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Distribution and Quantification of Infectious and Parasitic Agents in Managed Honeybees in Central Italy, the Republic of Kosovo, and Albania

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Abstract

This study, conducted jointly by diagnostic institutes from Italy and the Republic of Kosovo/Albania, aimed to determine the occurrence of relevant infectious and parasitic agents (IPAs) in managed honeybees from these geographical areas to assess the overall health status of local apiaries. *Paenibacillus larvae*, *Melissococcus plutonius*, *Nosema ceranae*, *Nosema apis*, acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus variants DWV-A and DWV-B, and the parasitoid flies *Megaselia scalaris* and *Senotainia tricuspis* were detected by quantitative polymerase chain reaction (qPCR) and reverse transcriptase qPCR (RT-qPCR) in clinically healthy adult honeybees collected from 131 and 56 apiaries in the Abruzzo and Molise regions of Central Italy in 2022 and 2023, respectively, from 140 apiaries in the Republic of Kosovo and 18 in Albania in 2022, and from 66 apiaries in the Republic of Kosovo in 2023. The percentages of positive samples and abundance levels for *N. ceranae*, *P. larvae* and DWV-B were significantly higher in the Republic of Kosovo and Albania, while the percentages of samples positive for *M. plutonius*, CBPV, DWV-A, and the parasitoid flies were higher in Central Italy. Additionally, *P. larvae* and some viruses showed significantly different occurrence rates between the two years in Italy and the Republic of Kosovo. The co-occurrence of IPAs also differed between the two geographic areas. Their varying distribution could depend on epidemiological dynamics, climatic factors, and management practices specific to each country, whose relative impact should be defined to guide targeted interventions to reduce honeybee mortality.

Keywords: infectious and parasitic agents; managed honeybees; regional screening; distribution; contamination levels; co-occurrence

1. Introduction

Honeybee health is threatened by diseases caused by infectious and parasitic agents (IPAs), which can lead to colony loss either directly, due to their high pathogenicity, or indirectly by weakening the colony and making it more susceptible to the harmful effects of concurrent infections, diseases, and environmental stresses [1]. According to European legislation, only certain honeybee diseases are subject to surveillance and are assigned to different categories of required prevention measures. Specifically, *Varroa destructor* is assigned to category C, meaning measures are needed to prevent its spread to parts of the Union that are officially disease-free or have eradication programs for this disease. *Tropilaelaps* spp., *Aethina tumida*, and American foulbrood caused by *Paenibacillus larvae* spores are assigned to category D, meaning measures are needed to prevent their spread due to entry into the Union or movement between Member States. All these IPAs are also included in category E, which requires their surveillance within the Union. Eradication is not mandatory for any honeybee disease agent listed above under EU regulation, but it can be adopted by Member States in emergency situations [2].

All other diseases caused by honeybee IPAs are not included in national surveillance plans and are often diagnosed only on a voluntary basis. These IPAs include the Gram-positive bacterium *Melissococcus plutonius*, which causes European foulbrood (EFB), which is not notifiable in all countries, viral diseases, *Nosema apis* and *N. ceranae*, and the parasitoid flies *Megaselia scalaris* and *Senotainia tricuspis*. Nevertheless, these agents can significantly impact the survival of honeybee colonies [3–10], to the extent that their proactive control was requested more than a decade ago by the European Commission to prevent the decline of pollinating honeybee populations [11].

Although authoritative veterinary guidelines still recommend diagnosing honeybee disease agents only after clinical symptoms appear [12], proactive screening of IPAs in clinically healthy hives could help identify the risk of disease development and allow timely intervention to limit or prevent disease manifestation and spread by applying appropriate countermeasures [13].

This study addresses the diagnosis and definition of IPAs infection levels affecting honeybees during the spring and summer of 2022 and 2023 in the Abruzzo and Molise regions of Central Italy, the Republic of Kosovo, and, to a lesser extent, four provinces of Albania. It was conducted jointly by the responsible institutes in these countries. The aim was to provide an overview of the pathogens and parasites threatening managed honeybees in these geographical areas. Abruzzo and Molise are located at nearly the same latitudes as the Republic of Kosovo and Albania but differ in distance from the coast and apiary density. In 2023, Abruzzo and Molise (Italy) had 84,576 hives, a number similar to 2022 [14], while the Republic of Kosovo had 208,898 hives [15], and Albania had 519,000 hives (https://www.instat.gov.al/en/themes/agriculture-and-fishery/livestock/#tab2/, accessed on 10 June 2025). Additionally, there are differences in regulations regarding the beekeeping sector in Italy compared to the Republic of Kosovo and Albania. In the Republic of Kosovo, the government provides financial compensation to beekeepers for each hive lost to AFB and EFB [16]. Monitoring over two consecutive years was conducted in the Abruzzo and Molise regions (Italy) and the Republic of Kosovo, while preliminary data for the Albanian regions were obtained only in 2022. The pathogens considered were the bacteria *P. larvae* and *M. plutonius*, *N. ceranae* and *N. apis*, acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing paralysis virus variants A (DWV-A) and B and (DWV-B) and the parasitoid flies *M. scalaris* and *S. tricuspis*. In a previous study, these flies were detected in the geographic areas examined [10], so they were also included in this investigation. Adult honeybees were chosen for IPAs detection because their suitability for the surveillance of all the pathogens and parasites considered was demonstrated [10,12,17–19].

