

Parasites-*Iflavirus* association and emergence of three master variants of DWV affecting *Apis mellifera intermissa* in Tunisian apiaries

Khaoula ABDI^{1,2}, Khaoula BELGUTH¹, Chadlia HAMD^{1,3}, Yasmine SOUSSI¹, Jihène ESSANAA^{1,3}, Walid DRIDI⁴, Tarek HAJJI¹, Amor MOSBAH¹, Taoufik BEN HAMIDA⁴, Ameer CHERIF^{1,3}

¹University Manouba, ISBST, BVBGR-LR11ES31, Biotechpole Sidi Thabet, Ariana, Tunisia

²Faculty of Sciences of Tunis, University of Tunis El Manar, Tunisia

³Laboratory of Microorganisms and Active Biomolecules, Faculty of Sciences of Tunis, University of Tunis El Manar, Tunisia

⁴Laboratory of Parasitology, Institute of Veterinary Research of Tunis, La Rabta, Tunis, Tunisia

Abstract

As a social insect living in large groups, the honey bee *Apis mellifera* is vulnerable to parasites and pathogens, which have become a major threat for apiculture and pollination services worldwide. The co-occurrence of parasites, mainly *Varroa destructor*, with viral pathogens is suspected to constitute a primary cause in colony mortality and population losses. In our study, we investigated the presence and possible association of five honey bee pathogens occurring in Tunisian apiaries. This included the endoparasitic microsporidia *Nosema ceranae* and *Nosema apis*, the ectoparasitic mite *V. destructor* and two honey bee RNA virus; deformed wing virus (DWV or DWV genotype A) and *Varroa destructor* virus-1 (VDV-1), otherwise known as DWV genotype B. There was no statistical association between the five biotic stressors. Interestingly, from the most *Varroa*-infested colony sample (A27.H4, 45%); we detected a co-infection with three pathogens, DWV, *N. apis* and *N. ceranae*. Using a haplotype network analysis, we revealed a high diversity of the Tunisian DWV genotypes A and B from *Apis mellifera intermissa* with twelve haplotypes showing similarities with various European, Asian, and South American genotypes. We detected double infection by DWV genotypes A and B in the Bizerte region. These co-occurring genotypes were suggested that such co-infection gives rise to recombinant virus with potential enhanced virulence. Here, we support the presence of DWV-VDV-1 recombinant in *A. m. intermissa*. Overall and despite the lack of a clear epidemiological link between honey bee pathogens and colony health, their interactions may lead to disease and colony losses.

Key words: *Apis mellifera intermissa*, honey bees, Pettis test, deformed wing virus, *Varroa destructor* virus-1, *Nosema* spp., VDV-1-DWV recombinant virus, DWV strains (genotype A, B and C).

Introduction

The beekeeping sector and pollination phenomenon are highly dependent on the honey bees dynamic in the environment (Calderone, 2012). Several abiotic and biotic factors such as pesticides, mites and viruses can greatly affect this balance and pose serious problems not only for beekeeping, but also for pollination services and agriculture in general (Evans and Hung, 2000; Martin, 2001; Hamdi *et al.*, 2013). Furthermore, interactions between parasites and pathogens are suspected to constitute the major risk for honey bee colonies and increase the risks of honey bee decline (Nazzi *et al.*, 2012; Vanbergen, 2013; Natsopoulou *et al.*, 2016). Hence, multiple causes are related to the depopulation phenomena, such as virus's diseases vectored by the *Varroa* mite (Hung *et al.*, 1995; Carneiro *et al.*, 2007) and *Nosema ceranae* (Paxton, 2010). The synergistic interactions between these parasites and pathogens, conjugated with the presence of pesticides, can directly cause colony losses (Nazzi *et al.*, 2012; Ryabov *et al.*, 2014; Abbo *et al.*, 2017). It was demonstrated that the immunosuppressive effects observed in *Varroa*-infested colonies, lead to the emergence and amplification of virulent viral strains and colony collapse (Nazzi *et al.*, 2012; Ryabov *et al.*, 2014). Such disorder was reported to be responsible of high morbidity and colony losses in Europe, Asia and North

America (De Figueiró *et al.*, 2016). It was estimated that there is 20% to 30% decline between 1997 and 2009 (Genersch and Aubert, 2010). However, no information was reported about colony losses amongst Africanized and African honey bees (De Figueiró *et al.*, 2016; Pirk *et al.*, 2016).

