Prevalence of pathogens in honey bee colonies and association with clinical signs in southwestern Quebec, Canada

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Abstract

Honey bees can be affected by a variety of pathogens, which impacts their vital role as pollinators in agriculture. A cross-sectional study was conducted in southwestern Quebec to: i) estimate the prevalence of 11 bee pathogens; ii) assess the agreement between beekeeper suspicion of a disease and laboratory detection of the causative pathogen; and iii) explore the association between observed clinical signs and pathogen detection in a colony. A total of 242 colonies in 31 apiaries owned by 15 beekeepers was sampled in August 2017. The prevalence of *Varroa destructor* detection was estimated as 48% for colonies and 93% for apiaries. The apparent prevalence of colonies infected by *Nosema* spp. and *Melissococcus plutonius* was estimated as 40% and 21%, respectively. At least 180 colonies were tested by polymerase chain reaction (PCR) for deformed wing virus (DWV), acute-Kashmir-Israeli complex (AKI complex), and black queen cell virus (BQCV), which were detected in 33%, 9%, and 95% of colonies, respectively. *Acarapis woodi, Paenibacillus larvae,* and *Aethina tumida* were not detected. Varroasis was suspected by beekeepers in 14 of the 15 beekeeping operations in which the mite was detected. However, no correlation was found between suspected European foulbrood and detection of *M. plutonius* or between suspected nosemosis and detection of *Nosema* spp. Colony weakness was associated with *Nosema* spore counts of at least 0.5×10^6 per bee. *Melissococcus plutonius* was more frequently detected in colonies showing scattered brood.

Résumé

Les abeilles mellifères peuvent être affectées par plusieurs agents pathogènes, impactant leur rôle vital de pollinisateur en agriculture. Une étude transversale a été réalisée dans le sud-ouest du Québec afin 1) d'estimer la prévalence de onze agents pathogènes de l'abeille, 2) d'évaluer l'accord entre la suspicion d'une maladie par l'apiculteur et la détection de l'agent causal, 3) d'explorer les associations entre les signes cliniques et la détection d'un agent pathogène dans une colonie. Au total, 242 colonies de 31 ruchers appartenant à 15 apiculteurs ont été échantillonnées en août 2017. La prévalence de *Varroa destructor* a été estimée à 48 % pour les colonies et à 93 % pour les ruchers. La prévalence apparente de colonies infectées par *Nosema* spp. ou *Melissococcus plutonius* a été estimée à respectivement 40 % et 21 %. Le virus des ailes déformées, le complexe viral AKI et le virus de la reine noire ont été détectés dans respectivement 33 %, 9 % et 95 % dans des 180 colonies testées par PCR. *Acarapis woodi, Paenibacillus larvae* et *Aethina tumida* n'ont pas été détectés. La varroase était suspectée par les apiculteurs de 14 des 15 entreprises où la mite a été détectée. Aucune corrélation n'a été trouvée entre la suspicion de loque européenne et la détection de *M. plutonius* ou entre la suspicion de nosémose et la détection de *Nosema* spp. La faiblesse des colonies a été associée à des comptes de *Nosema* d'au moins $0{,}5 \times 10^6$ spores par abeille. *Melissococcus plutonius* était plus fréquemment détecté parmi les colonies présentant du couvain en mosaïque.

(Traduit pas les auteurs)

Introduction

Honey bees *(Apis mellifera)* are important for honey production and their crucial role as pollinators in agriculture (1). The high winter colony mortality reported by beekeepers is therefore cause for widespread concern (1). The etiology of mortality in honey bee colonies is multifactorial, with pathogens being one of the most likely contributing causes (2).

In Canada, the parasitic mite *Varroa destructor* is thought to be the main contributor to colony mortality (3). The following viruses are most commonly associated with the decline of honey bees: black queen cell virus (BQCV); acute bee paralysis virus (ABPV); Israeli

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acute paralysis virus (IAPV); and deformed wing virus (DWV) (4–6). The tracheal mite *Acarapis woodi* and the fungi *Ascosphaera apis* can also cause significant production losses (7,8). Finally, the small hive beetle *(Aethina tumida)* can cause physical damage to the hive and brood that can lead to collapse in weak colonies (9).

Only a few prevalence studies have been conducted on honey bees in Canada (3,10). Moreover, as passive surveillance of honey bee pathogens relies primarily on the observations of beekeepers, their reports on disease status need to be validated. Furthermore, in the context of pathogen surveillance, determining the clinical signs that could help to identify colonies at greater risk of carrying a pathogen is relevant to targeted sampling.

The objectives of this study were to: i) estimate the prevalence of 11 honey bee pathogens in southwestern Quebec, Canada; ii) assess the agreement between beekeeper suspicion of a disease and laboratory detection of the causative pathogen; and iii) explore the correlation between observed clinical signs and pathogen detection in a colony.

