA), Buenos Aires, Argentina

⁵ Centro de Investigación en Abejas Sociales (CIAS) UNMdP, Mar del Plata, Argentina

⁶ Cátedra de Inmunología Veterinaria Aplicada, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (UNLP), La Plata, Argentina

also considered to be the most important pollinator because of the large number of individuals per hive [64]. According to Buchmann and Nabhan [32], 77% of the plants that produce food for the world's population are pollinated by *A. mellifera*. Moreover, some fruit, seed, and nut crop yields decrease by more than 90% without honey bee pollination [131]. Hünicken et al. [76] showed that in Patagonia, Argentina, the quality and quantity of pear and apple production is significantly higher with *A. mellifera* pollination service than without.

In recent decades, worrying rates of hive loss have been observed in many countries around the world, leading to the creation of associations such as the Prevention of Honey Bee Colony LOSSes (COLOSS), an international association made up of researchers, veterinarians, agricultural specialists, and students, with the aim of improving the welfare of bees worldwide [42]. According to data obtained from the colony loss monitoring group, after the winter of 2017-2018, the rate of loss was 16.4%, based on data collected from 25,363 beekeepers from 33 European countries plus Algeria, Israel, and Mexico [71, 138]. Moreover, the Bee Informed Partnership (beeinformed.org) has conducted voluntary surveys of US beekeepers since the 2006-2007 season. In the last season (April 1, 2018-April 1, 2019), 4,696 beekeepers who handle 11.9% of the total hive stocks in the USA were surveyed, and they reported a 40.7% loss of colonies [31]. In Latin America, the Beehive Loss Monitoring Group of the Latin American Society for Bee Research (SOLATINA) estimates losses between 13% (Peru and Ecuador) and 53% (Chile). Argentina had annual losses of 34% for the period of October 1, 2016 to October 1, 2017 [110].

The causes of these losses are the object of study by numerous researchers around the world. The most likely causes that have been postulated are pests and diseases, pesticides, nutrition, and beekeeping practices [84, 98, 132]. Adjlane and Haddad [2] have suggested that the main causes of mortality are diseases such as varroosis, acariasis, nosemosis, American foulbrood, European foulbrood, and a wide range of viruses [67, 83]. Although the ectoparasitic mite *Varroa* sp. has been detected throughout almost the entire world, with Australia being an exception [118], and is considered the main pest of *A. mellifera* [88, 119, 132], it is not the only cause of beehive depopulation, which in fact is caused by the synergistic effect of several factors [55, 79, 83, 98, 112]. *Varroa destructor* causes direct damage due to parasitism [3, 106, 119] and indirect damage by acting as a vector for several bee viruses [48, 56, 67, 94, 142]. Martin et al. [89] have suggested the transmission of certain viruses by the mite to be the main threat to bee health. For example, the association between *V. destructor* and the iflavirus deformed wing virus (DWV) reduces the lifespan of bees and is considered to be one of the main causes of colony loss [54, 97, 144]. Additionally, other viruses, such

as Israeli acute paralysis virus (IAPV) and Kashmir virus (KBV), alone or in combination, can also cause significant colony losses together with *V. destructor* mites [67, 142].

The aim of this review is to describe the characteristics of honeybee viruses detected in Argentina. In addition, we briefly discuss the relationship between honeybee viruses and *Varroa destructor*. Finally, we review the most common techniques that are available for the detection of honeybee viruses.

Overview of bee viruses

Viruses are obligate intracellular parasites and need cellular machinery to replicate. They consist of a single nucleic acid surrounded by a protein capsid [2]. To date, 24 different viruses that affect bees have been detected [142]. With the exception of *Apis mellifera* filamentous virus (AmFV) and *Apis iridescent virus* (AIV), which are DNA viruses, the vast majority of bee viruses have an RNA genome, surrounded by an icosahedral capsid 20-30 nm in size. Taxonomically, almost all of them are members of the order *Picornavirales* [37]. Among the viruses present in Argentina, DWV and sacbrood bee virus (SBV) belong to the family *Iflaviridae*, and their virions contain a linear, positive-sense, single-stranded RNA molecule with a single large open reading frame (ORF) [77]. Black queen cell virus (BQCV), acute bee paralysis virus (ABPV), and IAPV belong to the family *Dicistroviridae*. KBV also belongs to this family, but up to now, it has not been detected in Argentina. These viruses also have a linear, positive-sense RNA genome, but they have two ORFs that are non-overlapping, separated by an untranslated region. Chronic bee paralysis virus (CBPV) is heterogeneous in size, with a diameter between 20 and 30 nm, a length of 60 nm, and an unusual anisometric shape. It has not yet been classified taxonomically [77].