2. Materials and Methods

2.1. Sample Collection and Storage

Sampling was conducted from May to early July in 2022 and 2023 in all provinces of the Abruzzo and Molise regions, Italy, and in five provinces of the Republic of Kosovo. In Albania, samples were collected only in 2022 in four provinces. Approximately 40 honeybees were collected from the top of each hive by dragging a Falcon tube (Merck, KGaA, Darmstadt, Germany) along the frame edges while using a bee smoker. The insects were immediately refrigerated and transported within 48 hours to the laboratory, where they were divided into aliquots for DNA and RNA extraction. The aliquots not used promptly for analysis were immediately frozen at -80 °C. Samples collected in the Republic of Kosovo and Albania were placed in RNAlater solution (Thermo Fisher Scientific, Rodano, Italy) according to the manufacturer's instructions before being transported at room temperature to the

Campobasso branch of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM), Italy, for molecular analyses.

2.2. Nucleic Acids Extraction

DNA was extracted from two bees in the same tube using the "small-scale" method previously described [10] to detect bacteria and microsporidia, with the modification that 400 µL of T1 buffer (Carlo Erba, Cornaredo, MI, Italy), instead of 200 µL, was added to the sample before mechanical lysis. For the detection of *M. scalaris* and *S. tricuspis*, the large-scale method previously described [10] was applied to 20 bees. A DNA extraction control consisting of homogenized *T. molitor*, kindly provided by Dr Sauro Simoni (CREA - Consiglio per la Ricerca in Agricoltura – Difesa e Certificazione, Firenze, Italy), was added to the sample before extraction as described by Rossi et al. [10]. RNA extraction was carried out from two honeybees as previously described, using *T. molitor* as the RNA extraction control [22]. Positivity at the apiary level was defined based on the results obtained from the analyzed honeybee samples.

2.3. Quantitative PCR and RT-qPCR

A quantitative polymerase chain reaction (qPCR) or reverse transcriptase qPCR (RT-qPCR) was performed to determine the abundance of each pathogen in the samples using the extracted DNA or RNA, following previously described assays [10,17,22,23]. The *P. larvae*-specific qPCR protocol was based on Rossi et al. [24], with the modification of replacing Sybr Green with the Cy5-TGTAAGACCGGGATAACTT-MGBEQ probe at a 0.2 µM concentration. In the qPCR, DNA extracted from the bacterial type strain *P. larvae* ATCC 9545 was used as the positive control for *P. larvae*, while recombinant plasmids containing the pathogen-specific DNA fragment, supplied by GenScript Biotech (Rijswijk, Netherlands), were used for *M. plutonius*, *N. apis*, *N. ceranae*, *M. scalaris*, and *S. tricuspis* [10,17,23,24]. For viral pathogens, RT-qPCR positive controls consisted of synthetic RNA fragments corresponding to the specific target region of the investigated viruses (GenScript Biotech) [22]. Negative process controls (400 µL of nuclease-free water in place of the bee sample) and negative amplification controls (nuclease-free water in place of DNA or RNA) were included in each extraction and amplification run. The qPCR and RT-qPCR reactions for each pathogen were performed using a QuantStudio 5 instrument (Thermo Fisher Scientific) as previously described [10,17,22–24].

The quantification of viral pathogens in honeybee samples was performed as described by Rossi et al. [22], while *M. plutonius*, *N. apis*, *N. ceranae*, and *P. larvae* were quantified using standard curves constructed in this study, with their linearity range shown in Figure S1. The copy number of IPAs in samples with Ct values outside the linearity range was estimated by comparison with honeybee samples spiked in triplicate with copy numbers of pathogen-specific recombinant plasmids or cells external to the linearity range. For *M. scalaris* and *S. tricuspis*, calibration curves were not constructed because their levels in adult honeybees were previously found to be close to or below the LOD [10]. Therefore, approximate quantification was achieved by comparison with honeybee samples spiked in triplicate with 2 and 3 log copy numbers of the fly-specific recombinant plasmids. For *M. plutonius* and the microsporidia, the standards consisted of DNA extracts from honeybee samples spiked in triplicate with 10-fold serial dilutions from 1×10^8 to 1×10 copies of the pathogen-specific recombinant plasmids.

DNA extracts from honeybee samples inoculated in triplicate with 10-fold serial dilutions ranging from 1×10^8 to 1×10 CFU of *P. larvae* ATCC 9545 cell suspension were used as standards for *P. larvae* quantification. The *P. larvae* cell suspension was prepared by streaking a single colony of *P. larvae* ATCC 9545 on *Paenibacillus larvae* agar (PLA) [25], incubating at 37 °C for 2–5 days in a 9% CO_2 atmosphere incubator, and resuspending the colonies grown on the entire plate in 2 ml of sterile saline. DNA standards with known CFU numbers were prepared from serial decimal dilutions of this suspension, for which the number of CFU/ml was determined on PLA medium.

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The honeybee samples used for constructing all standard curves had previously been tested in triplicate by qPCR to verify the absence of IPAs.

