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Emergent and Known Honey Bee Pathogens through Passive Surveillance in the Republic of Kosovo

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Abstract: In recent years, honey bee colony losses in the Republic of Kosovo remained largely unknown. From 2019 to 2021, 81 apiaries with different disease suspicions were investigated in the framework of honey bee disease passive surveillance. Fifty-nine of the eighty-one apiaries were tested for *Vairimorpha ceranae*, *Vairimorpha apis*, trypanosomatids *Lotmaria passim*, and *Crithidia mellificae*. All samples were positive for *V. ceranae* (100%) whereas *L. passim* was found with a lower frequency (11.9%). *V. apis* and *C. mellificae* were not found. Thirteen of the eighty-one apiaries were tested for seven viruses (ABPV, CBPV, DWV, BQCV, SBV, IAPV, KBV) and five of them were found (ABPV, CBPV, DWV, BQCV, SBV). The most frequently detected viruses in honey bees and *Varroa* mites were DWV (100%) followed by BQCV, ABPV, SBV, and CBPV (92.3%, 69.2%, 30.8%, and 7.7%, respectively). *Varroa* mite samples had different degrees of co-infection by viruses. Nine of the eighty-one apiaries consisted of brood combs with larvae, eight of them were AFB positive, ERIC I genotype, and one EFB positive. This paper represents the first molecular investigation (PCR) and detection of the honey bee viruses ABPV, CBPV, DWV, BQCV, and SBV as well as *V. ceranae*, *L. passim*, and *M. plutonius* in the Republic of Kosovo.

Keywords: honey bee; *Vairimorpha* spp.; trypanosomatids; *Varroa destructor*; viruses; passive surveillance

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1. Introduction

Honey bee health has recently become a major topic due to the important role that bees play in pollination and food production [1]. In the last ten years, some regions of the world have suffered from a significant reduction in honey bee colonies [2]. It is believed that the reduction in honey bee populations is caused by a number of different biotic and abiotic factors, in particular pests, genetic factors, bee management, including beekeeping practices and breeding, climatic changes, malnutrition, agricultural practices, and the use of pesticides [2]. From emergent bee pathogens, the microsporidian species *Vairimorpha* (formerly *Nosema*) *apis* and *Vairimorpha* (formerly *Nosema*) *ceranae* [3] have been identified in European honey bees, *Apis mellifera*. *V. ceranae* is the most common gut pathogen in adult honey bees [4] and its infection could induce a degeneration of gut epithelial cells [5], a significant reduction in honey production [6], and a reduction in bee lifespan [7–10].

Two trypanosomatids *Lotmaria passim* and *Crithidia mellificae* (Kinetoplastea: Trypanosomatidae) are capable of colonizing the digestive tract of honey bees [11,12]. Both

pathogens are considered to alter bee physiology, behaviour, immune responses, and lifespan [13–16].

Several bee viruses, like acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Kashmir bee virus (KBV), Sacbrood virus (SBV), and Israeli acute paralysis virus (IAPV) have been documented to be transmitted and activated by *Varroa* mites in field conditions [17–27]. ABPV, BQCV, KBV, and IAPV belong to the *Dicistroviridae* family [28] while DWV and SBV are classified in the *Iflaviridae* family [28]. CBPV has not yet been classified into any taxa [29].

ABPV and IAPV cause trembling, inability to fly, rapidly progressing paralysis, and death of honey bees [30–32], whereas KBV in natural infections commonly persists within apparently healthy broods and adults [33–35]. CBPV causes massive worker bee losses, mostly in strong colonies, and its infection appears in two groups of clinical signs: one including inability to fly, clustering, trembling, and crawling; the other consisting of black hairless individuals with shortened abdomens [36]. BQCV and SBV are the most widely distributed of all honey bee viruses. BQCV does not cause visible symptoms in infected adult bees but it could lead queen larvae and pupae to death by turning their cells black. Two-day-old larvae appear to be most vulnerable to SBV infection and, once they are fed with contaminated larval food, they fail to pupate [37,38], acquiring a sac-like appearance. DWV causes deformed wings in honey bees and induces colony weakening and mortality. Typical disease symptoms of DWV infection include shrunken, crumpled wings, decreased body size, and discoloration in adult bees. Three genetic variants of DWV were discovered and identified as types A, B, and C, but DWV-A and -B are the most widespread variants [39,40].