Nosemosis is caused by the microsporidia parasites *Nosema apis* and *Nosema ceranae* (Heintz *et al.*, 2011). Both of these microsporidian species are intracellular parasites infecting the honey bees' digestive tract (Fries, 2010). Beside, *N. ceranae* was suggested to cause colony losses (Higes *et al.*, 2008). Diagnosis of nosemosis can be performed by light microscopy and molecular methods (Fries, 2010). These virulent pathogens have been detected in Asia, Europe, America and North Africa, especially in Algeria (Martín Hernández *et al.*, 2007; Higes *et al.*, 2009). The ectoparasite mite *Varroa destructor* Anderson et Trueman is also reported among the most detrimental honey bee parasites (Brodschneider and Crailsheim, 2010; Calderón *et al.*, 2010; Chauzat *et al.*, 2010; Genersch and Aubert, 2010; vanEngelsdorp *et al.*, 2011). This ectoparasite is considered as a virus vector, especially, deformed wing virus (DWV) (Evans and Huang, 2000; Chen and Siede, 2007; Boecking and Genersch, 2008; Genersch and Aubert, 2010; Wilfert *et al.*, 2016). Actually, 23 honey bee viruses have been described to infect honey bee worldwide (Allen and Ball,

1996; Chen and Siede 2007; Genersch and Aubert, 2010; de Miranda *et al.*, 2015; Gisder and Genersch, 2015). Hence, DWV is associated to several typical clinical symptoms such as malformed appendages, shortened and bloated abdomens, miscolouring (Ball and Allen, 1988; Martin, 2001; Yue and Genersch, 2005) and a severely shortened adult life span for emerging worker and drone bees (Kovac and Crailsheim, 1988). The isolation of DWV was reported worldwide (Allen and Ball, 1996; Ellis and Munn, 2005; Reddy *et al.*, 2013). In the meanwhile, many honey bee viruses are asymptomatic such as *Varroa destructor* virus-1 (VDV-1) (Chen *et al.*, 2005). Different studies reported that the coexistence of many viruses in the vector *V. destructor* and honey bee colonies may induce virus recombination (Genersch and Aubert, 2010; Gauthier *et al.*, 2011; Moore *et al.*, 2011; Wang *et al.*, 2013; Zioni *et al.*, 2011). Therefore, three VDV-1-DWV recombinant viruses (HM067437, HM067438 and KJ437447) were detected and identified from *Varroa*-infested colony in the United Kingdom (Moore *et al.*, 2011; Ryabov *et al.*, 2014). Concurrently, Zioni *et al.* (2011) reported the detection of a recombinant virus (JF440526) in Israeli apiaries. It was postulated that the presence of this recombinant virus is related to *Varroa*-infested colonies and to the appearance of viruses' virulent strains (Zioni *et al.*, 2011). Recently, a DWV-VDV-1 variant (KX373900) was isolated in Southern France (Dalmon *et al.*, 2017). In this report, it was proposed to name parental DWV strain "type A", VDV-1 strain "type B" and a "type C" variant of DWV-VDV-1. This latter is a recombinant between DWV and VDV-1, also named DWV-VDV-1 recombinant (Moore *et al.*, 2011; Zioni *et al.*, 2011; Mordecai *et al.*, 2016b; Dalmon *et al.*, 2017). This new variant can be more virulent than parental genotypes A and B and able to cause overt infection in *Varroa*-infested colonies (Zioni *et al.*, 2011; Ryabov *et al.*, 2014).

Honey bee colony fitness is associated with interac-

tion between abiotic and biotic stressors (Nazzi *et al.*, 2012; Ryabov *et al.*, 2014; Meixner *et al.*, 2015; Wilfert *et al.*, 2016). While virus-mite association has been widely investigated and many effects have been revealed (Shen *et al.*, 2005; Yang and Cox-Foster, 2005; Ryabov *et al.*, 2014), it was recently reported that *Varroa*-neonicotinoid pesticide synergism play key roles in colonies decline (Abbo *et al.*, 2017). In contrast, the endoparasite *N. ceranae* showed no interaction with pesticide stressors (Retschnig *et al.*, 2015), DWV infection (Martin *et al.*, 2013) or *Varroa* mite infestation (Hedtke *et al.*, 2011).

On the basis of these findings, at the extent that very few studies exist on DWV honey bees prevalence that cover the Middle East and North Africa (MENA) region (Haddad *et al.*, 2015) and as no data exist on screening and molecular characterization of DWV genotypes infecting African honey bee and especially Tunisian colonies, this study aimed their investigation. Thus, we performed a molecular survey of honey bee, more specifically, DWV genotypes A, B and C, in northern Tunisia, from four governorates (Beja, Jendouba, Bizerte and Siliana). Honey bee colonies infected with *V. destructor* were sampled from twenty eight different apiaries, during Autumn-Winter 2011. Screening for the presence of *Varroa*, *Nosema* beside *Varroa* associated viruses (DWV genotypes A and B) was achieved in order to assess the biotic interactions between honey bees and their parasites and pathogens.

