Prevalence of honeybee viruses in different regions of China and Argentina

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Summary

Honeybees are threatened by various pathogens and parasites. More than 18 viruses have been described in honeybees and many of them have been detected in China and Argentina. In China, both *Apis cerana* and *Apis mellifera* are raised. In Argentina, beekeepers raise different ecotypes of *A. mellifera*: European honeybees (in both temperate and subtropical regions) and Africanised honeybees (in subtropical areas only). A thorough study was carried out in both China and Argentina to analyse the current virus presence and distribution in different climatic zones and gather information on different bee species/subspecies. Adult honeybees were collected from apiaries in temperate and subtropical regions of China (including areas with exclusive populations of *A. mellifera*, areas where *A. mellifera* and *A. cerana* coexist, and areas with exclusive populations of *A. cerana*) and Argentina. Six viruses, namely, deformed wing virus (DWV), black queen cell virus (BQCV), sacbrood virus (SBV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV)

and Israeli acute paralysis virus (IAPV) were detected in China, both in *A. cerana* and in *A. mellifera*, while four viruses (DWV, BQCV, CBPV and ABPV) were present in Argentina. Interestingly, multiple infections were commonly found in China, with up to five different viruses co-circulating in some colonies without apparent abnormalities. In this study, no Chinese samples were positive for slow bee paralysis virus (SBPV). The most prevalent viruses were BQCV (China) and DWV (Argentina). Kashmir bee virus (KBV) was absent from samples analysed for both countries.

Keywords

Apiculture – *Apis cerana* – *Apis mellifera* – Argentina – China – Honeybee virus – Prevalence.

Introduction

Honeybees are threatened by various pathogens and parasites (1). Among these, viruses have been suggested as potential causes of colony losses (2) and can lead to severe disease or mortality at both the individual and the colony level (3, 4). More than 18 viruses have been described in honeybees, among which the most common are viruses of the genus *Iflavirus*: sacbroodvirus (SBV) and deformed wing virus (DWV); members of the genus *Cripavirus* (family Dicistroviridae): Kashmir bee virus (KBV), acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV), slow bee paralysis virus (SBPV) and black queen cell virus (BQCV) (5); and chronic bee paralysis virus (CBPV), which remains unclassified. According to epidemiological data, these viruses are found in honeybee colonies worldwide (6, 7).

Almost all of the above viruses were given names that reflect the clinical signs they produce. However, at both the individual and the colony level, bees have developed mechanisms that allow viruses to circulate within colonies under normal conditions without significant negative effects. Yet, if a disequilibrium occurs in the colony – which may be caused by several different factors, such as *Varroa* or *Nosema* infections, climatic conditions, genetic factors, use of pesticides, etc. –

pathogenic effects can be elicited. In these cases, the colony might reach a point where it cannot recover, and will deteriorate progressively. Thus, in the field, viruses can be present in honeybee colonies as overt infections with clear signs of disease or covert infections without clinical signs (8).

Deformed wing virus is one pathogen that can cause well-defined signs of disease and is associated with overwinter mortalities (9). This virus can spread by both horizontal and vertical transmission (7, 10), normally persisting at low levels as covert infections (10, 11). In addition, it has been widely demonstrated that *Varroa* mites can significantly increase the spread of DWV (12). Black queen cell virus follows a typical acute infection strategy, causing larvae to discolour and die. In the field, BQCV outbreaks have been linked to infection with *Nosema apis*, but there has been little agreement on the importance of *Varroa* mites as a vector of this virus (5, 13). Sacbrood virus affects the honeybee brood and results in larval death. It may also affect adult bees without clinical signs, but may sometimes decrease the lifespan of an infected worker (7); SBV infections appear more detrimental to *A. cerana* than to *A. mellifera* (7, 14). Two serologically distinct varieties, Thai sacbrood virus (TSBV) and Chinese sacbrood virus (CSBV), have produced serious outbreaks in *A. cerana* (3). Acute bee paralysis virus (ABPV), KBV and IAPV are closely related viruses that share a number of biological characteristics. They normally have a low but widespread prevalence with predominantly subclinical presentation, but can cause high mortality rates at elevated titres (15). It has been suggested that IAPV is associated with colony collapse disorder (CCD) (2). Acute bee paralysis virus and IAPV can also be vectored by *Varroa* mites (5, 16). Chronic bee paralysis virus mainly attacks adult bees, with two forms of ‘paralysis’ (7). The most common form is characterised by an abnormal trembling of the body and wings, inability to fly, a bloated abdomen and dislocated wings, while the other is identified by the presence of hairless bees that appear shiny and black and are sometimes rejected by members of their colony (17). Slow bee paralysis virus can also causes nervous system signs and is associated

with high mortality in colonies infested with *Varroa destructor*, but it usually persists as a covert infection (13).