Viruses reported in Argentina

To date, 10 viruses that infect *Apis mellifera* have been detected in Argentina (Table 1). Cloudy wing virus (CWV), SBV, and filamentous bee virus X (BVX) were the first to be reported in the country [7]. Fourteen years later, Reynaldi et al. [112], using molecular RT-PCR techniques, detected the presence of SBV, CBPV, and ABPV in bee samples from Buenos Aires Province. In 2011, the same group reported the presence of IAPV [113]. Two years later, using real-time PCR, Brasesco et al. [27] detected the presence of SBV in bee larvae as well as IAPV and DWV in adult bees and *V. destructor* mites from apiaries in the southeast of Buenos Aires. Others reports, such as one by Ding et al. [55], who observed the presence of BQCV, ABPV, CBPV and DWV,

Table 1 Viruses reported in Argentina according to region and diagnostic methodology used

Virus	Authors	Ref.	Region	Prevalence (number of hives sampled)	Diagnostic methodology
CWV	Allen & Ball 1996	[7]	-	-	Only overt viral infections in hives
BVX	Allen & Ball 1996	[7]	-	-	-
SBV	Allen & Ball 1996	[7]	-	-	Only overt viral infections in hives
	Reynaldi et al., 2010	[112]	Buenos Aires	9% (61)	RT-PCR
	Brascesco et al., 2013	[27]	Buenos Aires	9.5% (21)	RT-PCR
	Sguazza et al., 2013	[126]	Buenos Aires, Córdoba, Santa Fe, Entre Ríos,	13.6% (170)	RT-PCR
ABPV	Reynaldi et al., 2010	[112]	Buenos Aires	10% (61)	RT-PCR
	Sguazza et al., 2013	[126]	Buenos Aires, Córdoba, Santa Fe, Entre Ríos,	7.7% (170)	RT-PCR
	Ding et al., 2016		Balcarce, province of Buenos Aires 2011	44%	RT-PCR
		[55]	Balcarce, province of Buenos Aires 2012	4%-10%-6%*	RT-PCR
			Rafaela, province of Santa Fe	9%	RT-PCR
CVPV	Reynaldi et al., 2010	[112]	Buenos Aires	26.2% (61)	RT-PCR
	Sguazza et al., 2013	[126]	Buenos Aires, Córdoba, Santa Fe, Entre Ríos,	12.3% (170)	RT-PCR
	Ding et al, 2016	[55]	Balcarce, province of Buenos Aires	15%-30%-6% *	RT-PCR
IAPV	Reynaldi et al., 2011	[113]	Buenos Aires, Córdoba, Santa Fe, Entre Ríos, San Luis, Río Negro	41% (170)	RT-PCR
	Brascesco et al., 2013	[27]	Buenos Aires	4.8% (21)	RT-PCR
	Sguazza et al., 2013	[126]	Buenos Aires, Córdoba, Santa Fe, Entre Ríos,	45.9% (170)	RT-PCR
DWV	Brascesco et al., 2013	[27]	Buenos Aires	19% (21)	RT-PCR
	Sguazza et al., 2013	[126]	Buenos Aires, Córdoba, Santa Fe, Entre Ríos,	37.1% (170)	RT-PCR
	Ding et al., 2016		Balcarce, province of Buenos Aires 2011	83%	RT-PCR
		[55]	Balcarce, province of Buenos Aires 2012	12%-60%-56%*	RT-PCR
			Rafaela, province of Santa Fe 2012	9%	RT-PCR
			Formosa 2012	90%	RT-PCR
BQCV	Sguazza et al., 2013	[126]	Buenos Aires, Córdoba, Santa Fe, Entre Ríos,	3.5% (170)	RT-PCR
	Ding et al., 2016	[55]	Balcarce, province of Buenos Aires 2012	58%-25%-13%*	RT-PCR
AmFV	Quintana et al., 2019	[105]	Buenos Aires, Córdoba, Entre Ríos, Santa Fe, La Pampa, Mendoza, Chaco, Santiago del Estero	64.7% (34)	RT-PCR
LSV	Fernández de Landa et al., 2020	[61]	Buenos Aires, Córdoba, Santa Fe Entre Ríos, Mendoza, Río Negro	38.5% (26)	RT-PCR

CWV, cloudy wing virus; BVX, bee virus X; SBV, sacbrood virus; ABPV, acute bee paralysis virus; CBPV, chronic bee paralysis virus; IAPV, Israeli acute bee paralysis virus; DWV, deformed wing virus; BQCV, black queen cell virus; AmFV, *Apis mellifera* filamentous virus; LSK, Lake Sinai virus.