Materials and methods

Apiaries selection and colonies sampling

Fifty six colonies from twenty eight apiaries were collected during Autumn-Winter 2011 across four regions situated in northern Tunisia, Bizerte, Beja, Jendouba and Siliana (figure 1 and supplemental material table S1).

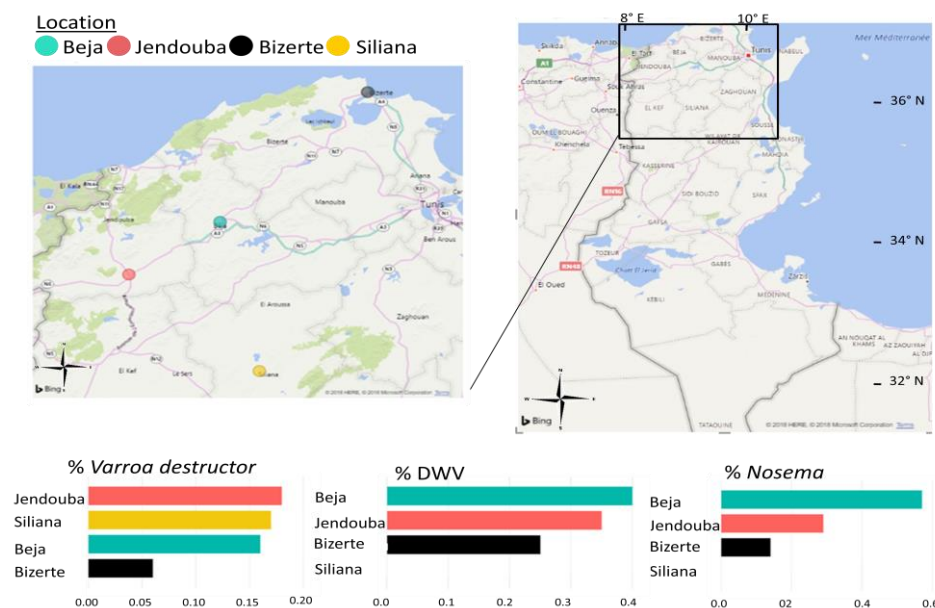


Figure 1. Sampling locations and parasites-Iflavirus infestation in Tunisian apiaries (Beja, Jendouba, Siliana and Bizerte).

The choice of samples was based on the distribution of apiaries in the North of Tunisia (registered at the office of development sylvo-pastoral in the North West, Tunisia). Each colony collected contains about 150 honey bee workers, in order to test the *Varroa* mite infestation, to detect *Iflavirus* (DWV genotypes A, B and C) and the two microsporidian pathogens (*N. apis* and *N. ceranae*). *V. destructor* infestation was observed in the majority of sampled colonies. All samples were looking healthy, except one colony where honey bees showed deformed wings and thus considered as positive control for DWV genotype A.

Varroa destructor infestation and assay for acaricide effectiveness

V. destructor rates were determined by using an acaricide treatment. According to the Pettis test (Pettis *et al.*, 1998), all colonies were treated with Amitraz[®], using one strip per hive box to rid honey bee of *Varroa* mites. The samples of approximately 150 bees per colony from 56 colonies collected inside the hive as described below were put into a jar with the section of Amitraz[®] strip and a sugar cube. All samples are incubated at 24 hours in 30 °C. The number of mites per 150 bees that dislodged from the bee's bodies was used to calculate the percentage of infestation.

DNA extraction and *Nosema* detection

We focused on two microsporidian *N. ceranae* and *N. apis*, as honey bee intracellular gut pathogens. First, a standard light microscopy was used to identify *Nosema* spp. spores from crushed abdomen of workers bee (Martín-Hernández *et al.*, 2007). We treated asymptomatic and symptomatic colonies with diarrhea signs. Owing to difficulties in differentiating spores of the two species *N. ceranae* and *N. apis* under a light microscopy (Martín-Hernández *et al.*, 2007; Michalczyk *et al.*, 2011), a PCR-based approach was adopted. For each colony, five worker bees were collected, pooled and used to extract DNA with universal salt-extraction protocol (Aljanabi and Martinez, 1997) and precipitated with ethanol. Two sets of primers targeting specific rRNA regions in *N. ceranae* and *N. apis* were used to allow amplification and differentiation of the two species (Chen *et al.*, 2008) (table 1). PCR reactions contained 1 µl of total DNA, 6 µl of non-acetylated BSA (1 mg/ml), 2.5 mM dNTP, 3 µM of each primer, 25 mM Mg Cl₂, 1.5 µl of reaction Buffer (10×), 5 U of

Taq DNA polymerase (Feldan-Bio) and sterile water to a final volume of 15 µl. PCR cycles consisted as follows: 2 min of denaturation at 95 °C, 29 cycles of each denaturation at 95 °C for 45 s, 45 s hybridization at 56 °C and extension at 72 °C for 30 s, followed by a final step of 5 min at 72 °C. Amplicons were analyzed by electrophoresis on a 2% agarose gel and visualized on a UV transilluminator.