In China, beekeepers keep both *A. cerana* and *A. mellifera*, and in Argentina beekeepers raise different ecotypes of *A. mellifera*: European honeybees in temperate and subtropical regions and Africanised honeybees in subtropical areas. Viruses have been detected in both China and Argentina (14, 18, 19) but, in most cases, it has not been possible to establish a clear association between these infections and clinical signs. Until now, no data have been made available for comparison of virus occurrence among different species and ecotypes located in different climatic regions. Given the importance of both countries as major honey producers and exporters, an understanding of the current situation with respect to virus prevalence could help in avoiding problems in the future. Therefore, the objective of this study was to determine the virus prevalence in different honeybee species and ecotypes distributed in temperate and subtropical regions of China and Argentina.

Materials and methods

Sample collection

Adult honeybees were collected from apiaries in temperate and subtropical regions of China and Argentina.

In China, samples were collected from three different beekeeping regions:

– areas with an exclusive population of *A. mellifera* (Xinjiang and Beijing)

– areas where *A. mellifera* and *A. cerana* coexist (Liaoning, Fujian and Hainan)

– areas with an exclusive population of *A. cerana* (Guangdong) (Fig. 1).

In Argentina, *A. mellifera* samples were taken from three locations: two situated in temperate regions (Balcarce and Rafaela), and one in a subtropical region where Africanised honeybees were present (Formosa) (Fig. 1).

With the exception of *A. cerana* apiaries in Liaoning province in China, where larvae showed signs of being affected by a sacbrood-like disease, none of the sampled colonies contained bees with specific clinical signs. A representative number of colonies (between 9 and 20) were selected randomly from each location on the basis of the total number of colonies in the apiaries. About 100 live adult bees from each colony were collected. In cases where sampling was repeated at different times in the same apiary, the later sampling included the previously chosen colonies plus their swarms. Samples were immediately sent to a laboratory and stored at –80°C until RNA extraction.

Virus detection

Pools of 30–32 bees were homogenised in 5 ml nuclease-free water or 15 ml phosphate buffer solution and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany) for Chinese samples or Trizol® (Invitrogen) for Argentinian samples, following the manufacturers' recommendations. Real-time polymerase chain reaction (RT-PCR) (one step in China and two steps in Argentina) was carried out to determine the presence of DWV, BQCV, SBV, SBPV, CBPV, ABPV, KBV and IAPV, using the primers described by Locke *et al.* (20). Negative (H_2O) and positive controls (recombinant plasmid DNA with virus inserted in the pGEM-T Easy vector) were included in each run of the RT-PCR reaction. After amplification, a melting curve analysis was performed to determine the specificity of the PCR products.

Statistical analyses

Statistical analysis was carried out with SPSS 17.0 (SPSS Inc., Chicago, Illinois, United States of America). The infection prevalence of viruses in different locations and in different honeybee species in

China and Argentina was compared using a chi-square test; *p*-values below 0.05 were considered significant.

Results

The results of this study showed that BQCV was the most prevalent honeybee virus in China. It was detected in all samples from both temperate and subtropical climates, in 2011 and 2012, with a prevalence of 100% in all but one region (Table I). In Argentina, BQCV was detected only in Balcarce (temperate climate), with a prevalence of 58% in March, 25% in April and 13% in May (Table II).

Deformed wing virus was detected in all locations in China, with a prevalence ranging from 41% to 100% (Table I). Xinjiang presented the lowest prevalence among the samples collected in 2012 ($p < 0.05$). In the samples from Argentina, DWV was the virus that showed the highest prevalence. As can be seen in Table II, DWV was present in 83% of colonies in Balcarce in 2011, but with lower prevalence in 2012 (12%, 60% and 56%). Among the Argentinian samples collected in 2012, Rafaela presented the lowest prevalence of DWV (9%) and Formosa the highest (90%; $p < 0.05$) (Table II).