Two bacterial pathogens affecting honey bee larvae but not adult bees are *Paenibacillus larvae*, the causative agent of American Foulbrood (AFB), and *Melissococcus plutonius*, responsible for European Foulbrood (EFB) [41]. The clinical symptoms of AFB are darkened, sunken, and perforated cell caps containing diseased larvae, a characteristic unpleasant odour, and sticky larval remains when drawn out with a matchstick. Instead, EFB usually affects young larvae that die while still coiled before they are capped. The younger larvae affected cover the bottom of the cell and are almost transparent, with a visible trachea and midgut; the latter, full of bacteria, appears as a yellow spot. Dead, flaccid discoloured larvae in uncapped cells show colour changes from pearly white to yellow to yellowish brown [42]. AFB and EFB are both widely distributed and potentially lethal to infected colonies [41–44].

According to the data from the Beekeepers' Association in the Republic of Kosovo, the beekeeping industry consists of around 135,750 honey bee colonies and 6453 beekeepers [45], with mild fluctuations over the years. In the last two years, there have been frequent reports from beekeepers about *Varroa destructor* mite infestation and increased requests for investigation due to colony losses supposedly caused by some honey bee pathogens, such as viruses, fungi, or other parasites, often positively correlated with *Varroa* infestation. Due to the lack of information about the presence of viral disease, we aimed to determine the presence and distribution of known and emergent honey bee pathogens such as ABPV, KBV, IAPV, DWV, BQCV, CBPV, SBV, *V. apis*, *V. ceranae*, *L. passim*, and *C. mellificae* in apiaries of the Republic of Kosovo based on reporting from beekeepers and veterinarians.

2. Materials and Methods

2.1. Sample Collection

From 2019 to 2021, based on complaints of beekeepers concerning weakened colonies, the presence of *Varroa* mites, honey bees with deformed wings, sac brood, and sticky larvae, sampling was performed on 89 apiaries suspected of disease by beekeepers and veterinarians and adult bee specimens, brood combs with larvae and pupae, as well as *Varroa* mites, were collected and sent to the Kosovo Food and Veterinary Agency, Veterinary Laboratory for analyses (Table 1, Figure S1).

Table 1. Municipalities involved in the passive surveillance, number of sampled apiaries per group, pathogens investigated, and results of laboratory investigations.

Municipality		Group 1			Group 2										Group 3		
		Vairimorpha spp. and Trypanosomatids			Honey Bee Viruses										American and European Foulbrood		
		N. of Sampled Apiaries	N. of Apiaries with Positive Results		N. of Sampled Apiaries	N. of Apiaries with Positive Results (Honey Bee Samples at Different Stages of Development)					N. of Sampled Apiaries	N. of Apiaries with Positive Results (*Varroa* Mite Samples)				N. of Sampled Apiaries	N. of Apiaries with Positive Results	
			Vairimorpha ceranae	*Lotmaria passim*		ABPV	CBPV	DWV	BQCV	SBV		ABPV	CBPV	DWV	BQCV		AFB	EFB
1	Suharekë	3	3	0	2	2	0	2	2	0	2	2	0	2	2	1	1	nt
2	Shtime	2	2	0	1	0	0	1	1	0	1	1	1	1	1	1	1	nt
3	Prishtinë	4	4	1	ns						ns					ns		
4	Deçan	14	14	3	ns						ns					1	nt	1
5	Kamenicë	4	4	0	1	1	0	1	1	1	ns					1	1	nt
6	Podujevë	3	3	0	ns						ns					ns		
7	Vushtri	4	4	0	ns						ns					ns		
8	Drenas	1	1	0	2	1	0	2	2	0	1	0	0	1	1	1	1	nt
9	Ferizaj	1	1	0	ns						ns					ns		
10	Prizren	1	1	0	2	0	0	2	1	0	2	1	0	2	0	ns		
11	Mitrovicë	2	2	0	ns						ns					ns		
12	Novobërdë	2	2	0	ns						ns					ns		
13	Hani i Elezit	1	1	0	ns						ns					ns		
14	Malishevë	3	3	2	ns						ns					1	1	nt
15	Gjilan	3	3	0	1	1	0	1	1	1	1	1	0	1	1	ns		
16	Lipjan	3	3	0	1	1	1	1	1	0	1	1	0	1	1	ns		
17	Graqanicë	1	1	0	ns						ns					ns		
18	Skenderaj	2	2	0	1	1	0	1	1	0	ns					ns		
19	Viti	2	2	1	ns						ns					2	2	nt
20	Fushë Kosovë	1	1	0	ns						ns					ns		
21	Obiliq	1	1	0	ns						ns					ns		
22	Pejë	1	1	0	ns						ns					ns		
23	Gjakovë	ns			1	1	0	1	1	1	ns					ns		
24	Junik	ns			1	1	0	1	1	1	ns					ns		
25	Istog	ns			ns						ns					1	1	nt
Total apiaries sampled per group		**59**			**13**						**8**					**9**		
Total pathogens detected per group			**59**	**7**		**9**	**1**	**13**	**12**	**4**		**6**	**1**	**8**	**6**		**8**	**1**
% of infections			**100**	**11.9**		**69.2**	**7.7**	**100**	**92.3**	**30.8**		**75**	**12.5**	**100**	**75**			
% of infection in honey bees and *Varroa* together						**84.6**	**15.4**	**100**	**92.3**	**30.8**								