2.4. Statistical Analyses

Principal Component Analysis (PCA), Pearson's correlation analysis, and graphical representations were performed using Past 4.17 software (https://www.nhm.uio.no/english/research/resources/past/). The data used in the PCA consisted of the ratios of positive samples to total samples for each pathogen in each province. The Student's t-test was conducted with Microsoft Excel 2016.

3. Results

In 2022, 131 honeybee samples were collected in the regions of Abruzzo and Molise (Italy), 140 in the Republic of Kosovo, and 18 in Albania. In 2023, 56 samples were collected in Abruzzo and Molise, and 66 in the Republic of Kosovo. In 2023, sampling was limited to one apiary representative for nearby sites that had shown identical pathogen presence/absence profiles in 2022. Samples examined for all IPAs except parasitoid flies consisted of two adult honeybees from one hive per apiary, rather than pooled samples, as higher sensitivity had previously been observed for non-pooled samples [20,21]. Table S1 shows the co-infection profiles observed by year and geographic area. No samples were free of IPAs, and the number of co-occurring pathogens or parasitic agents ranged from two to ten in Italy in 2022, four to eight in Italy in 2023, six to seven in Albania in 2022, and three to eight in the Republic of Kosovo in both years. Table 1 shows the distribution of pathogens and parasites observed in the different provinces or districts over the two years. *N. apis* was not detected in any sample, and *M. plutonius*, *M. scalaris*, and *S. tricuspis* were not detected in Albania.

Table 1. Number of positive samples for the honeybee IPAs investigated in this study in the provinces of the Abruzzo and Molise regions (Italy) and in the districts of the Republic of Kosovo and Albania.

Province/ district	Year	N. samp	*N. apis*	*N. ceran*	*M. pluto*	*P.*	ABPV	BQCV	CBPV	DWV-A	DWV-B	SBV	*M. scala*	*S. tricus*
				Abruzzo and Molise regions (Italy)										
				N. positive samples										
Campobasso	2022	26	0	7	10	6	3	21	11	15	9	11	7	4
	2023	10	0	2	5	3	1	10	9	8	8	3	4	2
Chieti	2022	26	0	6	6	1	1	21	16	22	17	12	8	11
	2023	10	0	1	3	3	0	10	8	10	4	6	6	1
Isernia	2022	18	0	8	8	6	3	16	10	9	11	7	5	2
	2023	7	0	0	4	7	0	7	6	7	3	5	2	0
L'Aquila	2022	25	0	4	18	15	2	23	16	15	4	17	14	4
	2023	10	0	1	4	1	0	9	7	9	5	8	5	2
Pescara	2022	17	0	11	3	1	2	16	11	12	12	7	2	4
	2023	8	0	0	1	6	0	8	8	6	5	5	2	0
Teramo	2022	19	0	5	9	2	4	19	3	7	2	3	4	2
	2023	11	0	2	4	7	3	11	8	11	8	6	1	1
				Republic of Kosovo										
				N. positive samples										
Gjakova	2022	45	0	41	2	32	07	36	17	27	38	28	6	2
	2023	19	0	14	0	16	30	18	3	11	16	12	0	0
Gjilane	2022	13	0	13	0	6	6	12	3	9	8	9	2	0
	2023	11	0	7	0	9	3	11	2	8	8	6	0	0
Mitrovica	2022	13	0	12	1	12	1	13	5	9	12	7	1	0
	2023	4	0	3	1	4	1	4	1	1	4	2	0	1
Peja	2022	29	0	24	1	18	4	23	13	16	26	16	5	0

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	2023	9	0	8	1	9	3	9	1	6	7	6	0	0
Pristina	2022	14	0	14	0	7	5	13	8	7	11	10	2	0
	2023	12	0	12	0	12	2	12	3	7	10	8	0	0
Prizren	2022	26	0	21	2	21	5	24	6	12	21	11	0	1
	2023	11	0	10	0	11	1	10	3	7	10	9	0	1
Albania														
N. positive samples														
Kukës	2022	4	0	3	0	2	1	4	3	4	4	4	0	0
Berat	2022	4	0	4	0	3	0	4	4	3	4	4	0	0
Tiranë	2022	4	0	4	0	4	0	4	4	4	4	4	0	0
Korçë	2022	6	0	6	0	6	0	6	5	6	6	6	0	0

The results of a principal component analysis (PCA), based on the ratios of positive samples to total samples for each pathogen in each province, are shown in Figure 1.