As can be seen from Table I, ABPV was detected in only a few colonies in China in 2011, in *A. mellifera* from Xinjiang (70%) and *A. cerana* from Guangdong (22%). In Argentina, ABPV was present in samples from the temperate region. In samples from Balcarce, the prevalence of this virus varied with sampling time: the infection rate was 44% in 2011 and 4% in March, 10% in April and 6% in May in 2012. In addition, ABPV was detected in 9% of colonies from Rafaela (Table II).

Chronic bee paralysis virus was detected in colonies located in Liaoning and Fujian in 2011, and in Beijing in 2012. The virus was detected in both *Apis* species, with significantly lower prevalence in *A. cerana* ($p < 0.05$). In Beijing, CBPV was detected only in the samples collected in July 2012, with a prevalence of 15%. The prevalence of CBPV infection in Balcarce, Argentina, also varied in

different months: 15% in March, 30% in April and 6% in May (Table II).

In China, IAPV was first detected in *A. mellifera* in 2008, and was found more recently in *A. cerana* (14, 21). In agreement with the earlier studies, IAPV was found in both honeybee species in this study. In areas of China with a subtropical climate, IAPV was detected in 2011 in *A. mellifera* in Fujian (11%) and in *A. cerana* in Guangdong (22%). In 2012, IAPV was detected in both species in Hainan, with a prevalence of 60% in *A. mellifera* and 40% in *A. cerana*. In the Beijing *A. mellifera* apiary, the IAPV prevalence was 30% in May and 35% in July. Although IAPV has been reported in Argentina (19, 22), it was not detected in this work.

Sacbrood virus was detected in China in *A. mellifera* in all areas (different years and climatic areas), with a prevalence of between 70% and 100%. Surprisingly, in *A. cerana*, SBV was not detected in Guangdong, and its prevalence in Hainan and Liaoning was significantly lower than that observed in *A. mellifera* ($p < 0.05$). Colonies from Argentinian samples were not analysed for SBPV and Chinese samples were not infected with this virus. Honeybees from both China and Argentina in this study were negative for KBV.

Simultaneous infections were detected in both countries, with most Chinese colonies positive for two to four viruses and some Argentinian samples infected with two viruses. For samples from both temperate and subtropical regions in China, more viruses were found in *A. mellifera* than in *A. cerana*: three viruses were detected in 39% of *A. mellifera* colonies, while two viruses were found in 37% of *A. cerana* in temperate regions; three viruses and four viruses, respectively, were found in 32% and 37% of *A. mellifera*, whereas two viruses were found in 54% of *A. cerana* in subtropical regions (Fig. 2). No viruses were found in 40% of Argentinian colonies, and 41% of the samples were infected with only one virus (Fig. 3).

Discussion

As they are important pollinators, the decline in populations of honeybees is concerning. The fall in their numbers is the result of multiple stressors, which are thought to include diseases, pesticide exposure, malnutrition, habitat loss and climate changes. Among these factors, viruses are emerging as a global threat to honeybee health and are suspected to be major contributors to CCD (1, 2). However, under normal conditions, defence mechanisms make bees resistant and allow virus circulation without apparent problems. Insights into the situation with respect to viral infections could help in evaluation of the possible risks related to a potential decrease in resistance.

Deformed wing virus was the most frequent virus detected in Argentina in this study, which is consistent with previous reports (19). In contrast, BQCV was the most prevalent virus in both *A. mellifera* and *A. cerana* in China. It should be noted that a variant of SBV, CSBV, which is highly virulent to *A. cerana*, is present in China, and the apiaries sampled in Liaoning had a recent history of clinical signs and mortalities caused by CSBV. Although CSBV is a variant of SBV, Ma *et al*. (3) reported heterogeneity between CSBV and SBV at the genomic level. Taking into account that the primers used here were selected from sequences of European SBV, it could be hypothesised that the technique used in this study detects mainly SBV and not CSBV.

Six viruses (DWV, BQCV, SBV, CBPV, ABPV and IAPV) were detected in China, whereas four viruses (DWV, BQCV, CBPV and ABPV) were present in Argentina. Co-infections were detected in both Argentina and China. Interestingly, multiple infections were found to a high degree in China, with up to five different viruses co-circulating in some colonies, without observable clinical signs. These results contrasted with those of vanEngelsdorp *et al*. (6), who found that a higher percentage of CCD colonies were co-infected with a greater number of disease agents than control colonies (odds ratio: 3.62, $p < 0.05$). Among the multiple factors that can lead to a decrease in immune competence, *Varroa* parasitism is the most important.