RNA extraction and RT-PCR detection of DWV genotypes A and B

For each sampled colony, total RNA was extracted from 5 pooled adult worker bees using Trigent (Total RNA Isolation Reagent, K516161), according to the manufacturer's instructions (BIO BASIC Inc). It was then stored at -80 °C until use. The extracted RNA's were converted to cDNA's and amplified using a one-step real-time RT-PCR, SYBR Green (Takara, RR086A). PCR reactions contained 0.4 µM of total RNA, 10 µl of 2× One Step SYBR RT-PCR Buffer 4, 0.8 µl of Prime Script 1 Step Enzyme Mix 2, 0.4 µl of ROX Reference Dye or Dye II, and 10 µM of one set of primers. We used two pairs of primers targeting specific regions in the polyprotein genes of DWV genotype A (DWV) and genotype B (VDV-1), allowing the amplification and differentiation of the two genotypes (Kukielka *et al.*, 2008, Zioni *et al.*, 2011) (table 1). Reactions were run on the Applied Biosystems[®] 7500 Fast Real-Time PCR system. The following cycling conditions were used: 48 °C at 30 min (RT) followed by 95 °C for 10 min and 40 cycles of (95 °C for 45 s, 61 °C for 1 min and 72 °C for 1 min). The final extension was 72 °C for 7 min. Lastly, a dissociation curve was performed at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. PCR products were then analysed on a 2% agarose gel and visualized on a UV transilluminator.

Sequencing and haplotype network analysis of RNA viruses

PCR products were purified and sequenced, using BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems[®]), according to the manufacturer's instructions on 3130xL Genetic Analyzer (Applied Biosystems[®]). The obtained nucleotide sequences were compared with the virus reference sequences available online using the NCBI Blast search in Genbank (<http://www.ncbi.nlm.nih.gov/>).

Sequences were aligned with MEGA7 (Kumar *et al.*,

Table 1. Primers sets used for the molecular detection of *Nosema* spp. and *Iflavirus*.

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Positions in the genome	Expected size (bp)	References
<i>N. ceranae</i>	CGGATAAAAGAGTCCGTTACC	TGAGCAGGGTCTAGGGAT	4186-4436	250	Chen <i>et al.</i> , 2008
<i>N. apis</i>	CCATTGCCGGATAAGAGAGT	CACGCATTGCTGCATCATTGAC	4396-4665	269	Chen <i>et al.</i> , 2008
DWV	CTGTATGTGGTGTGCCTGGT	TTCAAACAATCCGTGAATATAGTGT	8575-8801	226	Kukielka <i>et al.</i> , 2008
VDV-1	CCGTAGTTGGGAGATTGATG	GCGGGTACATCTTCAGCTA	1186-1390	204	Zioni <i>et al.</i> , 2011

2016) and the haplotype network was constructed using Median joining network (Bandelt *et al.*, 1999). Twelve partial polyprotein reference sequences were used in this analysis: 8 sequences of DWV genotype A (AY224602, KY327829, DQ434965, DQ434911, DQ434951, DQ434939, KT733631 and KT733632); one of DWV genotype B (KC786222) and three of DWV-VDV-1 recombinant (HM067437, HM067438 and KX373900).

Statistical analysis

The statistical analysis was performed to compare the prevalence of the honey bee pathogens between geographical regions. Correlations between parasites and pathogens were estimated, using Spearman tests (R core team, 2013).

Nucleotide sequences accession numbers

The viruses' sequences of our samples were deposited in the Genbank database under accession numbers KX279943 - KX279947, KX781204 - KX781209, KY428662 - KY428664 and KY514413 - KY514418.

Results

Detection and correlation between parasites and pathogens

Varroa destructor and *Varroa* associated viruses

Infestation grades differed among hives. Percentages of *V. destructor* were ranging from total absence (observed in two colonies from Bizerte), to 45% detected in

A27.H4 apiary in Beja.

The three northwestern governorates (Jendouba, Siliana and Beja) showed an approximate average rate of 18%; 17% and 15% respectively. Conversely, the northeastern coastal Bizerte governorate showed the lowest *Varroa* infestation rate average of 6%.

In order to detect the *Varroa* associated viruses (DWV genotypes A and B), all samples of worker bees were tested by RT-PCR using specific primers for each variant (table 1). Based on the obtained results, from fifty six non-deformed wings' (i.e., asymptomatic bees) worker bees tested, 15 samples were demonstrated to be positive for DWV genotype A (5 colonies in Jendouba, 6 colonies in Beja and 4 colonies in Bizerte), while four were positive for DWV genotype B (2 colonies in Jendouba, one colony in Beja and one colony in Bizerte). We report here for the first time the presence of DWV genotype B in the non-deformed wings' *A. mellifera intermissa*. No virus infection was detected in Siliana apiaries although its high *Varroa* infestation rate (17%).