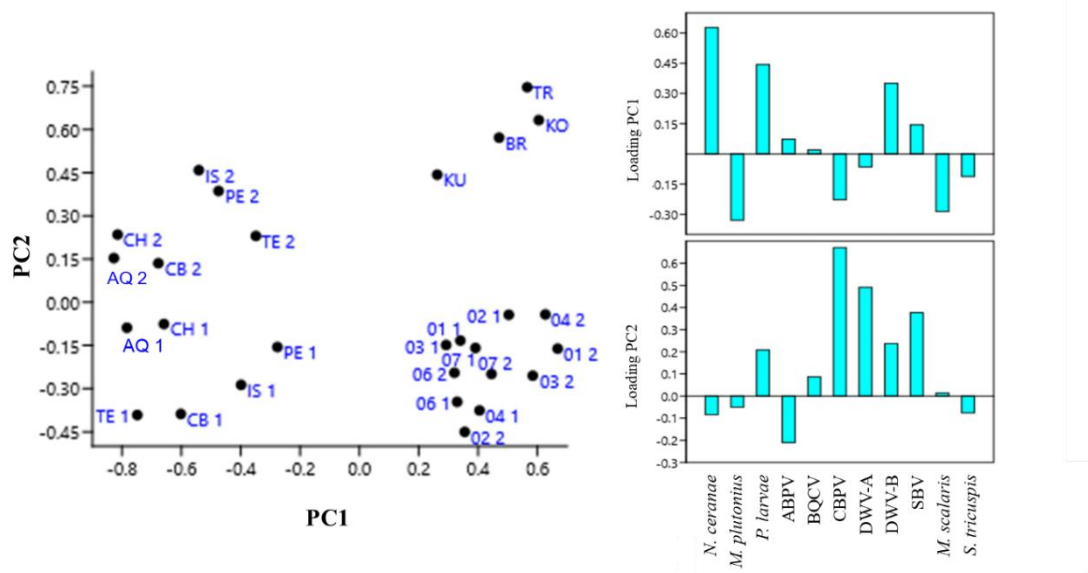

Figure 1. PCA of the ratios of positive samples to total honeybee samples for IPAs in the regions of Abruzzo and Molise (Italy) and in various provinces of the Republic of Kosovo and Albania in 2022 (1) and 2023 (2). AQ, L'Aquila; CB, Campobasso; CH, Chieti; IS, Isernia; PE, Pescara; TE, Teramo; 01, Pristina; 02, Mitrovica; 03, Peja; 04, Prizren; 06, Gjilane; 07, Gjakova; BR, Berat; KU, Kukës; TR, Tiranë; KO, Korçë.

PCA analysis showed that the occurrence of *N. ceranae*, *P. larvae*, and DWV-B mainly influenced sample distribution on PC1, while CBPV, DWV-A, and SBV, in that order, were the main variables influencing sample distribution on PC2. Samples from the Abruzzo and Molise regions formed distinct clusters along PC2 according to the sampling year, suggesting that one or more variables influencing PC2 changed between the two sampling periods. Conversely, samples from the Republic of Kosovo collected in the two years were intermixed along PC1 and PC2, indicating an unchanged situation in the variables that most influence the PCs between the two sampling periods. Furthermore, samples from the Italian regions for 2022 and those from the Republic of Kosovo were not separated along PC2, indicating that they did not differ significantly for the variables with positive loadings on this component. Samples from Albania were separate from those collected in the Republic of Kosovo on PC2, while they were not separate on PC1, indicating that the variance in the presence of *N. ceranae*, *P. larvae*, and DWV-B did not differ between the two neighboring countries.

Since samples from the two geographic areas clustered closely in the PCA, the percentages of positive samples for each pathogen in the two years for the Italian regions and the Republic of Kosovo

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were determined based on the total number of samples from each country, not for each province or district (Figure 2). Samples from Albania were not included due to their low number.

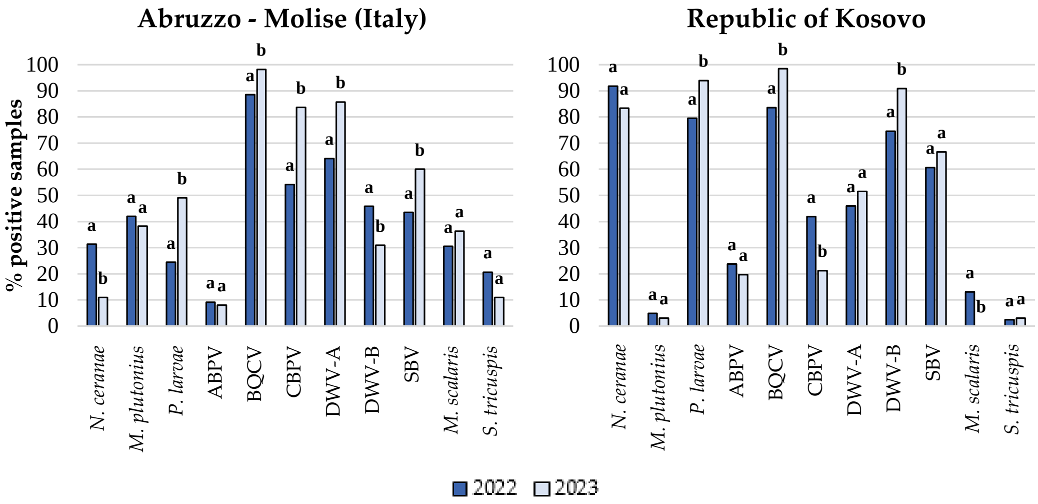

Figure 2. Percentages of apiaries positive for honeybee IPAs in 2022 and 2023 in the Abruzzo and Molise regions (Italy) and in the Republic of Kosovo. Different letters on the bars indicate significantly different infection or infestation rates between the two sampling periods ($p<0.05$).