*Corresponding to the months of March, April, and May, respectively

and one by Molinari et al. [93] showed that viral infections were more frequent in temperate and subtropical zones. In 2019, Quintana et al. [105] reported the first molecular detection of AmFV in South America in samples from eight

Argentine provinces, and recently, Fernández de Landa et al. [61] reported the presence of Lake Sinai virus (LSV) in bee-hives of the main honey-producing provinces of Argentina.

Briefly, the honey bee viruses detected in Argentina are characterized as follows:

Deformed wing virus (DWV)

In 1983, deformed wing virus was first isolated from apiaries in Japan from adult worker bees [23]. Subsequently, it was found in bees of all castes and stages of development [7, 135, 143]. This virus can sometimes be present without causing symptoms (covert infections) [39, 67, 125], or it can cause symptomatic infections (overt infections) with symptoms such as wing deformities, wrinkled appearance, swollen abdomen, reduced life span, and sometimes death [30, 135]. Some authors consider DWV to be the most harmful virus for bees [1, 92]. Perhaps this particularity and the fact that it is the most prevalent virus in the country [28, 55] have made DWV the most extensively studied virus worldwide [26]. There are three major variants of DWA (DWV-A, B, and C) [46], two of which, DWV-A and DWV-B, have been described in Argentina [28].

Sacbrood bee virus (SBV)

Sacbrood disease was first described in diseased larvae in the USA in 1913 [140]. Later, White [141] replicated the disease by inoculating larvae with extracts obtained from diseased larvae and suggested the viral nature of the disease. Using electron microscopy, Bailey et al. [18] observed isometric particles in reparations of diseased larvae, establishing the etiology of the disease. It is currently one of the most widely distributed viruses in the world [26, 81]. It is present in adult worker bees without causing any apparent signs, although it can significantly decrease their survival [38]. This virus accumulates in the head, mainly in the hypopharyngeal glands [95], which contributes to its spread when worker bees feed the young larvae [127]. Four days after the cells are capped, the infected larvae are unable to pupate, and consequently, liquid accumulates between the larva's body and the skin, forming a kind of characteristic sac, which gives it the name "sac brood". The color of the sac then begins to change from pearly white to pale yellow, finally turning dark brown [18]. In Argentina, SBV was first reported in 1996 [7], but clinical signs were seen 10 years earlier in Buenos Aires province (Raul Pérez, personal communication). The first molecular detection of SBV was done in 2010 with samples from Buenos Aires province by Reynaldi et al. [112].

Black queen cell virus (BQCV)

Black queen cell virus was first isolated from dead, pale yellow, hard-looking royal pre-pupae found within a cell with blackened walls, hence its name [20, 80]. This virus rarely

affects the larvae of worker bees, which could be explained by their lower ingestion of viral particles because workers receive less food and attention than royal larvae [16]. According to Tentcheva et al. [135], BQCV does not cause visible symptoms of infection in adult worker bees, but it does persist chronically through horizontal transmission between workers and from workers to larvae when they are fed [39]. Although this virus mainly affects queen breeders [49], recent studies have suggested that high viral loads could compromise the orientation ability of honey bees, increasing drift [111]. This virus was reported in Argentina by Sguazza et al. [126], who analyzed samples from all over the country. The results showed a low prevalence (3.5%) in comparison with the 91% reported in Uruguay by Antúnez et al. [14]. A recent study [55] showed 8% prevalence, similar to that reported by Sguazza et al. [126].

Cloudy wing virus (CWV)

Bailey et al. [21], while conducting laboratory tests for other viruses, observed the premature death of bees and discovered viral particles that were called "cloudy wing virus" because of their characteristic signs, including wing opacity. Years later, Carreck et al. [36] determined that only severely infected bees showed these characteristic signs, while those with low titers were asymptomatic, though they sometimes had a reduced lifespan. Cloudy wing virus is widely distributed over all continents [7]. Although it can contribute to the collapse of a colony, due to its high prevalence, it has been suggested that it is not highly pathogenic [25]. This virus was reported once by Allen and Ball [7], and until now, it has never been reported again.