To confirm the identities of the detected viruses, twenty positive PCR products were sequenced and then aligned with available sequences in Genbank using BLAST database search (Altschul *et al.*, 1990) on the NCBI. Alignment results showed homologies ranging from 94 to 100% for DWV genotype A and 93 to 99% for DWV genotype B (VDV-1) with respective viral polyprotein gene sequences from United Kingdom, France, Syria and Brazil (figure 2). The sole symptomatic sample from Beja used as positive control for DWV genotype A (KX279943), showed 99% identity with a DWV strain from South West England (DQ434951).

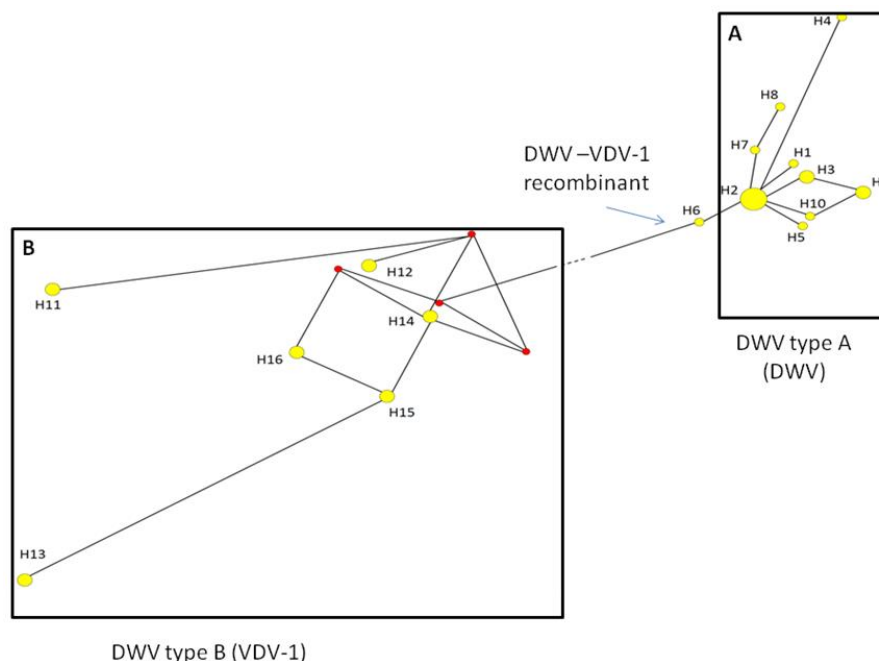


Figure 2. Identified haplotype of DWV gene sequences in *A. m. intermissa*. Intraspecific phylogeny was constructed by Median joining network (Bandelt *et al.*, 1999). H1-5 and H7-10: network of eight observed haplogroups shows the distribution of sixteen DWV genotype A sequences from different geographical regions. H6: haplotype of observed recombinant virus between DWV genotype A and B. H11-16: network of six observed haplotypes shows the distribution of four DWV genotype B sequences from different geographical regions.

The sixteen DWV genotype A and four DWV genotype B partial polyprotein sequences obtained from Tunisian honey bee samples and twelve closely related sequences obtained from the Genbank, were used for haplotype diversity of DWV gene sequences, based on Median joining network (Bandelt *et al.*, 1999). The obtained haplotype network showed two groups A and B clustering the DWV (genotype A) and the VDV-1 (DWV genotype B) sequences, respectively; linked with the haplotype 6 (VDV-DWV recombinant; KX373900, DWV genotype C) (figure 2). The group A encompassing DWV genotype A sequences is divided into eight haplogroups (H1-5 and H7-9). Haplotype H1 is the positive control (KX3279943). Haplotype H2 includes eight out of sixteen DWV genotype A sequences, including those from Jendouba, Beja and Bizerte and three published DWV sequences (DQ434965, AY224602 and KY327829) from south west England, France and Syria, respectively.

The DWV genotype A sequence detected in A1.H2 from Bizerte governorate was clustered separately with three sequences from United Kingdom (DWV genotype A: DQ434951, DQ434939 and DWV-VDV-1 recombinant HM06437) and formed the haplotype H3. Two DWV sequences (A27.H4 and A27.H6) from Beja region were clustered separately with two sequences from South of Brazil (DWV genotype A, KT733631 and KT733632) and formed the haplotype H9. These two colonies demonstrated the highest rate of *Varroa* destructor infestation (45% and 33%). The most distant DWV genotype A sequence was detected in sample A4.H3 from Bizerte region and formed an out-group-like clade (homology 95%) designated as haplotype H4. This hive showed a relatively high *Varroa* infestation rate of 23%.