Materials and methods

Study design and area

This cross-sectional study was conducted in the active surveillance zone for the small hive beetle *Aethina tumida* in Quebec, Canada, as it was defined at the time of the study (Figure 1).

Selection of apiaries

A total of 42 of the 75 apiaries located in adjacent regional county municipalities from western Montérégie region, in addition to 34 of the 51 apiaries located in Pontiac, were randomly selected for mandatory inspection. It was determined that these 2 sample sizes would be sufficient for detecting *A. tumida* in at least 1 apiary at a 95% confidence level, given a minimal prevalence of 5% and a finite population (11). Beekeepers owning these apiaries were invited to participate in the project on a voluntary basis.

Sampling

The sampling took place from August 7, 2017 to September 1, 2017. In apiaries of 10 colonies or less, all colonies were selected. In larger apiaries, 10 colonies were systematically selected. Sampling was conducted by 1 of 3 members of the research team, with the help of the beekeeper. Hives of the first 2 apiaries were inspected jointly by the investigators to limit inter-observer variability.

The top of each box and the floor of the hive were visually inspected for the presence of *A. tumida* (12). Colonies were considered strong when more than 30% of the surface of the brood frames was covered by bees; otherwise, they were considered weak. The 3 visually oldest frames in the brood nest were examined for the presence of mummies, dead larvae, scattered brood, deformed wings, and *V. destructor* on adult bees. Colonies were considered positive for *A. apis* when 1 or more mummies were present (7).

For each colony, approximately 300 nurse bees were collected from 2 brood frames in the bottom brood chamber and put in a plastic container with 70% isopropyl alcohol for *V. destructor* counts. Approximately 200 foraging bees were also collected and put in 70% isopropyl alcohol in order to detect *A. woodi* and *Nosema* spp.

Approximately 30 nurse bees (for detecting *M. plutonius* and *P. larvae*) and approximately 100 nurse bees (for detecting viruses) were collected from each colony and put into separate single-use polyethylene bags (Fisherbrand; Fisher Scientific, Waltham, Massachusetts, USA). The bees were immediately killed on dry ice and kept on dry ice until storage at −80°C. Weak or dead bees, if present in front of the hive, were collected in a sealed plastic container and kept at ambient temperature to investigate for *Apocephalus borealis.*

Questionnaire

A questionnaire was developed and pre-tested with 2 experienced beekeepers for clarity, thoroughness, and time to administer (13,14). Beekeepers were asked about their perceptions relative to the presence of diseases in their apiaries, *e.g.*, "To the best of your knowledge, is there currently or has there been varroosis in your apiaries in 2017?" This questionnaire was filled out through telephone interviews with participating beekeepers, who were blinded to laboratory results.

Laboratory analysis

Varroa destructor

Varroas were detected using the alcohol wash method (15). Bees and *V. destructor* mites in each sample were counted to estimate the infestation level.

Acarapis woodi

Within each apiary, 50 of the foraging bees kept in alcohol were selected across all selected hives. A thin thorax section was cut from each bee and immersed in 40 mL of potassium hydroxide (KOH) (8%). The solution was heated in a microwave at low power for 4 min. Each trachea was examined under the stereomicroscope (450X) for the presence of the parasite or its excreta, as described in a previous study (16).

Nosema spp.

For each colony, spores were counted on 60 of the foraging bees kept in alcohol and averaged per bee (17). When spores were observed, polymerase chain reaction (PCR) was used to detect *N. apis* and/or *N. ceranae* species. (See Appendix for additional information).

Foulbrood agents

Adult bees were cultured for the detection of *M. plutonius* and *P. larvae.* (See Appendix for additional information). For *Paenibacillus larvae,* this method was adapted from Lindström and Fries (18).

Viruses

Due to limited resources, 3 apiaries from 1 beekeeper with 9 selected apiaries in the study were randomly selected for virus testing. All colonies from the other beekeepers were tested. Total nucleic acids were extracted from 10 adult bees per colony and tested by PCR for the following: deformed wing virus (DWV); viruses of the AKI complex [acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), and Israeli acute paralysis virus (IAPV)]; and black queen cell virus (BQCV). (See Appendix for additional information).