nt = not tested. ns= not sampled.

Group 1: fifty-nine apiary samples taken from 22 municipalities (Table 1). For each apiary, a pool of 30 adult worker honey bees collected from 3 different colonies was prepared to be tested for *Vairimorpha* spp. since suspicion of infection was raised based on a weak colony condition. Sampling was carried out upon the request of beekeepers for suspected cases of weak colonies during the spring, summer, and autumn periods. For each of the three colonies of the apiary, 30–60 bees were taken randomly from the flight board and inside of the hive, and, once pooled, the samples were sent by the beekeepers to the laboratory for *Vairimorpha* spp., *L. passim*, and *C. mellificae* detection. All the samples were stored at −20 °C until analysis.

Group 2: thirteen apiary samples (adult and/or larvae and/or pupae and/or *Varroa*) taken from 10 municipalities (Table 1). Sampling was carried out after suspicion of viral infections due to the presence of *Varroa*, lack of body hair on the thorax, deformed wings, and sac brood in weak or lost bee colonies. Each colony sample consisted of a full frame with different stages of bee development (adult and/or larvae and/or pupae and/or *Varroa*). Each apiary sample consisted of a pool of frames from 3 to 6 colonies belonging to the same apiary. In the laboratory, samples were collected from the honey bee combs with disposable forceps, placed in 10 mL plastic vials, and stored at −80 °C until analysis.

Group 3: nine apiary samples taken from 8 municipalities (Table 1) of which eight were analysed for AFB and only one for EFB. For each apiary, the sample consisted of a pool of several brood combs (10 × 10 cm with larvae) with suspicion of AFB or EFB infection.

2.2. Clinical Inspection of Samples

Thirty honey bee abdomens from Group 1 specimens were homogenized and prepared on the same day for *Vairimorpha* spp. spore detection by light microscopy at 400× magnification.

Adult bees, larvae, and pupae as well as *Varroa* mites collected from Group 2 were examined for the presence of sac-brood-like larvae, bees with deformed wings, or *Varroa* mites.

Samples from Group 3, consisting of brood combs, were clinically examined for symptoms of AFB and EFB, and then eight suspected larvae were tested for AFB and only one for EFB with rapid immunochromatography and microscopy.

2.3. Vairimorpha spp. Spore Detection and Vairimorpha apis/Vairimorpha ceranae Species Identification

The abdomens of 30 honey bees were separated and macerated in about 2.5 mL of distilled water using a mortar and pestle and successively 27.5 mL of distilled water was added to a final volume of 1 mL per bee. A drop of this suspension was examined under a light microscope at 400× magnification to evaluate the presence of *Vairimorpha* spp. spores. *Vairimorpha* spp.-positive samples were analysed for *V. apis* and *V. ceranae* species identification after DNA extraction from 1 mL homogenate using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with a lysozyme pre-incubation step. The yield and purity (260/280 and 260/230 nm absorbance ratios) of DNA were determined using the Nanodrop™ OneC (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer. DNA was stored at −20 °C until use. Negative controls (negative process control—NPC: water for molecular biology applications instead of the sample) were included in each extraction session.

For *V. apis* and *V. ceranae* identification, two different sets of primers described by Martín-Hernández et al. [46] were used and PCR analysis was carried out as previously described by Bordin et al. [47]. Negative (negative template control—NTC: water for molecular biology applications instead of the DNA template) and positive (positive template control—PTC: DNA from *V. apis*- or *V. ceranae*-positive samples) controls were included in each PCR.