Chronic bee paralysis virus (CBPV)

Burnside et al. [33] experimentally replicated the disease caused by this virus in a group of caged bees after spraying, injecting, or feeding them with extracts from paralyzed bees. Twenty years later, Bailey et al. [17] isolated and characterized CBPV and confirmed it to be the causal agent of the disease. The virus can be detected in the queen and at all stages of bee development [2], but it mainly causes two well-defined syndromes in adult worker bees [25]. The first syndrome is characterized by abnormal tremor of the wings and body, dislocated wings, and loss of the ability to fly, which is why many bees are often observed crawling on the ground and may sometimes have a distended abdomen. They finally die within a few days of the onset of symptoms [38]. The second syndrome is called "black robbers" or "black hairless syndrome" [116]. At first, the bees maintain the ability to fly, but they turn dark, almost black, with a greasy and shiny appearance, which makes

healthy bees in the colony identify them as thieves and attack them. After a few days, they lose the ability to fly, become shaky, and die [24]. The prevalence of CBPV in Argentina was reported by Reynaldi et al. to be 26.2% [112], but it had dropped to 10% in 2016 [55].

Apis mellifera filamentous virus (AmFV)

Apis mellifera filamentous virus was first detected in the United States in adult worker bees [40]. It is a double-stranded DNA virus that infects bees [41]. It generally does not induce obvious signs, but sometimes, when it acts synergistically with other agents, it can manifest signs at the colony level [73]. In acute AmFV infections, the virus replicates rapidly in the fatty bodies and ovarian tissue of adult worker bees [60], causing lysis of the tissues, and consequently, the hemolymph is observed to have a milky white color due to a large accumulation of enveloped virions and cellular degradation, which are the characteristic signs of the disease [105]. It can be transmitted both horizontally by honey and pollen and vertically from queen to progeny [66]. Although AmFV was reported only recently in Argentina by Quintana et al. [105], the virus has been present in Argentina since 2006 according to analysis of three old samples processed in that laboratory.

Bee virus X (BVX)

During experiments in which they tried to purify Arkansas bee virus using sucrose gradients, Bailey and Woods [19] noticed a very large component, both in bees of the treated group and in the control group, that did not react with antisera against known bee viruses, and the virus was therefore named "bee virus X" (BVX). This virus multiplies experimentally when fed to newly emerged bees, and if they are kept at 30 °C, viral particles can be found in the abdomen and intestine [19] in the absence of characteristic signs. Infected bees can emerge and live for several weeks, although a reduction in the lifespan of infected bees has been shown [16]. It is not considered a very pathogenic virus, because it multiplies slowly, and therefore, winter bees, which have a longer life, are needed to establish the infection [57]. Colonies can thus be significantly affected or even die in early spring [7]. Like CBV, BVX was reported once by Allen and Ball [7], and until now, it has never been reported again.

Lake Sinai virus (LSV)

Lake Sinai viruses 1 and 2 (LSV-1, LSV2) were first found in bee samples near Lake Sinai, South Dakota, USA [121]. The following year, the LSV-3 variant was discovered by

Cornman et al. [43], and in 2015, the LSV-4 and LSV-5 variants were discovered by Ravoet et al. [107], and the LSV-6 and LSV-7 variants were discovered by Daughenbaugh et al. [48]. These viruses have been linked to hive collapse syndrome [43]. The specific pathology is unknown, and although LSV-2 was detected throughout the anatomy of infected bees, markedly higher titers were observed in the intestine and abdomen, suggesting fecal-oral transmission as the main route of infection [48]. There are reports of the presence of this virus in *V. destructor* mites [107], and even of their replication in these mites [48]. Although there are numerous variants, LSV-1 and LSV-2 are the ones with high prevalence and global distribution [48]. The LSV-3 variant has been reported recently in Argentina [61] and could be associated with losses that occurred in the country.

AIK complex

Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), and Israeli acute paralysis virus (IAPV) can be considered a viral complex because they are closely related to each other [53], though not identical. They can be distinguished by serology [87], protein profiles of the capsid [137], RT-PCR [51], and RT-mPCR [126].