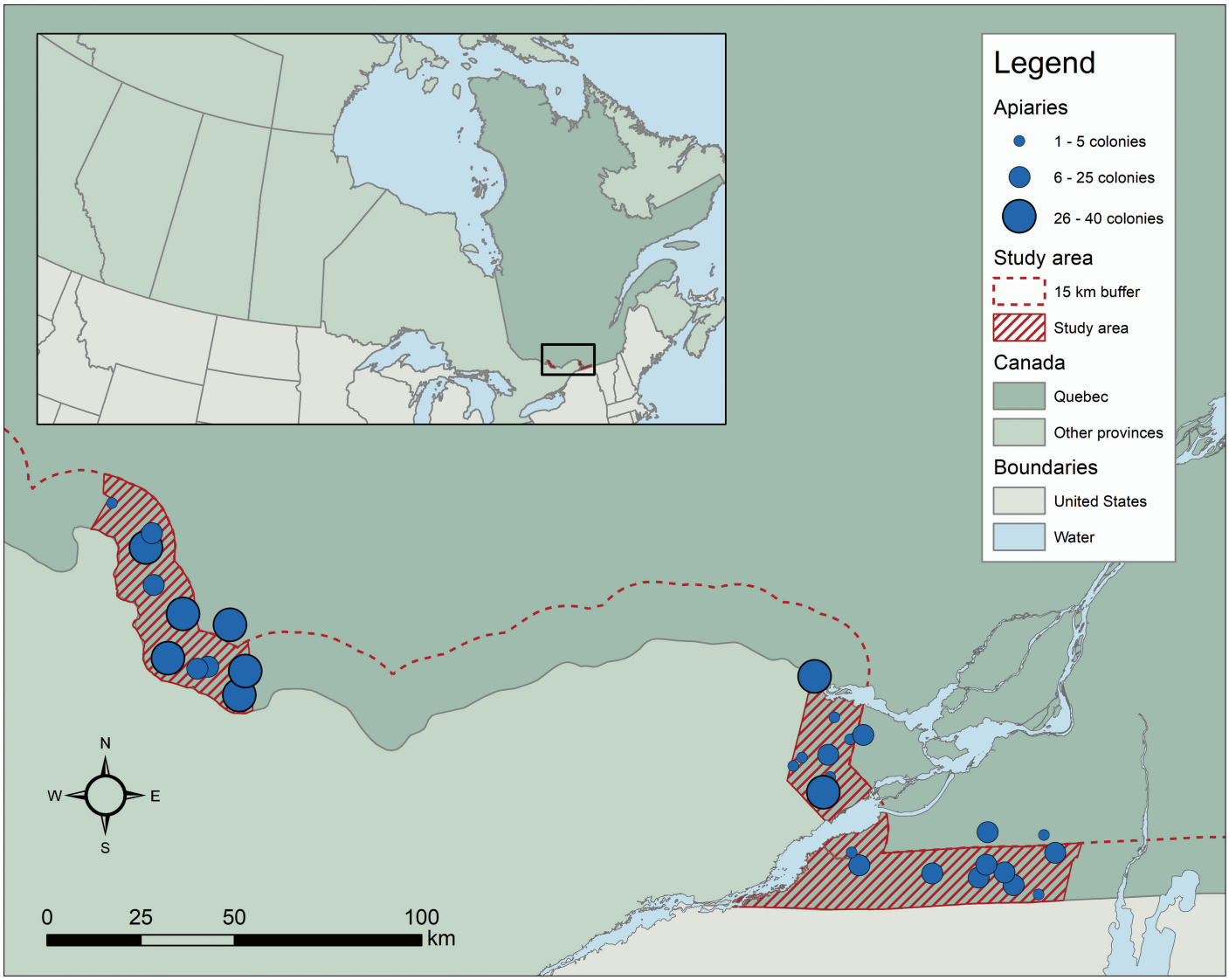

Figure 1. Area of active surveillance zone for the small hive beetle *(Aethina tumida)* in Quebec where the study of honey bee colonies was carried out.

Apocephalus borealis

The weak or dead bees collected were kept at room temperature for 15 d. Emerged larvae were speciated based on typical adult morphology (19,20) using a 4.7-150X stereomicroscope. For larvae that died before reaching adult form, PCR testing was conducted. (See Appendix for additional information).

Statistical analyses

All statistical analyses were carried out in SAS software, Version 9.4 (SAS Institute, Cary, North Carolina, USA). The apparent prevalence of each pathogen with 95% confidence intervals (CI) was estimated at the colony and apiary levels. For *V. destructor* and *Nosema* spp., different thresholds were used to define a positive case (Table I) based on recommendations for treatment or prediction of damage (21–24).

An apiary with at least 1 positive colony detected was considered positive. At the colony level, prevalence estimates were adjusted for the stratified (2 areas) multi-level sampling design by attributing a sampling weight to each colony. In addition, the variance estimate was adjusted for clustering of colonies within apiaries using the Taylor series method. For all prevalence of 0% or 100%, however, exact confidence intervals without adjustment were estimated using the Clopper-Pearson method.

The proportion of beekeepers who suspected the presence of a disease in their colonies was compared to the proportion of beekeepers for whom the causative agent was detected from the study samples using a McNemar test, which was carried out separately for each pathogen. The agreement between a beekeeper's suspicion of the disease and pathogen detection was estimated using the *Kappa* coefficient.