2.4. Lotmaria passim and Crithidia mellificae Detection

For *L. passim* and *C. mellificae* detection, two pairs of primers described by Bartolomé et al. [48] were used and PCR analysis was carried out as previously described by Bordin et al. [47]. Negative (NTC: water for molecular biology applications instead of the DNA template) and positive (PTC: DNA from *L. passim*- or *C. mellificae*-positive samples) controls were included in each PCR.

2.5. Honey Bee Viruses

Thirteen honey bee (adult and/or larvae and/or pupae) and eight *Varroa* mite samples were analysed for seven honey bee viruses relevant with respect to colony health status: ABPV, CBPV, DWV, SBV, BQCV, KBV, and IAPV.

For each sample, a pool of five specimens per development stage (adult, larvae, or pupae) or a pool of up to ten specimens of *Varroa* mites was homogenized by Tissue Lyser II (Qiagen, Hilden, Germany) (2 cycles, 1 min each, at 30 Hz) in the presence of a 5 mm stainless steel bead. Total RNA extraction was performed using the NucleoSpin RNA kit (Macherey Nagel GmbH & Co. KG, Dueren, Germany) according to the manufacturer's instructions. The yield and purity of RNA (260/280 and 260/230 nm absorbance ratios) were assessed with a Nanodrop™ OneC spectrophotometer. RNA was stored at −80 °C until use. Negative controls (NPC: buffer RA1 from the extraction kit instead of the homogenized sample) were included in each extraction session.

To detect viral RNA, real-time RT-PCR (rRT-PCR), or end-point RT-PCR were performed as described by Bordin et al. [47]. To detect the presence of DWV the molecular protocol described by Martinello et al. [49] was used. Negative (NTC: water for molecular biology applications instead of the RNA template) and positive (PTC: viral RNA from positive sample) controls were included in each PCR.

2.6. Paenibacillus larvae (AFB) Detection by Field Tests, Isolation, and PCR

A first visual inspection of brood combs was carried out to search for symptoms of AFB: irregular and patchy brood pattern, unpleasant odour, perforated and sunken cell caps, dark-coloured larvae, ropy larvae, and stickiness. Larvae with AFB symptoms were then analysed with the AFB lateral flow test—Diagnostic Test Kit (Vita Europe, London, UK) according to the manufacturer instructions (https://www.vita-europe.com/beehealth/products/afbdiagnostic-test-kit/) (accessed on 20 January 2024). The samples were stored at −20 °C for further analyses.

The presence of *P. larvae* in the eight samples of suspected larvae was also determined by the culture method and PCR.

The isolation of *P. larvae* and the following colony identification (catalase test and Gram staining) was performed on Columbia sheep-blood agar (CSA), supplemented with nalidixic acid and pipemidic acid, according to the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, chapter 3.2.2 (2023) [50].

To assess the presence of *P. larvae* by the PCR method, DNA extraction was performed from a larvae homogenate using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with a lysozyme pre-incubation step. The yield and purity (260/280 and 260/230 nm absorbance ratios) of DNA were determined using a Nanodrop™ OneC spectrophotometer. DNA was stored at −20 °C until use. Negative controls (NPC: water for molecular biology applications instead of the sample) were included in each extraction session.

For *P. larvae* detection, the primers described by Dobbelaere et al. [51], targeting a 1096 bp region of the 16S rRNA gene, were used. PCR was carried out on a Veriti™ 96-Well Thermal Cycler (Applied Biosystems™, Waltham, MA, USA), using the AmpliTaq™ Gold kit (Applied Biosystems™, Waltham, MA, USA). In a final volume of 50 µL, 200 ng of DNA was amplified using a final concentration of 2 mM $MgCl_2$, 1 µM for each primer, 0.2 mM of dNTPs, and 1 U AmpliTaq Gold DNA polymerase. The thermal cycling profile consisted of an initial activation step at 95 °C for 10 min followed by 35 cycles at 93 °C for 60 s, 55 °C

for 30 s, 72 °C for 60 s, and a final elongation step at 72 °C for 5 min. Negative (NTC: water for molecular biology applications instead of the DNA template) and positive (PTC: *P. larvae* DNA) controls were included in each run of PCR. The presence and the size of amplification products (1096 bp) were evaluated by electrophoresis in 7% acrylamide gel after silver staining or capillary electrophoresis on a LabChip® GX Touch HT Nucleic Acid Analyzer (Perkin Elmer, Waltham, MA, USA).