Varroa mites are not only highly damaging to bees, but the parasites can also act as both mechanical and biological vectors for the transmission of multiple viruses to bees. It should be noted that *Varroa* infestation was controlled in the apiaries of *A. mellifera* sampled in China, but not in *A. cerana* apiaries because this species is naturally resistant (23, 24, 25, 26). In Argentina, the beehives sampled in this study did not receive acaricides for *Varroa* control.

The results of this study confirm the presence of honeybee viruses in China and Argentina. As seen in previous studies, the viruses circulated in both *A. cerana* and *A. mellifera* colonies, and even in unique and isolated populations of *A. cerana* or *A. mellifera* (Africanised and European). The prevalence of DWV, BQCV, ABPV and IAPV appears similar in both species, while *A. cerana* generally has lower infection rates of SBV and CBPV. Africanised bees in Formosa seem to be less susceptible to virus infections because only DWV was detected, and even in cases of high prevalence no signs of infection were observed in subtropical colonies; similarly, no signs of disease were detected in samples of European honeybees in temperate regions.

In samples taken from the same zones at different times of the year (DWV, BQCV, CBPV, ABPV in Balcarce in 2011 and 2012; CBPV and IAPV in Beijing in May and July 2012), the presence and prevalence of virus varied. Thus, it can be presumed that these viruses were present in the colonies at low levels, near the detection threshold of the diagnostic technique, producing both positive and negative results depending on the sampling time. These results are in agreement with those obtained by Tentcheva *et al*. (5), who found variability in the detection of virus in both adults and pupae.

Conclusion

Variation in virus prevalence was found between distinct species (*A. mellifera* and *A. cerana* in the same climatic region), and among different regions (*A. cerana* and *A. mellifera* in temperate and subtropical areas) and countries (variable virus distribution and co-infection pattern). Further research on virus infections, with sequential

samples and measurement of virus loads, and interactions with other factors (such as honeybee species, *Varroa* mites and other pathogens) is needed and will be very helpful for improving apiculture.