In the group B, the retrieved Tunisian DWV genotype B sequences (n = 4), formed distinct haplotypes (H11-14) and clustered separately with two published sequences/haplotypes (H15 and H16) recovered from United Kingdom (DWV genotype B: KC78622 and VDV-1-DWV recombinant HM067438, DWV genotype C).

Interestingly, two sequences were recovered from sample A1.H2 from Bizerte that showed a double infection with both DWV genotypes A and B. While the DWV genotype A related sequence fall in haplotype 3 as described above, sequence related to DWV genotype B (KX279945) formed the haplotype H12 separately clustered with two sequences/haplotypes from United Kingdom: haplotype H15 relative to a DWV genotype B (VDV-1 sequence, KC786222) and haplotype H16 generated from a recombinant virus sequence DWV genotype C (VDV-1-DWV, HM067438). Additional experiments based on cloning and sequencing of clones or next-generation sequencing must be conducted to confirm these overall results, particularly the occurrence of a double infection in the same worker bees.

Statistical analyses were performed to better understand the connection between these biotic stress factors occurring in the hive ecosystem. Spearman's rank correlation rho was performed to reveal associations between parasites and DWV genotypes (A and B). Using Spear-

man test ($p = 0.036$, $\rho = 0.1225$, $S = 2567$), a significant association was found between DWV genotype A and its presumable vector *Varroa*.

In fact, most of the areas are infected with the *Varroa* mite, except two hives in Bizerte region, A1.H4 with no infection by DWV genotypes A or B, and interestingly, A3.H4 infected by DWV genotype A. Conversely, several *Varroa*-infected apiaries do not harbour any DWV variants such as at the Siliana apiaries (figure 1).

Nosema spp.

In the current work, microscopic observation and PCR-based detection of *Nosema* microsporidia revealed their presence in Tunisian apiaries.

Throughout this study, *N. ceranae* was the dominant species detected in 6 colonies (Bizerte, Jendouba and Beja) whereas *N. apis* was shown to be present in a single colony in Beja region (A27.H4).

This latter presents a double infection by both microsporidian species. Interestingly, A27.H4 showed the highest *Varroa* infestation rate (45%), beside a positive detection of DWV. Considering the visibly weak state of the colony, it is clear that the co-occurrence of several pathogens and parasites increases the risk of colony collapse.

Statistical analysis, indicates evidences for a significant association ($p < 0.05$) between DWV genotype A and *N. ceranae* (Spearman $p = 0.004$, $\rho = 0.3768$, $S = 1823$). No other correlations were found between viruses and between DWV and *N. apis*.

Discussion

Honey bee pathogens and parasites, and their association, are thought to represent the major routes of colony collapsing (Nazzi *et al.*, 2012; Vanbergen, 2013; Ryabov *et al.*, 2014). It is actually assumed that honey bee colony losses are generated by the interaction between the mite *V. destructor*, the associated deformed wing virus (DWV) and others pathogens and stress factors (Le Conte *et al.*, 2010; Ryabov *et al.*, 2014; Meixner *et al.*, 2015). DWV and its vector might be classified as the two important pathogens to the world's honey bee (Ball, 1983; Ball and Allen, 1988; Hung *et al.*, 1995; Martin *et al.*, 2012; Ryabov *et al.*, 2014; Wilfert *et al.*, 2016). Therefore, there is an urgent need to understand the diversity of RNA viruses and their epidemiological link with parasites and pathogens (*Varroa* and *Nosema*) in order to better manage honey bee's diseases and colony collapse.

In the present study, we investigated *Varroa* infestation rates in northern Tunisia. Sampling from infested apiaries allowed us to distinguish two groups of colonies with low level in Bizerte and high *Varroa* mite infestation in the other governorates. The major *Varroa* infestation is located in two colonies respectively from Beja and Jendouba (45% in A27.H4 and 44% in A12.H4). The A27.H4 sample showed multiple infections by both microsporidia parasites (*N. apis* and *N. ceranae*) and DWV, beside the *Varroa* mite. In A27.H4 colony, a significant decrease in honey produc-

tion and honey bee population is observed suggesting that parasites and pathogens association could lead to honey bee decline as reported earlier (Martin *et al.*, 2012; Nazzi *et al.*, 2012; Vanbergen, 2013).