2.7. Genotyping of Paenibacillus larvae Isolates

For *P. larvae* genotyping, DNA was extracted from catalase-negative and Gram-positive colonies using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for Gram-positive bacteria, and the DNA was eluted in a final volume of 120 µL. The yield and purity of DNA were determined using the Nanodrop™ OneC spectrophotometer and the nucleic acid was stored at −20 °C until use. ERIC-typing PCR was performed using the primers published by Genersch et al. [52]. The PCR was carried out on a Veriti™ 96-Well Thermal Cycler (Applied Biosystems™, Waltham, MA, USA) in a final volume of 50 µL using the AmpliTaq™ Gold kit and containing a final concentration of 2.5 mM $MgCl_2$, 800 µM of dNTP mix, 1 µM of each primer, 2.5 U AmpliTaq Gold DNA polymerase, and 50–100 ng of DNA. The thermal cycling amplification profile consisted of an activation step at 95 °C for 10 min, followed by 50 cycles at 94 °C for 1 min, 53 °C for 1 min, 72 °C for 2.5 min, and a final elongation step at 72 °C for 10 min. Negative (NTC: water for molecular biology applications instead of the DNA template) and positive (PTC: *P. larvae* DNA) controls for ERIC I, II, and IV genotypes were included in each PCR. After amplification, about 16 µL of the PCR reaction was electrophoresed in a 1.7% SeaKem® Gold Agarose gel (Lonza Rockland, Inc., Rockland, ME, USA) and the PCR products were visualized after ethidium bromide staining on a UV trans-illuminator.

2.8. Melissococcus plutonius Detection by Field Tests and PCR

Brood combs were examined for EFB signs such as dead, flaccid discoloured larvae in uncapped cells or changes in their colour (from pearly white to yellow, yellowish to brown), and the presence of dry and dark brown scales that can easily be removed from the cells.

Larvae with EFB symptoms were tested with the EFB lateral flow test—Diagnostic Test Kit (Vita Europe, London, UK) according to the manufacturer's instructions (https://www.vita-europe.com/beehealth/products/efb-diagnostic-test-kit/) (accessed on 20 January 2024). After analysis, all samples were stored at −20 °C until further examination.

A pool of larvae was analysed to detect the presence of *M. plutonius* by the PCR method, using the primers described by Govan et al. [53], targeting an 832 bp region of the 16S rRNA gene. DNA extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (protocol for bacteria—isolation of genomic DNA from Gram-positive bacteria). The yield and purity of DNA were determined using the Nanodrop™ OneC spectrophotometer and the nucleic acid was stored at −20 °C until use. Negative controls (NPC: water for molecular biology applications instead of the sample) were included in the extraction session.

The PCR was carried out as described above in a final volume of 50 µL containing a final concentration of 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM of each primer, 2 U AmpliTaq Gold DNA polymerase, and 50–100 ng of DNA. The thermal cycling amplification profile consisted of an activation step at 95 °C for 10 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final elongation step at 72 °C for 10 min. Negative (NTC: water for molecular biology applications instead of the DNA template) and positive (PTC: *M. plutonius* DNA) controls were included in each PCR. The presence and the size of the amplification product (832 bp) were evaluated by electrophoresis in 7% acrylamide gel after silver staining.

3. Results

The results from 89 investigated apiaries are shown in Table 1 and Figures 1 and 2.