These viruses were discovered during viral propagation tests in white-eyed pupae, and ABPV was discovered in experimental tests where CBPV was studied [17]. Inoculated, fed or sprayed CBPV and ABPV in healthy bees can cause tremors and paralysis [17]. However, unlike what happens with CBPV, the disease caused by ABPV occurs so rapidly that bees die without showing signs, even more so in the presence of varroa [8].

The first report of KBV was in 1974, when studies on iridescent virus of bees showed that extracts of Asian *Apis cerana* bees were contaminated with KBV. Bailey and Woods [20], by inoculating or feeding bees with these viral particles, caused their death, calling the agent Kashmir bee virus because of the place of origin of the samples.

IAPV was isolated for the first time from samples of dead bees from Alon Hagalil, Israel, that showed signs similar to those caused by ABPV. The virus was therefore called "Israeli paralysis virus" [87]. IAPV causes a similar pathology and is generally found subclinically with low titers, but it can be extremely virulent when healthy bees are inoculated or fed with it [115]. For example, injection of larvae with IAPV causes 80% mortality after 4 days [87]. Likewise, ABPV and IAPV may cause signs of tremor, inability to fly, gradual darkening, chest hair loss, paralysis, and death [87]. In contrast, KBV, which does not cause characteristic clinical symptoms [75], is normally associated with an abrupt decrease in the adult bee population and consequent

appearance of diseased larvae and pupae due to lack of brood care [115, 136].

ABPV, KBV and IAPV all cause high mortality of adult worker bees, associated with colony collapse disorder (CCD) [44], a syndrome characterized by the sudden, rapid disappearance of adult bees from apparently healthy colonies, leaving the queen with a small group of young worker bees that is insufficient to care for a large number of brood, increasing their mortality [139].

In Argentina, ABPV has been described several times [27, 55, 112], as has IAPV [27, 113]. In contrast, KVB has not yet been described in the country, although Riveros et al. [117] have reported its presence in apiaries in Chile.

Viruses and *Varroa destructor*

Varroa destructor is an ectoparasite that affects *Apis cerana* and *Apis mellifera* [11] and is considered one of the greatest threats to beekeeping in most of the world [88, 119]. Historically, several authors have claimed that it feeds on honey bee hemolymph, causing direct damage to all individuals in the colony [15]. However, recently, Ramsey et al. [106] discovered that it feeds on fatty bodies. The ectoparasite can sometimes cause the death of larvae and pupae, but generally, individuals survive with a reduced life span, smaller size, stunted wings, and less ability to fly [120]. Moreover, in adult honey bees, life expectancy decreases, and sometimes, less development of the hypopharyngeal glands, difficulty in flying, and disorientation are observed [63]. To make matters worse, numerous studies have shown that it can act as a mechanical or biological vector for various pathogens [45, 62]. There is evidence that *V. destructor* behaves as a mechanical and biological vector of DWV-B and IAPV and as a mechanical vector for ABPV, DWV-A, KBV, and CBPV [142]. Moreover, although there are no conclusive results yet, it may also act as a vector for LSV [48] and SBV [56]. The combination of varroa and viruses such as KBV, ABPV, or IAPV can trigger serious colony losses [68]. In addition, the synergy between the mite and DWV can neutralize the immune barriers of bees [54] and is considered to be one of the main causes of colony losses worldwide [144]. Particularly, the virulent variant of DWV (type B) can replicate in mites [100], while the transmission of DWV type A occurs in a non-propagative way [103].

Diagnosis

The first viral diseases discovered were recognized by clinical symptoms. A clear example is DWV, in which adult bees have wrinkled or vestigial wings, swollen abdomen, and later die [135]. Clinical signs are used to recognize viral diseases

because this type of diagnosis is fast, cheap, and concrete [51]. However, this method has numerous disadvantages: many viral diseases, such as the bee X virus disease, do not present obvious signs [16], and even those viruses that do present signs can also be present as inapparent infections, as occurs with covert DWV infections [67, 125]. To complicate this situation further, not all stages of honey bee development are affected in the same way, as is the case with SBV, which especially affects larvae [22]. Another issue is that some viruses cause similar signs. For example, ABPV and IAPV may both cause tremor, inability to fly, gradual darkening, chest hair loss, paralysis, and death [87]. In addition, multiple viral infections are frequently detected in the colonies [57, 112], confusing the symptoms [52].