DWV and VDV-1 were initially considered two different genotypes. Recently, DWV was named parental species that includes different strains or genotypes (A, B and C) (Mordecai *et al.*, 2016b; Dalmon *et al.*, 2017). All DWV positive samples were detected in *Varroa*-infested hives except the A3.H4 sample from Bizerte. In the other hand, *Varroa*-infected honey bees from Siliana apiaries were shown to not harbour any virus. As reported earlier, when colonies were treated against the *Varroa* mite, DWV became undetectable (Martin *et al.*, 2010). Statistical analysis showed significant correlation between *Varroa* and DWV. These findings do not support the general assumption that *V. destructor* is the primary virus vector (Genersch and Aubert, 2010; Francis *et al.*, 2013). Indeed, horizontal transmission of DWV vectored by *Varroa* is usually associated with clinical symptoms and colony collapse (Chen *et al.*, 2006; Yue *et al.*, 2007; Martin *et al.*, 2010; Francis *et al.*, 2013; Ryabov *et al.*, 2014). Independently to the *Varroa* mite, transmission of DWV via larval food or from the queens favours virus persistence with low virulence and as latent benign infection (Chen *et al.*, 2006; Yue *et al.*, 2007). Therefore, the mode of transmission appears to be an important strategy to involve the dynamics of host-pathogens interaction.

Overall, we found a lower occurrence of DWV genotype B (VDV-1, 7%) compared to DWV genotype A (27%). The DWV genotype B lower prevalence could be explained by the fact that this variant has much less efficient replication in bees with normally developed wings as reported in previous studies (Zioni *et al.*, 2011; Dalmon *et al.*, 2017). This observation is also in agreement with the hypothesis suggesting that DWV genotype B is less virulent than other master variants (Mordecai *et al.*, 2016b; Benaets *et al.*, 2017; Kevill *et al.*, 2017).

These results represent the first report of DWV genotype B infection in Tunisia or even most in the North African Region. Sequencing results showed high similarity between detected viruses and viruses' references genomes in the NCBI. RNA sequences showed that Tunisian DWV genotype A can be clustered into eight haplotypes (figure 2), with different DWV genotype A sequences occurring in the same apiary. This was the case of A3.H2 and A3.H4 in Bizerte; A12.H2 and A12.H4, A13.H7 and A13.H5 in Jendouba apiaries; and A27.H4 and A27.H6 from Beja hives.

The majority of DWV genotype A sequences were assembled with the symptomatic one while all these genotypes were asymptomatic. Based on these results, we can support previous assumptions suggesting that the appearance of symptoms is related to multiple factors which lead to high level of infection triggering the infection process (Yang and Cox-Foster, 2005; Highfield *et al.*, 2009; Zioni *et al.*, 2011; Dalmon *et al.*, 2017). The ectoparasite *Varroa* mite has a damage effect on honey bee immunity via viruses replication causing obvious symptoms (Yang and Cox-Foster, 2005; Nazzi *et al.*,

2012). The question remains not completely elucidated on how *Varroa*-viruses affect the immunity and viral amplification. *Varroa* mite infestation contribute to the induction of the immunosuppression by reducing the expression of gene encoding antibacterial peptides driven by viral replication (Yang and Cox-Foster, 2005; Nazzi *et al.*, 2012; Ryabov *et al.*, 2014). This interaction harbours asymptomatic workers with silent infection or symptomatic infection showed by elevated virus level and/or introducing virulent virus strain (Ryabov *et al.*, 2014).

The host subspecies of honey bee seems to not affect the occurrence of the virus. In fact, DWV genotypes reported from United Kingdom, France, and South of Brazil were related to *Apis mellifera ligustica* Spinola (Yue and Genersch, 2005; de Miranda and Genersch, 2010) and *Apis mellifera syriaca* Skorikov from Syria (Alburaki *et al.*, 2013). Similarly, local sampled honey bees *A. m. intermissa* as well as Algerian honey bees (Loucif-Ayad *et al.*, 2013) harbour closely related DWV genotypes. It would be worthy to achieve in-depth investigation of the genetic diversity of DWV in relation to the virulence, the *Varroa* and honey bee host species. It is however worthy to note that, due to limitations in the Sanger sequencing of PCR products from viruses, additional experiments should be achieved to confirm our findings. This would shed light on the pathogenesis of these pathogens on *A. m. intermissa* species in relation to their genetic background.

Unlike DWV genotype A, the four DWV genotype B detected amplicons (figure 2) from fifty six colonies of honey bee showed a relatively high conserved sequences (93%-99%) compared to those of United Kingdom genotype (GenBank: KC786222). In that same genotype B, a high sequence similarity (93%-97%) with a VDV-1-DWV recombinant virus genotype (GenBank accession number: HM067438) was also shown respectively with A22.H7 (haplotype H11) and A1.H2 (haplotype H12) from Beja and Bizerte samples (figure 2). Most importantly, the occurrence of double infection in the same apiary was observed in this later A1.H2 sample where the DWV genotype A sequence was closely related to another VDV-1-DWV recombinant, with 99% nucleotide identity (HM067437). These results suggest that the A1.H2 sample contained recombinant between two closely related viruses in African honey bee.