The association between colony weakness and pathogen detection was tested for each pathogen with at least 1 positive colony. The association between specific clinical signs, *i.e.*, dead larvae, scattered brood, and detection of *M. plutonius*, as well as between deformed

Table I. Apparent prevalence of pathogens according to visual inspection or laboratory testing of samples in 242 colonies from 31 apiaries owned by 15 beekeepers in southwestern Quebec, Canada, August 2017.

	Colony				Apiary				Beekeeper	
Case definition for a positive colony	Number tested	Number positive	Prevalence %	Prevalence 95% CI	Number tested	Number positive[a]	Prevalence %	Prevalence 95% CI	Number tested	Number positive[a]
Visual detection										
Varroa destructor										
≥ 1 mite	242	134	48	(36 to 60)	31	29	93	(77 to 99)	15	15
≥ 1 mite/100 bees	242	53	16	(10 to 25)	31	16	49	(31 to 68)	15	12
≥ 5 mites/100 bees	242	8	2	(0 to 5)	31	4	12	(3 to 29)	15	4
≥ 10 mites/100 bees	242	4	1	(0 to 3)	31	3	9	(2 to 25)	15	3
Acarapis woodi[b]			—		31	0	0	(0 to 11)	15	0
Nosema spp.										
≥ 1 spore/bee	242	88	40	(31 to 50)	31	24	78	(60 to 91)	15	11
≥ 0.5×10^6 spores/bee	242	32	15	(9 to 23)	31	17	56	(37 to 74)	15	10
≥ 1×10^6 spores/bee	242	13	6	(3 to 10)	31	10	35	(19 to 54)	15	5
Apocephalus borealis	242	1	0	(0 to 2)	31	1	4	(0 to 19)	15	1
Aethina tumida	242	0	0	(0 to 1)	31	0	0	(0 to 11)	15	0
Clinical sign detection										
Ascosphaera apis[c]	241	13	7	(2 to 17)	31	5	18	(7 to 36)	15	1
Culture positivity										
Melissococcus plutonius	242	57	21	(12 to 32)	31	17	55	(36 to 73)	15	8
Paenibacillus larvae	242	0	0	(0 to 1)	31	0	0	(0 to 11)	15	0
PCR positivity										
Deformed wing virus	182	61	33	(17 to 50)	25	22	88	(68 to 98)	15	13
AKI complex	182	19	9	(3 to 20)	25	7	28	(12 to 50)	15	4
Black queen cell virus[c]	180	168	95	(88 to 98)	25	25	100	(86 to 100)	15	15

[a] An apiary or beekeeper is considered positive if 1 ≥ colony is positive.

[b] Pooled samples (1 per apiary).

[c] Data missing on *A. apis* for 1 colony and on black queen cell virus for 2 colonies.

Table II. Association between suspicion of European foulbrood and nosemosis by beekeepers and pathogen detection in their operation during the 2017 season in southwestern Quebec, Canada.

a. European foulbrood

	European foulbrood	
Melissococcus plutonius	Not suspected	Suspected
Culture-negative	6	1
Culture-positive	5	3

b. Nosemosis

	Nosemosis	
Nosema spp.	Not suspected	Suspected
Not detected	3	1
≥ 1 spores	5	6

McNemar $P = 0.10$; *Kappa:* Estimate = 0.22; $P = 0.31$.

wings and detection of DWV, was also evaluated. Rao-Scott chi-square exact tests were used, taking colony clustering by apiaries into account. The *alpha* value was fixed at 5% for interpretation.

Results

Of the 27 beekeepers selected for mandatory *A. tumida* inspection, 15 agreed to participate in the study. The primary reason for refusal to participate was a lack of time. Due to time constraints and restrictions due to weather conditions, not all the selected apiaries could be sampled. Nevertheless, at least 1 apiary was sampled per beekeeper. A total of 11 apiaries was sampled in the Pontiac area and 20 apiaries in western Montérégie, for a total of 242 colonies.

Pathogen prevalence

Varroa destructor was detected by alcohol wash in 134 colonies (Table I), in 2 of which, mites were observed on adult bees during inspection. *Acarapis woodi* was not found in any bees. Among the 88 colonies positive for *Nosema* spp., only *N. ceranae* was detected in 66 colonies (75%), only *N. apis* was detected in 2 colonies (2.3%), and both species were detected in 2 colonies (2.3%). We were unable to identify the species for the 18 (20%) remaining cases. *Melissococcus plutonius* was found in 57 colonies (24%), whereas *P. larvae* was not isolated in any of the 242 colonies.

A total of 40 samples was PCR positive for DWV, 10 of which were sequenced and had > 98% homology with known DWV sequences. In addition, 21 samples generated cycle threshold (Ct) values of 34 to 36. All were considered positive after confirmation on a subset of 17 samples by agarose gel electrophoresis or sequencing (> 98% identity with known DWV sequences).