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Fig. 1

Maps of the study areas in China and Argentina

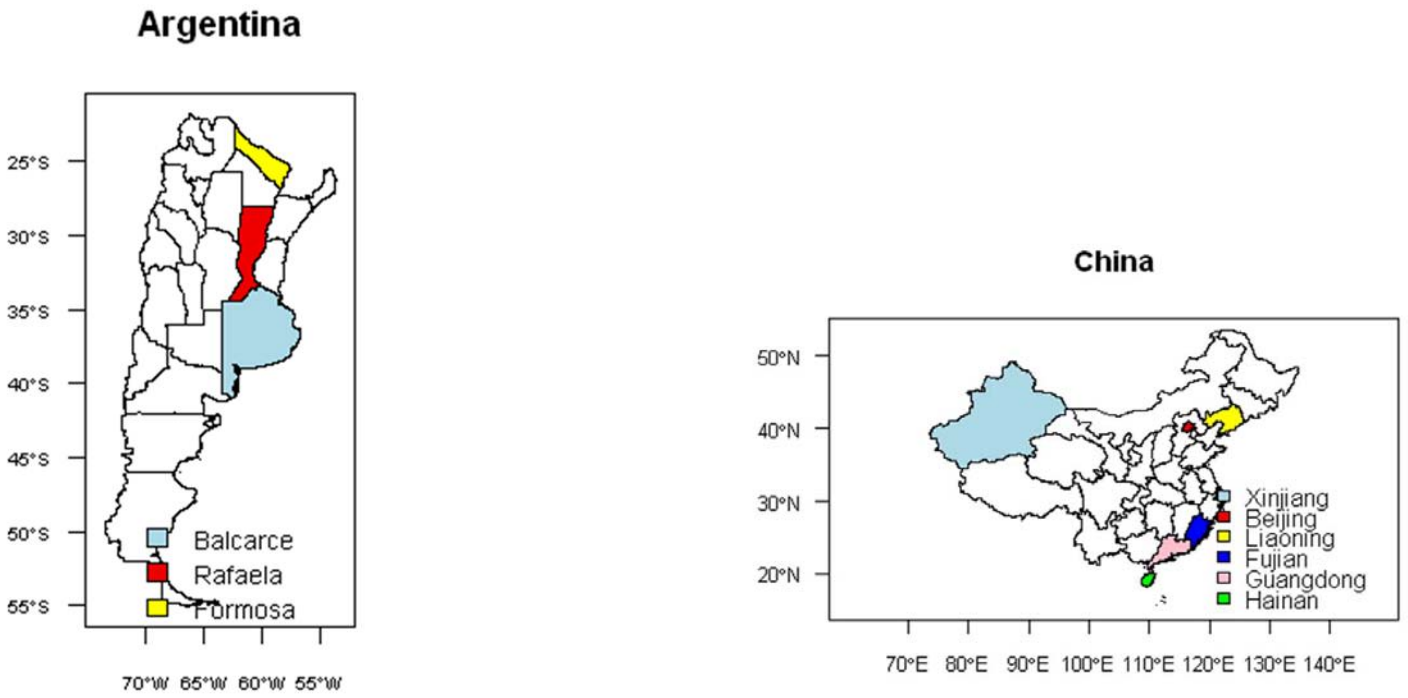