Bioassays or infectivity tests are very useful for demonstrating Koch's postulates [16, 35], and they have even competed with molecular techniques [104]. Most viruses replicate rapidly when inoculated into white-eyed pupae [47]. However, bioassays are laborious, and their main disadvantage is the lack of availability of specific-pathogen-free pupae, so some the pupae that are inoculated can already contain certain viruses at low levels that can replicate, interfering with the result [10, 53]. Indeed, certain viruses, such as ABPV [17], KBV [20], and BXV [19], were accidentally discovered when performing bioassays.

Electron microscopy is also a useful tool, especially for visualizing the morphology and size of viral particles [47]. Since almost all honey bee viruses have icosahedral symmetry and sizes ranging from 17 nm (CWV, CBPV) to 30 nm (BQCV, DWV, KBV, SBV) or 35 nm (BXV, BYV) [24, 52], it is often possible to determine the presence of viral particles but not to identify the agent [7]. However, filamentous virus and iridescent virus of honey bees, which measure 500 nm and 150 nm, respectively, can be differentiated using this technique [24].

Serological tests have been useful in the diagnosis of certain viruses, the most frequently used being immunodiffusion, ELISA, and Western blot [4]. Immunodiffusion on agar gel (IDGA) is based on the visualization of an insoluble precipitate due to the formation of antigen-antibody complexes [137]. For diagnostic purposes it is quick, simple, and inexpensive [4, 12, 24]. It produces only qualitative data [12], and although it is a specific test, it has relatively high sensitivity, since only approximately 10^9 viral particles are required to form complexes that produce a clearly visible line of precipitation [24]. Samples from honey bees that have died due to replication of the virus will contain large numbers of particles, but many viral infections continue without showing signs, and viral titers remain low.

ELISA was probably the most commonly used serological method [8, 99] until the advent of molecular techniques [124]. It has relatively low cost, can be used for quantification, is easy automated, and can be scaled up for high

throughput [52]. There are many variants of ELISA, those most widely used for the diagnosis of viruses that affect honey bees being direct ELISA and sandwich ELISA [99], which has greater sensitivity but requires another antibody and a greater number of steps [72]. The technique involves sensitive enzyme developers, significantly increasing the opportunity for error and for obtaining false positive or false negative results [58]. Possible sources of error include residual enzyme activity in the samples, defective enzymes or substrates, or antibody failure. To minimize these errors and validate the technique, it is advisable always to check enzymes, primary antibodies, viruses, and substrates [58]. Another disadvantage is the difficulty in diagnosing infection with multiple viruses, since ELISA usually detects only a single target [52].

The Western blot technique is based on the transfer of proteins to a nitrocellulose or nylon membrane [122] and then allowing them to bind to specific antibodies, which are subsequently detected using secondary antibodies [78]. It is more sensitive than IDGA but much more time-consuming [4], limited for the diagnosis of multiple viruses, and can yield false negatives if not all the structural proteins of the virus are exposed or strongly antigenic. In addition, the secondary and tertiary protein structure of viral proteins can be destroyed, resulting in a loss of antigenic sites [24].

Antibody production is one of the most critical points in serological techniques; polyclonal antibodies have been used for the diagnosis of many viruses, for example against the VP4 protein of the ABPV and KBV viruses [133] or against IAPV [87]. However, the main disadvantage of polyclonal antibodies is their lack of specificity. The technique depends to a great extent on the purity of the antigen, and because many viruses share similar characteristics, the purification of viral particles can be complicated, and thus, the antiserum obtained may contain antibodies against more than one virus [72]. This drawback can be overcome by producing monoclonal antibodies in HeLa cells [72], phage [102], or yeast [65]. However, this is much more laborious, slower, and considerably more expensive [52].

Nowadays, molecular methods, particularly RT-PCR, have displaced serological tests. In RT-PCR, viral RNA is converted into complementary DNA, followed by PCR amplification [52]. Compared to ELISA, RT-PCR is a more sensitive and accurate method [124]. There are one-step and two-step RT-PCR techniques; generally, the former minimizes errors due to less manipulation but is less sensitive [34]. Once the amplification products have been obtained, they are revealed by agarose gel electrophoresis and staining, providing qualitative results [58]. However, it is also possible to obtain quantitative results using real-time PCR (q-PCR), accurately determining the initial number of viral particles [34]. Another variant is multiplex PCR (m-PCR), although it requires complex optimization [58] and is less

sensitive than simplex PCR [74], enabling the simultaneous detection of several viruses in the same reaction and thereby speeding up the diagnosis [58, 126]. A critical point of the technique is the high genetic variability of RNA viruses, which results in the primers not recognizing the binding site, in which case no amplification occurs [101].