Of the 16 PCR-positive samples for the AKI virus complex, 9 were sequenced and confirmed > 85% identical to available sequences. In addition, 13 of the 16 samples positive for AKI, which were selected to represent all positive apiaries, were reamplified using 1 or more of the 3 conventional reverse transcription (RT)-PCR as described in the Appendix.

All samples generated RNA fragments of the appropriate size on agarose gel electrophoresis and had > 97% homology to known Israeli acute paralysis virus (IAPV) sequences. Three doubtful samples were considered positive after 2 of them were retested with different primers using conventional RT-PCR and gel electrophoresis. Among the 19 colonies positive for the AKI virus complex, IAPV was detected by PCR in 18 colonies (95%). The presence of acute bee paralysis virus-Kashmir bee virus (ABPV-KBV) was tested in 17 (89%) of AKI-positive cases and an amplicon of the appropriate size was generated in a total of 9 colonies. After sequencing these amplicons, however, it was determined that the virus identified in all of these samples was IAPV.

A total of 168 (93%) of the 180 tested colonies was reported as positive for BQCV. Sequencing of 17 samples from 15 apiaries showed over 98% homology to known BQCV sequences.

Dead bees were found in front of 109 colonies from 22 apiaries. Larvae emerged from the dead bees from 14 colonies. *Apocephalus borealis* was confirmed by PCR in 1 sample.

Aethina tumida was not visually detected.

Agreement between beekeeper suspicion and laboratory diagnosis

Varroasis was suspected by 14 out of 15 of the beekeepers and was detected in all operations. Although 3 beekeepers suspected American foulbrood in their apiaries during the 2017 season, *P. larvae* was not detected by culture in any sample. The agreement was not tested for these 2 diseases due to their sparse distribution. No significant agreement was detected between beekeepers' suspicion of European foulbrood or nosemosis and pathogen detection (Table II).

Association between clinical signs and pathogen detection

Colony weakness was not associated with detection of pathogens, except for *Nosema* spp. for positivity thresholds ≥ 0.5×10^6 spores (Table III). With regard to other clinical signs, only scattered brood was associated with *M. plutonius* colony status ($P < 0.010$). Bees with deformed wings were observed in only 1 colony, which tested positive for DWV.

Discussion

Our study provides an initial benchmark for the prevalence of honey bee pathogens in southwestern Quebec. The validity of our results is supported by the probabilistic sampling strategy and the overall good participation rate (56%) of beekeepers. However, considering the reported seasonal occurrence of many bee pathogens, including *V. destructor* (25) and *Nosema* (26), the inference should be limited to the study area and timeframe.

Varroa destructor was the most common pathogen of clinical importance detected and was detected in 48% of the colonies and 93% of the apiaries. In Ontario, Guzmán-Novoa *et al* (3) reported a higher prevalence (76%) of infested colonies in the fall. In participating beekeeping operations, 56% of colonies were treated in the previous spring with amitraz, an acaricide with > 90% efficacy (13,27). As our detection method did not allow for the detection of mites in capped broods (25), it should be noted that the actual prevalence of *V. destructor* is likely underestimated in our study.

Although a negative correlation between colony strength and *V. destructor* infestation level was expected (28), this was not

Table III. Distribution of presence of various pathogens according to clinical signs at 242 colonies (181 for viruses) in southwestern Quebec, Canada, August 2017.

	With clinical sign		Without clinical sign		
Clinical signs and pathogen	Number tested	Number (%) pathogen-positive	Number tested	Number (%) pathogen-positive	*P*-value (Rao-Scott χ^2)
Clinical sign: colony weakness					
Varroa destructor					
≥ 1 mite	33	16 (48)	206	117 (57)	0.38
≥ 1 mite/100 bees	33	6 (18)	206	47 (23)	0.44
≥ 5 mites/100 bees	33	1 (3)	206	7 (3)	0.91
≥ 10 mites/100 bees	33	0 (0)	206	4 (2)	1.00[a]
Nosema spp.					
≥ 1 spore/bee	33	16 (48)	206	0 (34)	0.19
≥ 0.5×10^6 spores/bee	33	9 (27)	206	22 (11)	0.04
≥ 1×10^6 spores/bee	33	4 (12)	206	8 (4)	0.048
Melissococcus plutonius	33	11 (33)	206	45 (22)	0.15
Deformed wing virus	27	9 (33)	154	52 (34)	0.96
AKI complex	27	5 (19)	154	14 (9)	0.20
Black queen cell virus	27	25 (93)	154	142 (93)	0.83
Ascosphaera apis	32	1 (3)	206	12 (6)	0.46
Clinical sign: Detection of dead larvae					
Melissococcus plutonius	29	8 (28)	212	48 (23)	0.58
Clinical sign: Detection of scattered brood					
Melissococcus plutonius	36	13 (36)	205	43 (21)	< 0.01
Clinical sign: Detection of deformed wings					
Deformed wing virus	1	1 (100)	180	60 (33)	0.34

[a] *P* from Exact Pearson chi-Square test (not taking clusters into account), given data distribution.

observed. As *V. destructor* population increases with the bee population, we hypothesized that heavy infestations were more likely to occur in strong colonies during the summer. Therefore, our sampling in late summer could have been too early to detect a detrimental impact of the parasitic load on the colony that could occur later in the fall. All but one of the 15 beekeepers suspected the presence of *V. destructor* in their colonies, which suggests a high level of awareness of this disease in Quebec.