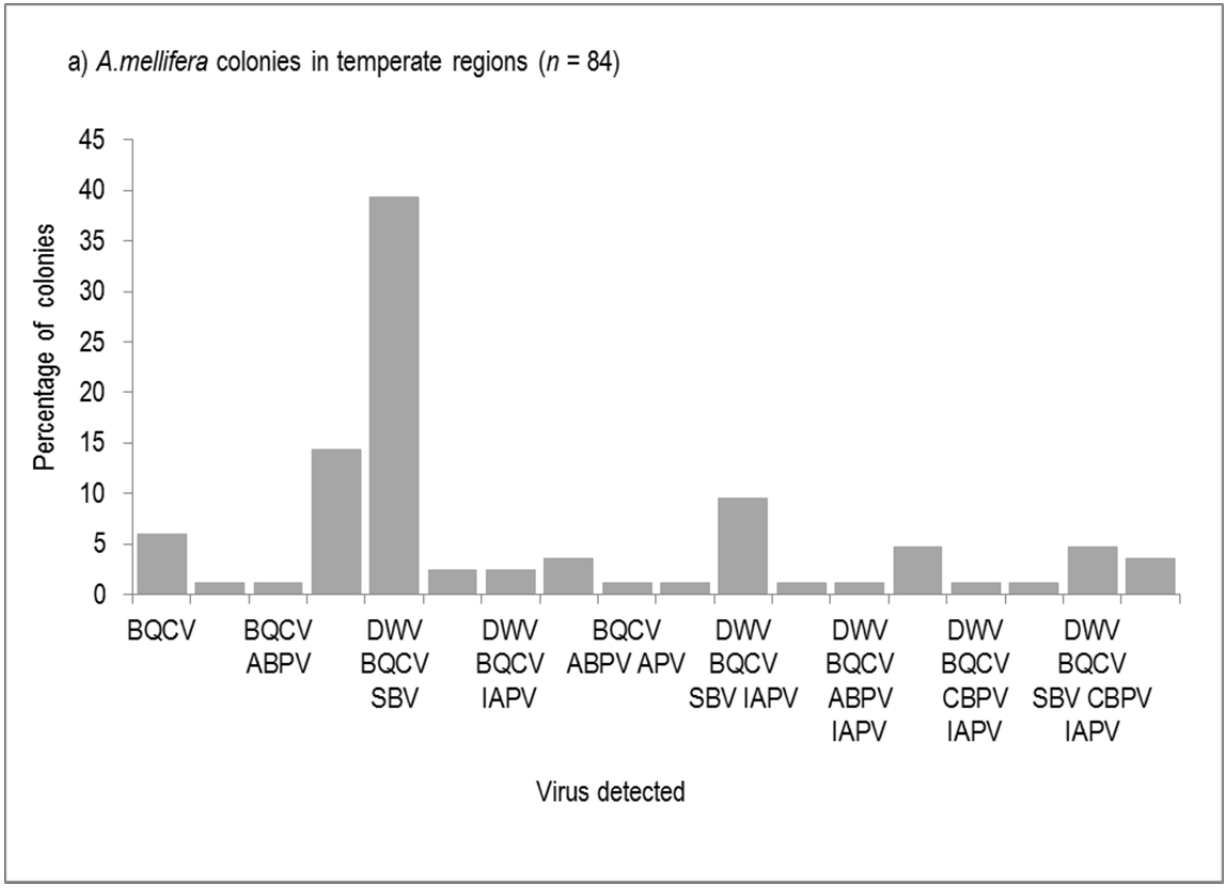
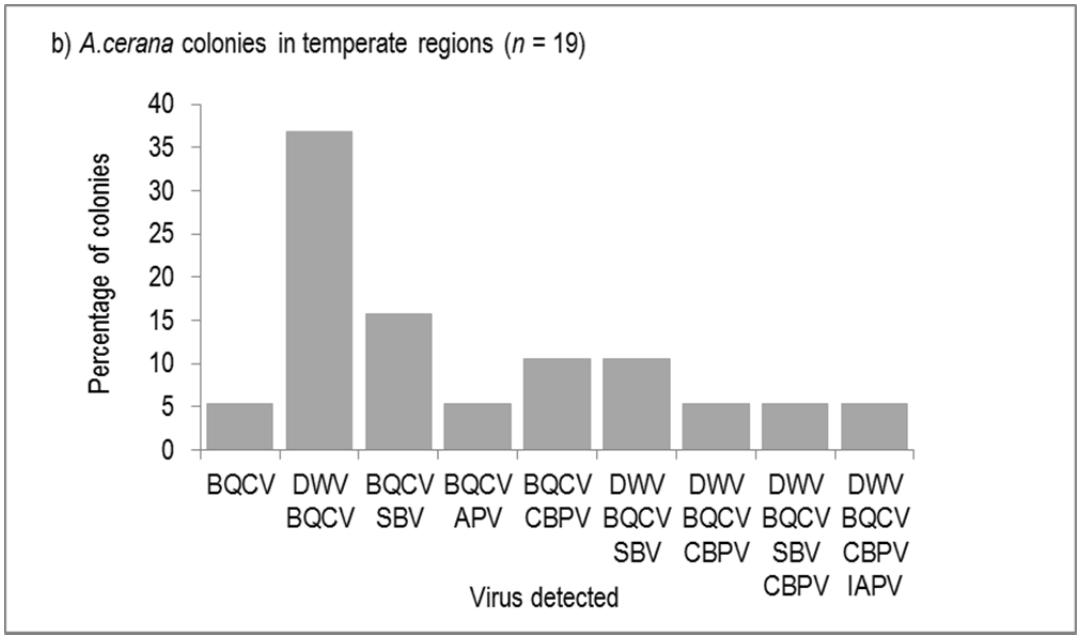
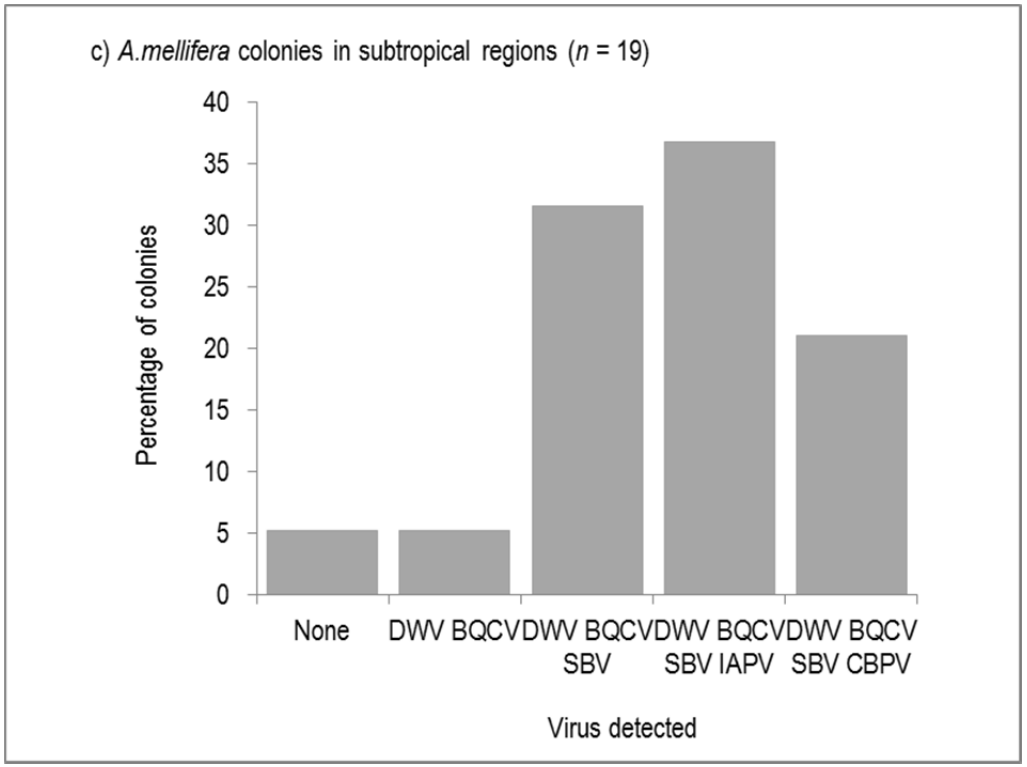