Based on the Student's t test, a significant increase in the number of samples positive for *P. larvae*, BQCV, CBPV, DWV-A, and SBV was observed in 2023 in the Italian regions, while the detection frequency for *N. ceranae* and DWV-B significantly decreased (Figure 2). In 2023 in the Republic of Kosovo, the number of samples positive for *P. larvae*, BQCV and DWV-B significantly increased, while those positive for CBPV and *M. scalaris* significantly decreased. *N. ceranae*, *P. larvae* and DWV-B were detected more frequently in the Republic of Kosovo in both years, whereas *M. plutonius* was more frequently detected in the Italian regions, with a much lower occurrence in the Republic of Kosovo. CBPV, DWV-A, and the parasitoid flies occurred more frequently in Italy, while DWV-B was more frequently detected in the Republic of Kosovo.

The quantification of IPAs, determined by qPCR and RT-qPCR in Italy and the Republic of Kosovo, is shown in Figure 3 along with the respective percentages of positive samples. Samples from Albania are presented separately, showing the number of samples per quantification level, but only for the IPAs detected in that country (Figure 4).

A striking difference between the geographic areas was observed in the abundance of *N. ceranae* and *P. larvae*. In some samples collected in the Republic of Kosovo, *N. ceranae* exceeded 8 log n. copies/g and *P. larvae* exceeded 7 log CFU/g in 2022, while in 2023, *N. ceranae* exceeded 7 log n. copies/g and *P. larvae* exceeded 8 log CFU/g. In contrast, in the Italian regions, *P. larvae* reached a maximum of 5 to 6 log CFU/g in both years and *N. ceranae* reached a maximum of 3 to 4 log n. copies/g in 2022 and less than 2 log n. copies/g in 2023.

M. plutonius reached levels between 6 and 7 log n. copies/g in a few Italian samples in 2022, while all samples collected in 2023 showed a maximum of 3 log n. copies/g, indicating a decrease in its abundance. This pathogen was always below 2 log n. copies/g in samples from the Republic of Kosovo in both years.

The BQCV was present at viral loads higher than 8 log n. copies/g in some samples from both geographic areas in both years, but in the Italian regions, the percentage of samples with low viral loads was higher. The DWV-A and DWV-B reached the highest viral loads, above 8 log n. copies/g, in samples from both geographic areas in 2022, while in 2023, the DWV-A and DWV-B reached maximum levels between 6 and 7 log n. copies/g in Italy. The percentage of samples with the highest levels of DWV-B was higher in the Republic of Kosovo in both years.

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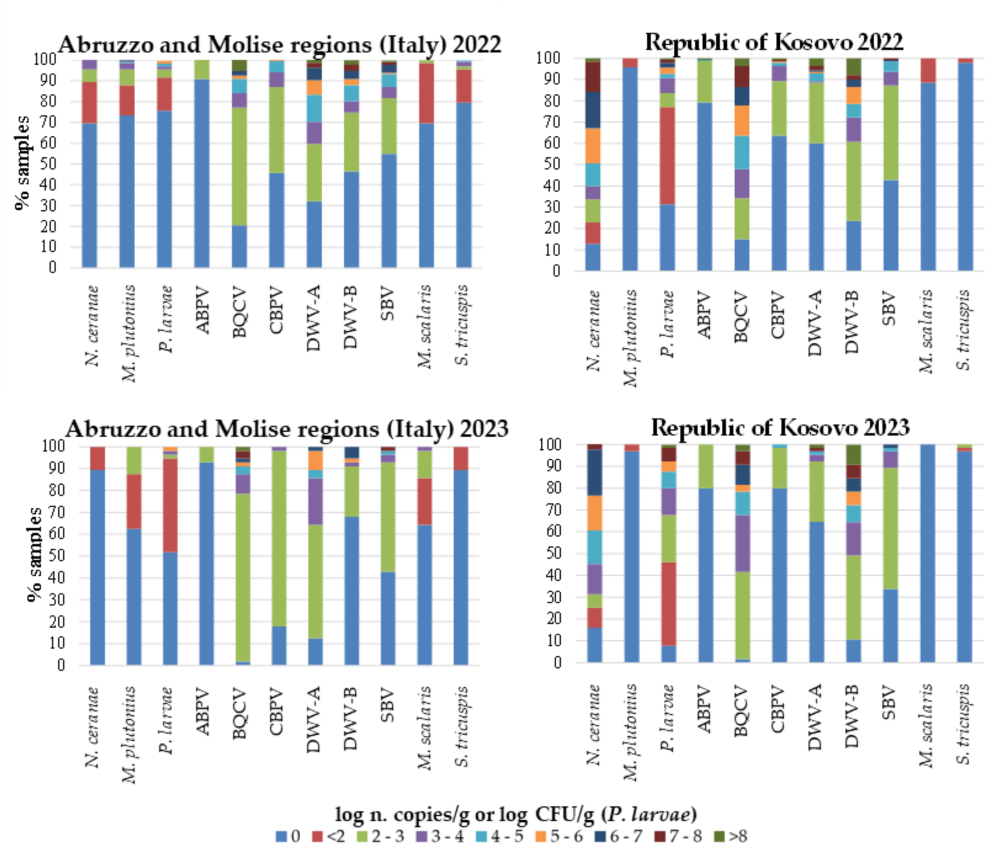

Figure 3. Quantification of honeybee IPAs determined by qPCR and RT-qPCR, and percentages of samples per range of pathogen loads in 2022 and 2023 in the Abruzzo and Molise regions (Italy) and in the Republic of Kosovo. For viruses, levels below 2 log n. copies/g were not considered, as they are below the limit of detection of the methods used [22].