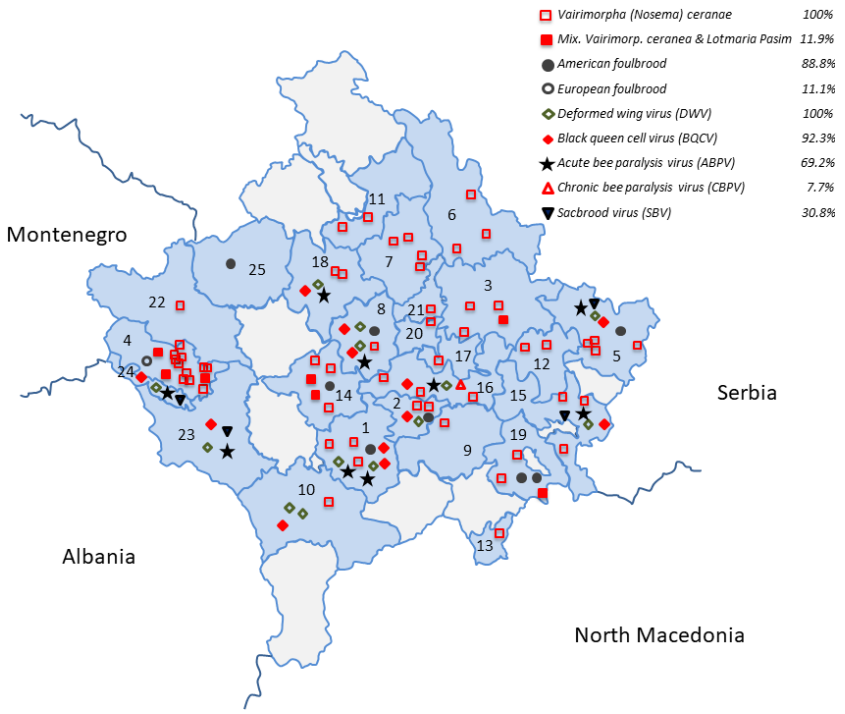

Figure 1. Distribution of detected pathogens by municipality in the Republic of Kosovo. The numbers correspond to the 25 involved municipalities (1—Suharekë; 2—Shtime; 3—Prishtinë; 4—Deçan; 5—Kamenicë; 6—Podujevë; 7—Vushtrri; 8—Drenas; 9—Ferizaj; 10—Prizren; 11—Mitrovicë; 12—Novobërdë; 13—Hani i Elezit; 14—Malishevë; 15—Gjilan; 16—Lipjan; 17—Graqanicë; 18—Skenderaj; 19—Viti; 20—Fushë Kosovë; 21—Obiliq; 22—Pejë; 23—Gjakovë; 24—Junik; 25—Istog). Each symbol indicates the presence of a different pathogen individually or in combination. Their percentage of detection is also indicated.

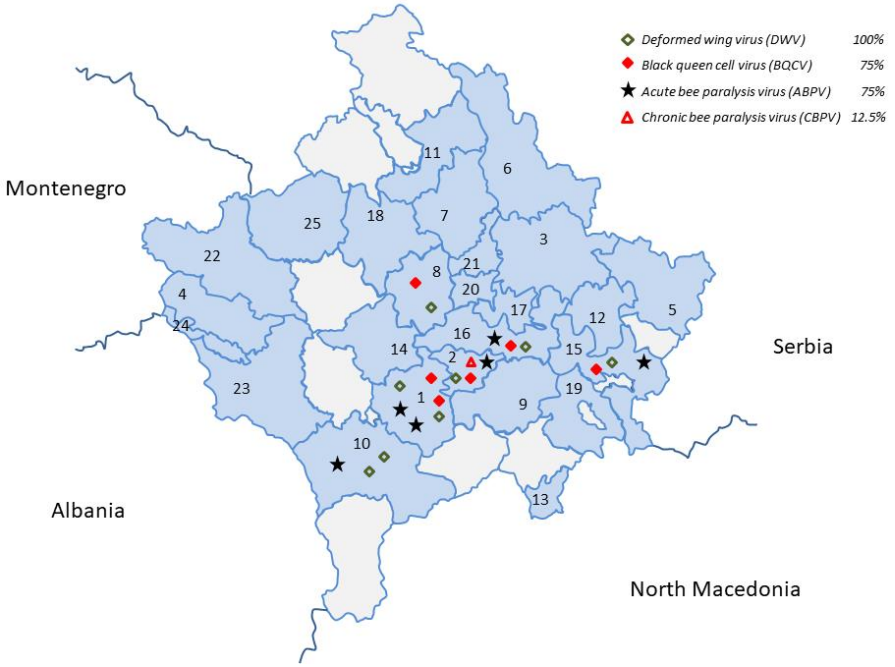

Figure 2. Distribution of detected honey bee viruses in *Varroa* mites by municipality in the Republic of Kosovo. The numbers correspond to the 25 involved municipalities. Each symbol indicates the presence of a different virus detected in *Varroa* specimens. Their percentage of detection is also indicated.

3.1. Group 1—*Vairimorpha spp. and Trypanosomatids L. passim and C. mellificae*

Of 59 investigated apiaries from 22 municipalities, *V. ceranae* infections were found in all apiaries (100%), whereas all were negative for *V. apis*. The trypanosomatid *L. passim* was detected in seven apiaries (11.9%) from four municipalities, while *C. mellificae* was never detected (Table 1, Figures 1 and 3A–C,E).