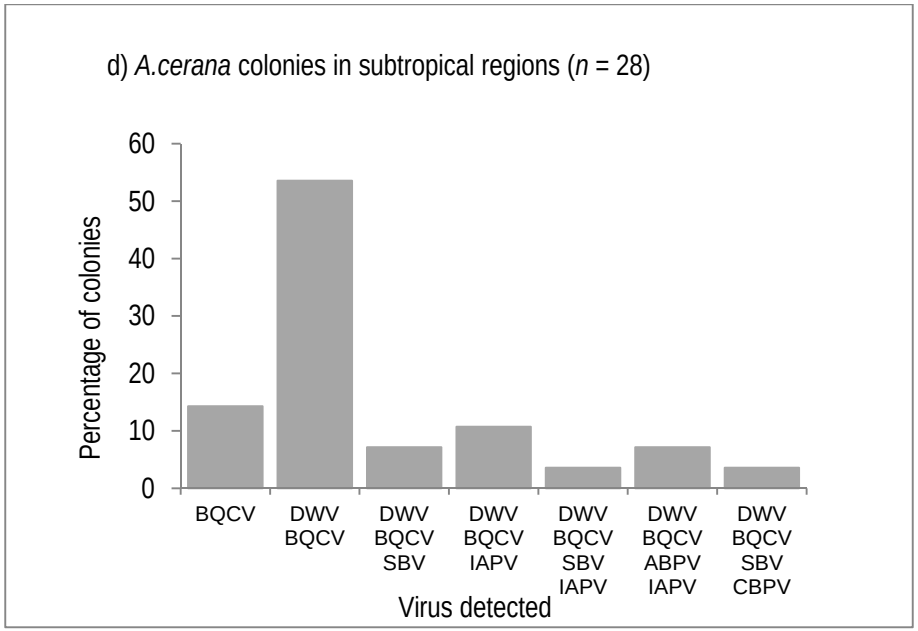
Fig. 2

Percentage of colonies infected with viruses in temperate and subtropical regions of China




b) A.cerana colonies in temperate regions (n = 19)
Percentage of colonies
40
35
30
25
20
15
10
5
0
BQCV
DWV BQCV
BQCV SBV
BQCV APV
BQCV CBPV
DWV BQCV SBV
DWV BQCV CBPV
DWV BQCV SBV CBPV
DWV BQCV CBPV IAPV
Virus detected


c) A.mellifera colonies in subtropical regions (n = 19)
Percentage of colonies
40
35
30
25
20
15
10
5
0
None
DWV BQCV
DWV BQCV SBV
DWV BQCV SBV IAPV
DWV BQCV SBV CBPV
Virus detected