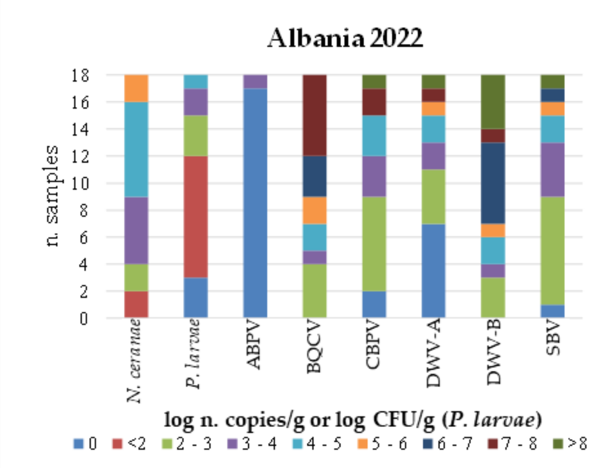

Figure 4. Distribution of honeybee samples collected in Albania in 2022 based on the quantification of each detected IPA.

The percentage of samples positive for the parasitoid flies was higher in Italy in both years. However, in 2023, an increase in *S. tricuspis* abundance to above 2 log n. copies/g was observed in one sample from the Republic of Kosovo.

Although only a few samples from Albania were examined, some differences in IPA abundance were observed compared to samples from the Republic of Kosovo. Specifically, the abundance of *N. ceranae* and *P. larvae* was lower in Albania, not exceeding 6 log n. copies/g and 5 log CFU/g, respectively. In contrast, the abundance of CBPV and SBV was higher in Albania, with both reaching loads above 8 log n. copies/g. Furthermore, the proportion of DWV-B positive samples exceeding 4 log copies/g was higher in Albania (Figure 4).

A correlation analysis based on the presence or absence of the IPAs was conducted to identify possible co-occurrence trends in Italy and the Republic of Kosovo, as shown in the graphical representation in Figure 5.

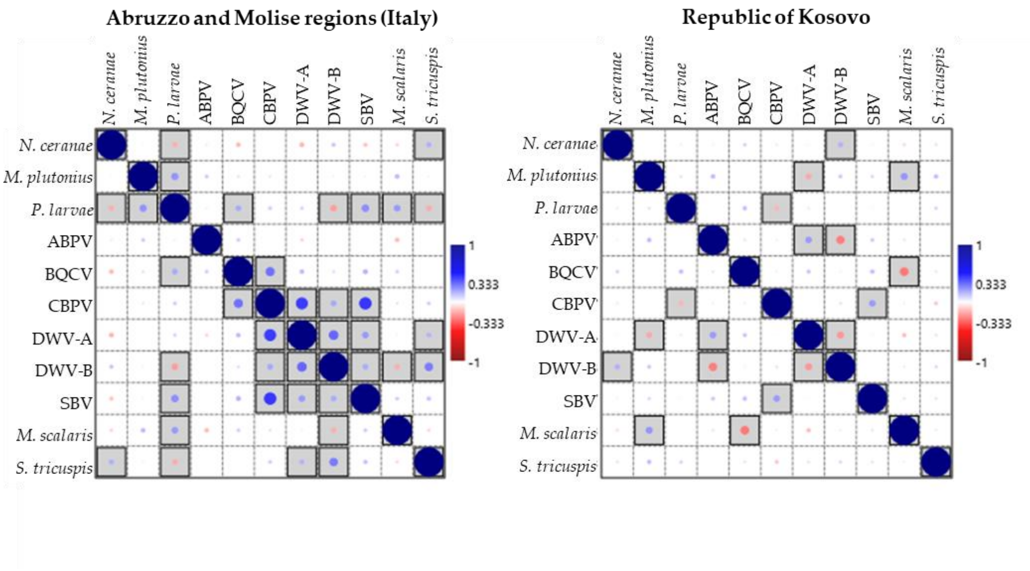

Figure 5. Pearson's correlation analysis of the presence or absence of honeybee IPAs in the Abruzzo and Molise regions (Italy) and the Republic of Kosovo. Bordered boxes indicate significance at P<0.05.

The significant correlations observed for the two geographic areas differed in most cases, except for the positive correlation between CBPV and SBV. Additionally, a positive correlation was observed between *M. scalaris* and *M. plutonius* as well as between the DWV-B and *N. ceranae*. While this correlation was not statistically significant for the Italian regions, it was significant for the Republic of Kosovo. The BQCV and *P. larvae* were positively correlated in both countries, but this correlation was not statistically significant for the Republic of Kosovo. Similarly, *P. larvae* and *M. plutonius* were negatively correlated in both countries, but the correlation was not significant for the Republic of Kosovo. In the Italian regions, significant positive correlations were observed for DWV-A with CBPV and DWV-B; for *S. tricuspis* with DWV-B, CPBV and BQCV; for SBV with *P. larvae* and both DWV variants; for *M. scalaris* and *P. larvae*; and for *S. tricuspis* and *N. ceranae*. Significant negative correlations (P<0.05) were observed for *P. larvae* with *N. ceranae* and DWV-B and between DWV-B, and *M. scalaris*. For the Republic of Kosovo, a significant positive correlation was observed between DWV-A and ABPV, while ABPV was negatively correlated with DWV-B, BQCV and *M. scalaris*, CBPV was negatively correlated with *P. larvae*, and DWV-A was negatively correlated with DWV-B and *M. plutonius* (Figure 5).