Currently, genomic studies consist of massive DNA sequencing to determine and reconstruct entire sections of the viral genome and then decipher the functionality of each region [58]. Sanger et al. [123] described the first method of DNA sequencing. Later, Smith et al. [130] automated the method, making it less laborious and more economical. With the emergence of high-throughput sequencing (HTS) methods, progress has been exponential, enabling thousands to millions of sequences to be sequenced quickly and at an even lower cost [128]. Advances in bioinformatic analysis and sequencing technology have led to the discovery of a wide range of new asymptomatic viruses [129].

Conclusion

In Argentina, as in several countries around the world, beekeeping is an activity of great economic and ecological importance. The large losses of bee colonies in recent years have seriously affected production worldwide. Specifically, the annual decrease of 34% estimated in Argentina [110] is based on voluntary surveys and may be even greater. The causes of these losses are not clear but probably involve multiple stressors that can act simultaneously [70, 132]. Among these stressors, changes in agricultural practices, inappropriate use of agrochemicals, and the increase and spread of pathogen loads from honey bees have been proposed as the main contributing factors to honey bee mortality [94]. Undoubtedly, the combination of the *Varroa destructor* mite and its associated viruses plays an important role [83, 142, 144]. Mondet et al. [94] have suggested that the presence of *V. destructor* increases the number of viruses that can be detected in a colony. This may be due, as mentioned above, to the capacity of the varroa mite to transmit viruses, including DWV, KBV, SBV, ABPV, CBPV, and IAPV [56, 144], all of which are present in Argentina except KBV [55, 113]. Another factor favoring the dispersion of honey bee viruses could be the ability of varroa to limit the immune response of bees, such as through alterations in signaling pathways, which leads to weakening of the colony and an increase in viral load as secondary infections occur due to immunosuppression [13, 94].

Thus, one of the main causes of loss of bee colonies worldwide is infestation by varroa mites in combination with viral infections, particularly DWV. This may be due to the key role played by the ectoparasite in the transmission and replication of the virus in addition to the fact that

it destabilizes viral immune control in the honey bee by feeding on its host [13]. Therefore, DWV is considered the most virulent and the most prevalent virus globally, which may be almost entirely due to its transmission by varroa [94, 96]

One alternative for controlling honey bee viruses could be the efficient control of varroa mites, which could considerably reduce the impact of these coinfections [119]. There are numerous physical, chemical, biological and biotechnical treatments [119], of which chemical treatments are the most accessible and easiest to apply, which is why they are the most widely used worldwide [50]. However, numerous authors have demonstrated the resistance of *V. destructor* to certain commonly used acaricides [69, 85, 86, 91]. Although *V. destructor* enhances the virulence of many viruses, high prevalence of certain viruses has also been detected in absence of the mite [118].

Viruses differ in their geographic distribution [53, 68]. In Argentina, up to now, 10 viruses that affect *A. mellifera* have been detected, among which DWV and the AIK complex are the ones most often associated with loss of hives worldwide [44, 142, 144]. However, it is not yet clear what the impact of these viruses has been on the losses that have occurred in Argentina.

In the same way, the presence of some viruses has been demonstrated in other insect species that cohabit the environment of pollinators, such as bumblebees, solitary bees, hoverflies, wasps, and ants. Taking into account their high mutation rates and short generation time, RNA viruses are likely to cross species barriers and adapt rapidly to new host environments. Moreover, relatedness and shared foraging habits may increase the risk of disease transfer among managed honeybees and wild bumblebees [5]. This scenario can explain the spillover of an RNA virus from honey bees (reservoir population) into a naive host population (insects of the hive environment). Particularly in Argentina, bee viruses have been detected in various hosts, including *Bombus pauloensis* [114], *Bombus brasiliensis* and *B. morio* [29], stingless bees [9], the carpenter bee *Xylocopa augusti* [82], and another non-hymenopteran insect, the green bug *Nezara viridula* [134]. These hosts could act as important natural reservoirs for viruses and play an important role in their dispersal in the environment [142].

In conclusion, the prevalence of viruses and their ability to cause signs of disease is the result of multifactorial events, among which concomitant infections stand out. Further studies to understand the different mechanisms by which viruses spread in the environment will enable us to develop various strategies for the control of infected colonies and the spread of viruses in the habitat where they are found.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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