A predominance of *N. ceranae* over *N. apis* was observed, as previously reported in other Canadian provinces (29). Even though our sampling took place in summer when spore loads were found to be the highest in eastern Canada (30), very few colonies showed spore levels above the suggested threshold of 1 000 000 spores/bee required to predict negative impacts on the colony (30).

In the present study, weak colonies were more likely to present high infection loads of *Nosema* spp. than strong colonies, but it is not known if the infection was the cause or the consequence of the colony weakness. The absence of clear clinical signs could partly explain why beekeepers in half of the operations in which the pathogen was detected did not suspect nosemosis. Also, beekeepers might not have been inclined to search for these signs as there is no consensus on the usefulness of treatments for nosemosis (31).

Melissococcus plutonius was detected in 21% of the hive samples. *Melissococcus plutonius* is often present in asymptomatic colonies, even in areas in which no clinical outbreak has been reported (32). Our study corroborates that scattered brood is indicative of *M. plutonius* infection and suggests that targeting colonies with a clear pattern of scattered brood could increase the likelihood of its detection in an apiary. Some negative-culture colonies also had scattered brood. Since scattered brood results from an episode of larvae mortality, it is possible that this clinical sign remained visible after the disease outbreak was resolved or that it was secondary to other health issues.

Paenibacillus larvae was not isolated or suspected in our study, despite previous reports of infection in Canada (10). This bacterium has been detected in Quebec every year since 2017 according to honey and larvae samples submitted for various reasons to the MAPAQ diagnostic laboratory. As *P. larvae* is a very contagious agent that can lead to severe disease outbreaks, it is extremely important to

identify and manage infected colonies (33). It is therefore important to evaluate the analytical sensitivity of the method used in this study using adult bee samples if it is to be used for surveillance purposes.

Both BQCV and DWV were detected in 95% and 33% of colonies, respectively, in our study. This is similar to previous reports of 62% of colonies testing positive to BQCV and from 4% to 33% testing positive for DWV in Israel and Germany (4–6).

The relatively low prevalence of AKI complex viruses (9%) reported in our study concurs with results obtained in Germany (6). Weak colonies tend to be more frequently infected by viruses from the AKI complex than strong colonies. As IAPV had been associated with the phenomenon of colony collapse disorder (34), this association should be further explored.

Both BQCV and DWV have previously been associated with a reduction in worker bee population (4,28,35). The viral loads of DWV and BQCV in the present study, which were not quantified, might have been insufficient to cause detectable weakness. It has also been reported that the clinical outcome of DWV infection depends on the genotype involved (36) and the co-presence of *V. destructor* (37).

Acarapis woodi was not detected in this study despite the previous report of this parasite in 1% of bees among 408 sampled colonies in the neighboring province of Ontario (3). Our results are consistent with the absence of *A. woodi* reported by passive surveillance for many years in the study area. The widespread use of formic acid and thymol in Quebec to control *V. destructor,* which also controls *A. woodi*, could be a reason for our results.

Ascosphaera apis was not detected in our study. Given that fungal growth is enhanced in cool and humid beehives (38), it is possible that the prevalence obtained in this study, during which samples were collected in late summer, was at its lowest point of the year.

Sampling for *A. borealis* was not optimal, as parasitized bees can be hard to detect; the use of a light trap at night is recommended to increase the likelihood of detection (39). The prevalence of *A. borealis* in our study should therefore be interpreted as evidence of its presence in Quebec. Although the impact of this parasitic fly on honey bee colonies remains to be clarified, it could act as a vector for other pathogens (40).

Aethina tumida was not detected during the annual monitoring of honey bee colonies in Quebec in 2017, which was not unusual, as the hive beetle was scarce in this area before the study. It has emerged in southern Quebec since then.

In the absence of reliable estimates of sensitivity and specificity for the various diagnostic tests used, only apparent prevalence estimates were presented. Although the method used to assess colony strength was rapid and replicable in the field (28), it was prone to subjectivity and could lead to misclassification bias.

Finally, no conclusion can be reached for when beekeepers suspected a disease in their operation, but no causative pathogen was detected, since the pathogen may only have been present earlier in the season or in unsampled colonies.