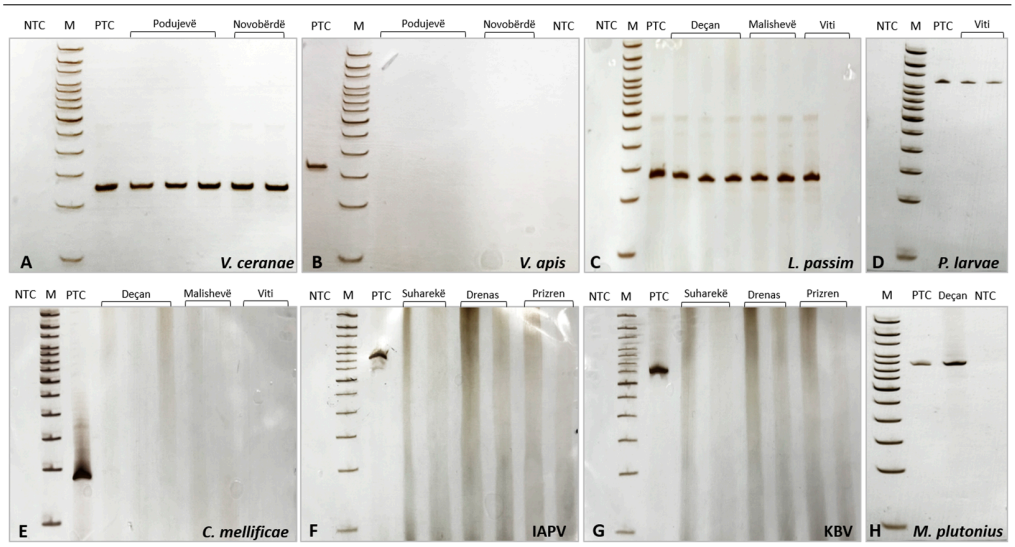

Figure 3. PCR products of eight bee pathogens after 7% acrylamide gel electrophoresis and silver nitrate staining. In this figure, PCR products from some of the analysed apiaries are shown: Suharekë, Drenas, Prizren, Podujevë, Novobërdë, Deçan, Malishevë, Viti. Eight bee pathogens were amplified by PCR: (**A**) *V. ceranae* (218 bp); (**B**) *V. apis* (321 bp); (**C**) *L. passim* (254 bp); (**D**) *P. larvae* (1096 bp); (**E**) *C. mellificae* (177 bp); (**F**) IAPV (767 bp); (**G**) KBV (659 bp); (**H**) *M. plutonius* (832 bp). M: 100 bp DNA Ladder (Invitrogen™); PTC: pathogen positive control; NTC: no template control.

3.2. Group 2—*Honey Bee Viruses (ABPV, CBPV, DWV, SBV, BQCV, KBV, and IAPV)*

The following honey bee viruses were detected in samples (adults/larvae/pupae) from the 13 apiaries in the 10 municipalities: DWV in all thirteen apiaries (100%), BQCV in twelve apiaries (92.3%), ABPV in nine apiaries (69.2%), SBV in four apiaries (30.8%), and CBPV in one apiary only (7.7%). IAPV and KBV were not detected in any apiary (Figure 3F,G). Viruses were found in all the developmental stages of honey bees and in particular, adults specimens tested positive for DWV, BQCV, ABPV, CBPV, and SBV; pupae for DWV, BQCV, ABPV, and SBV; and larvae for DWV, BQCV, and ABPV (Table 1 and Figure 1).

In the eight *Varroa* samples derived from eight apiaries from six municipalities, four out of seven viruses (ABPV, CBPV, DWV, BQCV) and different co-infections were detected. In particular, only the sample from one municipality (Shtime) was found to be co-infected with all four viruses, and four *Varroa* mite samples from three municipalities (Gjilan, Lipjan, Suharekë) tested positive for three viruses (ABPV, DWV, BQCV), whereas two samples from Prizren and one sample from Drenas tested positive for ABPV and DWV, and for DWV and BQCV, respectively (Table 1 and Figure 2).

3.3. Group 3—*Paenibacillus larvae and Melissococcus plutonius Detection*

In the third group, all 8 larvae samples from the brood comb (1, Suharekë; 1, Kamenicë; 1, Drenas; 1, Malishevë; 1, Shtime; 2, Viti; 1, Istog) investigated for the presence of the causative agent of AFB tested positive for *P. larvae* on an AFB lateral flow test—Diagnostic Test Kit (Vita Europe, London, UK), PCR, and the culture method (Table 1, Figures 1 and 3D). All *P. larvae* isolates were identified as the ERIC I genotype. The only sample from the Deçan municipality analysed for *M. plutonius* presence was positive for

EFB on a lateral flow test—Diagnostic Test Kit (Vita Europe, London, UK) and PCR (Table 1, Figures 1 and 3H).