Our results indicate that individual bees may harbour a mixture of viruses with no apparent symptoms or pathology. A1.H2 was a complex recombinant between the original DWV genotype A and the DWV genotype B. The ability of the two master variants A and B to infect the same host provides the opportunity of the exclusion of other common honey bee virus A/B recombinant or viral recombinants of DWV genotypes (Moore *et al.*, 2011; Wang *et al.*, 2013; Mordecai *et al.*, 2016a; McMahon *et al.*, 2016). The emergence of this recombinant variant was recently observed to be different variant that belongs to a single species (Mordecai *et al.*, 2016b; Dalmon *et al.*, 2017; Natsopoulou *et al.*, 2017).

The emergence of this double infection may appear to perturb the balance between honey bee colonies and viruses. Here we provide evidence that the ability of the

three DWV master variants (A, B and C) to infect the same colony is responsible for the decline in colony health (McMahon *et al.*, 2016; Kevill *et al.*, 2017).

The current investigation was complemented by a molecular diagnosis of *Nosema* spp. Our data showed the emergence of the two microsporidian *N. apis* and *N. ceranae* in Tunisian honey bee colonies. We found evidence of *N. ceranae* and *N. apis*, respectively, detected in 16% and 2% of the samples. *N. ceranae* species was previously related to colony collapse disorder (Paxton, 2010). This species was isolated from *A. cerana* in China (Fries *et al.*, 1996), then in *Apis mellifera* L. in Taiwan and Spain (Huang *et al.*, 2007; Fries, 2010). Beside its prevalence in *A. m. intermissa*, our data indicate a significant association between *N. ceranae* and DWV (Spearman $p = 0.004$). The closely related honey bees gut pathogen *N. apis* produced diarrhea signs (Bailey and Ball, 1991). Due to its very low incidence, no relationship between *N. apis* and DWV was found. To our knowledge, this is the first detection of this species in *A. m. intermissa* from Tunisian honey bee colonies.

Taken as a whole, none DWV genotype B or *N. apis* showed significant associations with DWV genotype A or *Varroa* whereas significant correlations between DWV/*Varroa* and DWV/*N. ceranae* were revealed. Such synergistic association could be observed in the A27.H4 Beja sample considered as case scenario of multiple infections (*Varroa* 45%, *N. apis*, *N. ceranae* and DWV genotype A) that may lead to honey bee decline. In the MENA region and especially in Tunisia the collapse of honey bee colonies phenomenon exists but is not very marked (Adjlane *et al.*, 2012; Haddad *et al.*, 2015). These abnormal losses of honey bee colonies have multiple causes and are often reported by beekeepers. In preceding studies, in areas where DWV strains emerge with the invasion of the mite *V. destructor*, collapse of honey bee colonies is established (Yang and Cox-Foster, 2007; Schroeder and Martin, 2012). Beside this pathogenic interaction, other associated abiotic stressors, mainly related to climate and pesticide use, could also be involved in colony losses and may give explanation on the relative resistance of African honey bees.

Conclusion

This study may constitute a first reporting for the presence of *Nosema* spp. and the genotypic diversity of DWV in Tunisia apiaries. These biotic stressors are investigated with absence of clinical signs observable at the individual bee and/or bees' populations. Transmission of honey bee pathogens can be explained by the contact between infectious beekeeping equipment, infected and uninfected individuals. Our finding supports the presence of recombinant variant between DWV genotypes A and B. Therefore, additional experiment should be performed to confirm the emergence of DWV-VDV-1 recombinant (DWV genotype C) in Tunisia and worldwide.

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Authors' addresses: Ameer CHERIF* (corresponding author, e-mail: cherif.ameur@gmail.com), Khaoula ABDI**, Khaoula BELGUITH, Chadlia HAMDJ*, Yasmine SOUSSI, Jihène ESSANAA*, Tarek HAJJI, Amor MOSBAH, LR Biotechnology and Bio-Geo Resources Valorization (LR11ES31) Higher Institute for Biotechnology - University of Manouba, BiotechPole of Sidi Thabet, 2020 Sidi Thabet, Ariana, Tunisia; Walid DRIDI, Taoufik BEN HAMIDA, Laboratory of Parasitology, Institute of Veterinary Research of Tunis, La Rabta, 1006 Tunis, Tunisia.

Additional addresses:

- * - Laboratory of Microorganisms and Active Biomolecules, Faculty of Sciences of Tunis, University of Tunis El Manar, 2092 Tunis, Tunisia.
- ** - Faculty of Sciences of Tunis, University of Tunis El Manar, 2092 Tunis, Tunisia.

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