In conclusion, *Varroa destructor* was the most common pathogen found in this study. Only *Nosema* spore counts were associated with colony strength. Scattered brood in a colony increased the likelihood of detecting *M. plutonius.* And finally, with the exception of *V. destructor,* beekeepers often did not observe clinical signs of diseases in their apiaries when a pathogen was present.

Appendix — Additional information for laboratory protocols

PCR protocol for Nosema

For DNA extraction, the last 3 tergites of 5 bees per sample were cut to remove the intestines. They were put in 1.5 mL tubes, dried for 20 min at 37°C to remove the residual ethanol, and then frozen at −80°C for 15 min. Then, 440 µL of saline extraction buffer, 44 µL of 20% sodium dodecyl sulfate (SDS), and 8 µL of proteinase K (20 mg/mL) were mixed into each sample, which was then incubated for 1 h at 60°C, while being vortexed every 20 min.

After adding 300 µL of 6M saline solution, samples were vortexed and then centrifuged at 16 660 × *g* for 20 min at 4°C. The supernatant was removed and centrifugation was repeated to remove debris. Next, 600 µL of precooled isopropanol (−20°C) was mixed into the supernatant. After incubation at −20°C for 30 min, the sample was centrifuged at 15 800 × *g* for 20 min at 4°C and the supernatant was removed.

Then 200 µL of 70% precooled ethanol (−20°C) was added to the sample. After centrifugation at 15 800 × *g* at 4°C for 10 min, the supernatant was removed and dried overnight at room temperature. Pellets were resuspended overnight at 4°C in 100 µL of water. Extracted DNA was stored at −20°C.

A PCR was realized with the following 15 µL mix: 3 µL Q5 reaction buffer (5×); 0.3 µL deoxynucleotide triphosphate (dNTP) (10 mM); 0.75 µL forward primer (10 µM); 0.75 µL reverse primer (10 µM); 3 µL Q5 High GC enhancer (5×); 0.3 µL Q5 High-Fidelity DNA polymerase (2U/µL); 4.9 µL double-distilled water (ddH_2O); and 2 µL of extracted DNA.

Primers for *N. apis* and *N. ceranae* were developed in the laboratory of Dr. Nicolas Derome at Université Laval (Quebec) and actin primers were used to assess the quality of extracted DNA, as described by Cox-Foster *et al* (1).

For *N. apis,* the forward primer was 'CCATTGCCGGATAAGA GAGT' and the reverse primer was 'CACGCATTGCTGCATCA TTGAC'. For *N. ceranae*, the primers were 'CGGATAAAAGAGTCC GTTACC' for forward and 'TGAGCAGGGTTCTAGGGAT' for reverse.

Expected fragment lengths were 401 bp for *N. apis* and 250 bp for *N. ceranae*. Samples for *N. apis* and *N. ceranae* were processed in a Biometra T1+ thermocycler according to the following program: 94°C for 2 min; 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 30 s; and 72°C for 5 min. Positive (confirmed cases) and negative (water sample) controls were included in each run. The results were visualized on agarose gel electrophoresis.

Culture method for foulbrood agents

For each colony, 30 bees were homogenized in 20 mL of phosphate buffer (PBS, pH 7.2) using a stomacher for 30 s at 560 paddle beats per minute (bpm). The homogenate was filtered through Whatman No. 1 paper and the filtrate was centrifuged at 1500 × *g* for 10 min. The pellet was suspended in 3 mL of sterile PBS. The suspensions were stored at −80°C until being sent to the LSA for culture.

For *P. larvae* culture, each preparation was separated into 3 vials, each containing 1 mL, and treated as follows: i) without heat treatment; ii) heat-treated at 80°C for 10 min; and iii) heat-treated at 95°C for 3 min in a heating block (Isotemp; Fisher Scientific, Waltham, Massachusetts, USA).

After heat treatment, a MYPGP agar plate media supplemented with nalidixic acid (final concentration of 10 μg/mL) was inoculated with a cotton swab. Positive controls of sporulated *P. larvae* (ATCC 25747) were used to monitor the heat treatment of samples. MYPGP plates were incubated for 7 d in aerobic conditions at 35°C.

Bacterial isolates were identified by MALDI-TOF mass spectrometry. For *M. plutonius* culture, suspensions without heat treatment were inoculated on basal media plates and incubated anaerobically for 7 d at 35°C. All plates with bacterial growth were analyzed by PCR for detection of *M. plutonius*. *Melissococcus plutonius* (ATCC 35311) was used as a positive control.

The sensitivity of the culture technique was tested on 1 homogenate of 30 bees from a colony with clinical signs of European foulbrood and 1 homogenate of 30 bees with signs of American foulbrood, which both tested positive for their respective agent.