ABPV: acute bee paralysis virus

BQCV: black queen cell virus

CBPV: chronic bee paralysis virus

DWV: deformed wing virus

IAPV: Israeli acute paralysis virus

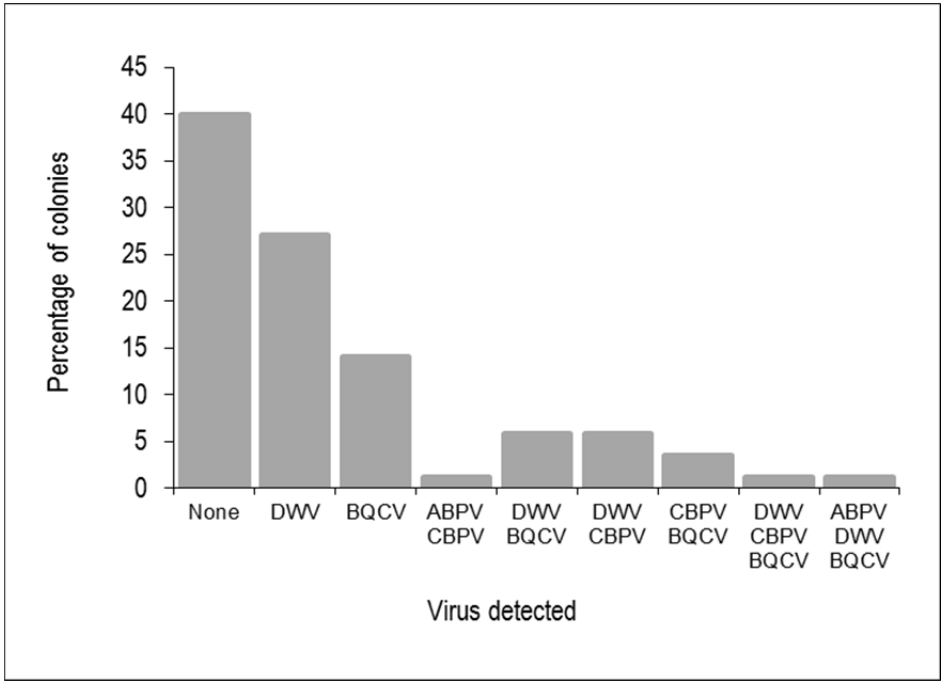
KBV: Kashmir bee virus

SBPV: slow bee paralysis virus

SBV: sacbrood virus

Fig. 3

Percentage of colonies (*n*=85) infected with viruses in temperate regions of Argentina



ABPV: acute bee paralysis virus

BQCV: black queen cell virus

CBPV: chronic bee paralysis virus

DWV: deformed wing virus

IAPV: Israeli acute paralysis virus

KBV: Kashmir bee virus

SBPV: slow bee paralysis virus

SBV: sacbrood virus

Table I

Frequency of virus detections in Chinese *A. mellifera* and *A. cerana* apiaries in temperate and subtropical regions

Climate	Year	Region	No. of colonies	Species	Rate (%)							
					DWV	BQCV	SBV	SBPV	CBPV	ABPV	KBV	IAPV
Temperate	2011	Xinjiang	10	*A. mellifera*	50	100	70	0	0	70	0	50
		Beijing	9	*A. mellifera*	100	100	78	0	0	0	0	11
		Liaoning	19	*A. cerana*	63	100	32	0	26	0	0	11
		Liaoning	18	*A. mellifera*	67	100	72	0	72	0	0	33
	2012	Beijing (May)	10	*A. mellifera*	100	100	100	0	0	0	0	30
		Beijing (July)	20	*A. mellifera*	85	100	95	0	15	0	0	35
		Xinjiang	17	*A. mellifera*	41	100	82	0	0	0	0	0
Subtropical	2011	Fujian	9	*A. mellifera*	89	89	89	0	44	0	0	11
		Fujian	9	*A. cerana*	67	100	22	0	11	0	0	0
		Guangdong	9	*A. cerana*	100	100	0	0	0	22	0	22
	2012	Hainan	10	*A. cerana*	90	100	20	0	0	0	0	40
		Hainan	10	*A. mellifera*	100	100	90	0	0	0	0	60

ABPV: Acute bee paralysis virus

BQCV: Black queen cell virus

CBPV: Chronic bee paralysis virus

DWV: Deformed wing virus

IAPV: Israeli acute paralysis virus

KBV: Kashmir bee virus

SBPV: Slow bee paralysis virus

SBV: Sacbrood virus

Table II

Frequency of virus detections in Argentinian *A. mellifera* apiaries in 2011 and 2012

Climate	Year	Region	No. of colonies	Rate (%)							
				DWV	BQCV	SBV	SBPV	CBPV	ABPV	KBV	IAPV
Temperate	2011	Balcarce	18	83	0	0	nd	0	44	0	0
	2012	Balcarce (March)	26	12	58	0	nd	15	4	0	0
		Balcarce (April)	20	60	25	0	nd	30	10	0	0
		Balcarce (May)	16	56	13	0	nd	6	6	0	0
	2012	Rafaela	11	9	0	0	nd	0	9	0	0
Subtropical	2012	Formosa	10	90	0	0	nd	0	0	0	0

nd: not determined

ABPV: Acute bee paralysis virus

BQCV: Black queen cell virus

CBPV: Chronic bee paralysis virus

DWV: Deformed wing virus

IAPV: Israeli acute paralysis virus

KBV: Kashmir bee virus

SBPV: Slow bee paralysis virus

SBV: Sacbrood virus