4. Discussion

This study, which focused on detecting and quantifying multiple honeybee IPAs by qPCR and RT-qPCR in relatively small geographic areas, can be compared with a similar investigation conducted in 2016 and 2017 around Stuttgart, Germany, where the same techniques were applied to individual honeybees [27]. In that study, as in this one, among 1,064 honeybee samples from colonies without clinical or pathological symptoms, none were free from pathogens (Table S1). Similar results were obtained for the occurrence of BQCV, but the other viruses were less prevalent than in this study, except for ABPV in Italy and DWV-B in the Republic of Kosovo. The occurrence of *N. ceranae* was similar to that in the Republic of Kosovo and Albania. *M. plutonius* showed an intermediate

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occurrence rate between Italy and the Republic of Kosovo, and *P. larvae* had a similar detection rate as in Italy. The reported differences indicate that the distribution of biotic risk for honeybees is not uniform, as also shown by the PCA conducted in this study, which revealed a distinction between the study periods. This observation suggests that periodic screening studies of local pathogens may be useful for early detection of disease threats, thereby improving honeybee health and productivity.

The high prevalence of the pathogens *P. larvae* and *N. ceranae* in the Republic of Kosovo could be a consequence of the hive compensation policy for honeybee colonies lost to AFB and EFB [27]. This policy likely results in reduced infection control by some beekeepers, facilitating the intense circulation of contagious agents, which is further aggravated by the high density of apiaries [15]. This undermines the efforts of conscientious beekeepers to maintain high levels of hive hygiene. The high prevalence of *N. ceranae* in the Republic of Kosovo is consistent with previous results from passive surveillance of weak honeybee colonies, which showed an infection rate of 100% [28]. Additionally, the results of recent interviews conducted with 200 beekeepers in the Republic of Kosovo confirm that the lack of adoption of good beekeeping practices is perceived as one of the main factors affecting honeybee colony health and apiculture productivity in the country, along with climate change, land use, and pesticide exposure [29]. Therefore, it is possible that a change in legislation regarding AFB control in the Republic of Kosovo could promote the adoption of good beekeeping practices and, in general, contribute to a reduction in IPA loads.

The relatively low frequency and levels of infection observed for *N. ceranae* and *P. larvae* in the Italian regions indicate effective control of these pathogens through adherence to good beekeeping practices. Moreover, plant-based feed supplements containing natural antimicrobials may be used to counter *N. ceranae* infections [30]. However, other studies conducted in Italy have reported a high frequency of detection of this parasite, suggesting the need to continue monitoring its presence [31,32].

Compared to a previous survey in the Abruzzo and Molise regions conducted in 2018 [33], the detection rate of *P. larvae* decreased in 2022 and then increased again in 2023, showing a fluctuating trend for this pathogen whose causes should be investigated. The most probable reason for its intermittent increase could be the varying levels of virulence among circulating strains. This should be confirmed by strain isolation and whole genome sequencing (WGS) to identify virulence traits and sources of infection for sequence variants, in order to improve AFB prevention strategies.

The frequent occurrence of *M. plutonius* in Italy may reflect the epidemiological dynamics of this highly destructive brood pathogen, its dependence on climatic conditions, and the existence of transmission routes specific to the region. Previous studies have shown that the distribution of *M. plutonius* is influenced by climatic factors such as precipitation and temperature [34]. In the Republic of Kosovo, *M. plutonius* was positively correlated with parasitoid flies, which also had low occurrence in that region. Therefore, the possible involvement of these or other insects in its spread in apiaries should be investigated. In a previous study [10], these flies were found to host microbial honeybee pathogens, so the possibility that they also propagate *M. plutonius* should be explored. Climatic factors are most likely responsible for the higher incidence of parasitoid flies in Italy compared to the Republic of Kosovo, according to predictive models [35,36], and this could contribute to the spread of *M. plutonius* in Italy. The detection of *M. plutonius*, *M. scalaris*, and *S. tricuspis* in Albania might have been missed because of the small number of samples examined. Therefore, their presence in the country cannot be excluded, though it is likely low.

According to the most recent data on the prevalence of *M. plutonius* in Europe, it reached a 46% detection frequency in areas with high apiary density [37]. Additionally, a high prevalence of *M. plutonius* was reported in Michigan (US) in 2021 and 2022, where it varied seasonally with a peak in June, and different genetic variants were identified within the same beekeeping operation [38]. A high occurrence rate (32.4%) of different strains of this pathogen, with variability among sites, was also observed for *A. cerana* in Guangxi (China) and stingless bees in Brazil [39,40]. Therefore, this pathogen represents a significant threat to the productivity of the apicultural sector in different countries, the causes of which should be defined and appropriately addressed. A four-gene

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multilocus sequence typing (MLST) scheme, used to discriminate the clonal complexes with a higher probability of causing EFB, and the detection of the plasmid-encoded *mtx*A gene for melissotoxin A production [41], could be applied to define the virulence of the genotypes infecting hives and to differentiate interventions based on the pathogenicity of the detected genotype.