4. Discussion

In the last decade, a reduction in honey bee colonies has been frequently reported around the world [2,54]. Among the causes for mortality and global threat for honey bee colonies are several pathogens and parasites [55].

Our study was based on passive surveillance on weak honey bee colonies aiming to determine the occurrence and distribution of known and emergent honey bee pathogens in the Republic of Kosovo. An investigation based on passive surveillance was already carried out by Hulaj et al. [45] focusing on AFB.

In all the 59 apiaries tested for *Vairimorpha* spp., a high percentage of infection (100%) with *V. ceranae* was found, while no samples tested positive for *V. apis*. These data confirm what has already been reported in Italy [47,56] and in the neighbouring countries to the Republic of Kosovo, such as Serbia, Croatia, Bosnia–Herzegovina, Montenegro, North Macedonia, and Bulgaria, where *V. ceranae* dominates in microsporidia infections in honey bees [57–61]. Furthermore, this finding confirmed the previously reported higher diffusion of *V. ceranae* in other European countries and worldwide [62–65]. The presence of *V. ceranae* infection could reduce honey bee lifespan [7], significantly increase honey bee worker mortality, and could be one stressor responsible for elevated colony losses [66].

In the Republic of Kosovo, *L. passim* was only detected in seven of fifty-nine apiaries (11.9%) from four municipalities during the passive surveillance. In the Veneto region (northern Italy), *L. passim* was detected in almost all of the apiaries analysed with an overall positivity rate of 48.8% in 2020, while an increase was observed in 2021 with an overall value of 62.2% [47]. In Serbia, *L. passim* was detected during 2007–2015 every year. In the Republic of Kosovo, *C. mellificae* was never reported, as well as in neighbouring Serbia [67].

In our study, five honey bee viruses were found (ABPV, CBPV, DWV, BQCV, SBV) and the most frequent were DWV and BQCV with 100% and 92.3% prevalence in the 13 apiaries analysed, respectively. ABPV was detected in nine apiaries (69.2%), SBV in four apiaries (30.8%) from four municipalities, and CBPV in only one apiary (7.7%). No positivity for IAPV and KBV was observed in the investigated apiaries.

When comparing our research on honey bee viruses with other countries, we found that the same viruses (ABPV, CBPV, DWV, BQCV, and SBV) have also been detected in neighbouring Serbia, Austria, Greece, and other countries [68–71], whereas KBV was also detected in France [20]. The presence of DWV is often associated with *V. destructor* infestation, and the role of this mite in viral transmission has already been experimentally demonstrated [72]. It is known that DWV, vectored by the *Varroa* mite, adversely affects humoral and cellular immune responses and promotes the reproduction of this parasitic mite [27].

Both BQCV and SBV are not known to be transmitted by *V. destructor* [73]. However, a significantly higher prevalence of BQCV and SBV in colonies infested with *V. destructor* mites has been detected, hypothesizing the role of *V. destructor* in their transmission [74]. In our study, considering not only honey bee but also *Varroa* mite samples, the virus frequency was increased for ABPV (from 69.2% to 84.6%) and CBPV (from 7.7% to 15.4%).

Furthermore, larvae collected from the brood comb tested for AFB were positive for the *P. larvae* ERIC I genotype in eight out of nine apiaries. This finding is in line with the results of the passive survey carried out during 2007–2019 that revealed a wide diffusion of AFB of the ERIC I and II genotypes in the country [45], thus suggesting the necessity of applying active disease surveillance strategies. The single positivity detected for EFB suggests that the disease is present in the country but further investigations are needed to understand its true distribution and possible impact on honey bee colonies.

5. Conclusions

Although there have been clinical cases of suspected emergent honey bee diseases in the Republic of Kosovo, they have never been investigated; this paper represents the first molecular detection of honey bee viruses (ABPV, CBPV, DWV, BQCV, SBV), the microsporidian *V. ceranae*, the trypanosomatid *L. passim*, and the bacterium *M. plutonius*. Furthermore, we also confirmed the presence of *P. larvae* in the apiaries of the Republic of Kosovo.

We hope that the results presented herein could open the path to further investigations in order to consolidate and update the knowledge on the health status of honey bee colonies in the Republic of Kosovo.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app14030987/s1, Figure S1: Map with the involved municipalities and the type of beehive matrices that have been sampled.

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