PCR protocol for viruses

Total nucleic acids were extracted from 10 adult bees per colony. Bees were first put in a mortar with liquid nitrogen and crushed for 1 to 2 min until a fine powder was obtained. An extraction control consisting of mouse norovirus [10^3 genome equivalent of murine norovirus-1 (MNV-1)] was added to 1 sample/extraction cycle. A total of 4 mL of PBS (pH 7.4) was mixed to the bee homogenate and centrifuged at 3800 × *g* for 10 min. A total of 140 μL of the supernatant was used for nucleic acid extraction using the QIAamp Viral RNA Mini Extraction Kit (Qiagen, Hilden, Germany). Total extracted nucleic acids were stored at −80°C until RT-PCR testing.

Qualitative one-step RT-PCR reactions were conducted using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). RT-PCR reactions were done in a final volume of 20 μL containing 2X QuantiTect Master Mix (Qiagen), a mix of reverse transcriptase and Taq polymerase, 0.6 μM of each primer, and 2 μL of total nucleic acid. All reactions were carried out on a Roche LightCycler 96 Instrument (Roche Diagnostics, Rotkreuz, Switzerland). To ensure specificity, amplification reactions were followed by a melting curve analysis (fluorescence was read at each 0.5°C increment from 70 to 95°C to record the dissociation point), ensuring amplicon fidelity.

For all viruses, samples with Ct values ≤ 34 were considered positive. To confirm the identity of the PCR products, subsamples of PCR-positive samples were sequenced. Samples generating Ct values between 34 and 36 were considered doubtful and their positive or negative status was determined after evaluating a random subset of samples either separated on a 1.5% agarose gel stained with SYBR Safe DNA Gel Stain or sequenced by Sanger sequencing and searched for sequence similarity against GenBank viral database. Samples generating Ct values > 36 were considered negative.

Conventional RT-PCR for the MNV-1 extraction control, BQCV, and confirmative RT-PCR for selected samples used the OneStep RT-PCR Kit (Qiagen). Primers and RT-PCR conditions for MNV-1 were as described by Kingsley (2). Following 35 cycles of RT-PCR amplification, 10 μL of the RT-PCR reaction was separated on agarose gel. In the case of BQCV, samples were analyzed using a QIAxcel instrument (Qiagen) following the manufacturer's recommendations. All samples spiked with 10^3 MNV-1 as an extraction control generated a DNA fragment of the expected size and were confirmed by sequencing.

For DWV, the primer pair designed by Li *et al* (3) was used. The RT-PCR conditions were: 30 min at 50°C; 15 min at 94°C, followed by 40 cycles at 94°C for 15 s; 58°C for 15 s; and 72°C for 15 s.

For the closely related ABPV, KBV, and IAPV, a "universal" primer pair (AKI) located in a highly conserved region of the genome was used as described by Francis and Kryger (4). The RT-PCR conditions were: 30 min at 50°C; 15 min at 94°C, followed by 40 cycles at 94°C for 15 s; 58°C for 15 s; and 72°C for 15 s.

Positive and doubtful samples from each positive apiary were reamplified using one or more of the following systems. Primers for KBV and ABPV, as designed by Tentcheva *et al* (5), were used as described by the authors, in addition to primers for IAPV, as designed by Di Prisco *et al* (6). If one or more of these 3 RT-PCR systems produced a band of the appropriate size (400 to 600 bp) on gel agarose, the sample was considered positive and the amplicon was sequenced for confirmation.

For black queen cell virus, the method designed by Benjeddou *et al* (7) was used. The RT-PCR conditions were: 30 min at 50°C; 15 min at 94°C, followed by 35 cycles at 94°C for 15 s; 58°C for 30 s; and 72°C for 30 s.

PCR protocol for *Apocephalus borealis* detection

Extraction was done using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions. Primer sequences were 'ATTCAACCAATCATAAAGATAT' for LEP-F1, 'TAAACTTCTGGATGTCCAAAAA' for LEP-R1, 'GGTCAACAAAT CATAAAGATATTGG' for LCO1490, and 'TAAACTTCAGGGTGA CCAAAAAATCA' for HCO2198.

Expected fragment lengths were 648 bp for LEP-F1/LEP-R1 and 710 bp for LCO/HCO. Samples were processed in a thermocycler according to the following program: 94°C for 1 min; 5 cycles of 94°C for 40 s, 45°C for 40 s, and 72°C for 60 s; 35 cycles of 94°C for 40 s, 51°C for 40 s, and 72°C for 60 s; and 72°C for 10 min.

The results were visualized on agarose gel electrophoresis. Amplicons were sequenced to obtain a chromatogram of the genes of interest, using the services of the Genome Sequencing and Genotyping Platform at the Centre Hospitalier Universitaire de Québec. Each sample comprised 2 reactions (sequences) for a pair of primers (5′ to 3′ reaction and 3′ to 5′ reaction). To clean and process the sequences, the software Geneious R8 (8.0.5) was used. The sequences obtained were compared with the reference databases NCBI and BOLD.

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