All honeybee viruses, except the BQCV, were reported to be associated with the *V. destructor* mite, both by experimental demonstration [28,42] and by statistical inference [43]. Consequently, their different distribution most likely reflects the prevalence of this parasite and its viral loads. The common source of infection can explain the multiple positive correlations in occurrence patterns found in this study for viruses in Italy, which indicate a high probability of co-infection. The impact of viral co-infections on overall hive health should be more clearly defined, taking into account both the identity and viral load of co-occurring viruses.

A different occurrence rate of the DWV-A and DWV-B was observed in this study between the two geographic areas examined, and the higher occurrence of DWV-A in Italy did not align with the general increasing trend of DWV-B over DWV-A reported in other countries [43–45]. The frequency of DWV-A detection was also slightly higher than that of DWV-B in the Veneto, Campania, and Sicily regions of Italy, according to recent studies [31,45]. Additionally, DWV-A was recently reported as the most abundant DWV variant in Michigan [46], indicating that specific variants of the DWV-A may outcompete DWV-B in different geographic locations. The use of a recently described amplicon sequencing strategy that links specific amplicon sequence variants (ASVs) to virulence [47] could experimentally verify this hypothesis.

The results on the presence of honeybee viruses in the Republic of Kosovo partly agree with previous findings from a study of 13 apiaries, where the DWV and BQCV were most frequently detected, followed by the ABPV, SBV, and CBPV in that order [28]. This study showed a lower occurrence of the ABPV and a higher occurrence of the CBPV and SBV, but the observed differences likely result from examining clinically healthy honeybee colonies, unlike the previous study, which involved hives suspected of disease.

For the Italian regions, detection results for the BQCV, DWV, and CBPV viruses were consistent with previous investigations conducted in other areas of Italy. A maximum infection rate of 84% for the CBPV in 2021–2023, similar to that found in this study in 2023, was previously reported [32,48]. The ABPV showed similar prevalence and low abundance in the Veneto and Piemonte regions [32,49], while in the present study its detection frequency was even lower, probably because the sampling period did not coincide with the peak prevalence of this pathogen, which occurs in late summer or autumn [50]. For the SBV, a study conducted in the Piemonte region during 2017–2020 is available for comparison and reported higher infection rates than those found in the present study [49].

5. Conclusions

This investigation showed that the distribution of honeybee IPAs can vary even between neighboring countries and that threats to apiary health and apiculture productivity require specific countermeasures based on those most frequently occurring in a given area. Regular local monitoring of IPAs and defining contamination trends can enable timely interventions. Moreover, the use of rapid, sensitive, and specific detection methods, even in the absence of clinical signs, is crucial for disease prevention.

This study also showed that regulation of the beekeeping sector in the Republic of Kosovo needs substantial revision to encourage the adoption of good management practices aimed at reducing the occurrence and abundance of *P. larvae* and *N. ceranae*, which significantly compromise productivity and help prevent their spread to neighboring countries.

A limitation of this study is the sampling of only a single hive per apiary and the analysis of only two bees, except for parasitoid flies, from each hive. This may have led to an underestimation of the actual occurrence rates of IPAs. Future investigations will define the optimal number of hives to be sampled at the apiary level and the optimal number of replicate samples per hive to exclude the presence of IPAs with certainty.

Another limitation was not identifying IPAs with high genotype resolution to explain virulence and spread dynamics. In particular, DWV-A variants, *M. plutonius*, and *P. larvae* strains circulating in Italy should be characterized by WGS to identify the specific physiological traits that determine their selective success.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Calibration curves constructed in this study to quantify IPAs in honeybee samples with DNA extracted from honeybee samples negative for these pathogens and inoculated in three biological replicates with serial decimal dilutions of plasmid pUC57 containing the target region of *N. apis*, *N. ceranae*, *M. plutonius* or with known numbers of *P. larvae* cells using previously reported qPCR methods [17,23,24]. Only the linearity ranges are shown; Table S1: IPAs co-infection profiles observed in honeybee samples from the Abruzzo and Molise regions (Italy), the Republic of Kosovo, and Albania in 2022 and 2023.

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Abbreviations

The following abbreviations are used in this manuscript:

ABPV	Acute bee paralysis virus
AFB	American foulbrood
ASV	Amplicon sequence variants
BQCV	Black queen cell virus
CBPV	Chronic bee paralysis virus
DWV-A	Deformed wings virus A
DWV-B	Deformed wings virus B
EFB	European foulbrood
IPA	Infectious or parasitic agent
LOD	Limit of detection
MLST	Multilocus sequence typing
PCA	Principal component analysis
PC1	Principal component 1
PC2	Principal component 2
qPCR	Quantitative PCR
RT-qPCR	Reverse transcriptase qPCR
SBV	Sacbrood virus
WGS	Whole genome sequencing

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