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ORIGINAL RESEARCH ARTICLE

Molecular characterization and phylogenetic analysis of acute bee paralysis virus collected from some important beekeeping provinces of Iran

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ABSTRACT

Viruses are the most infectious agents in honey bees (*Apis mellifera* L.). Acute bee paralysis virus (ABPV) is one of the honey bee viruses. ABPV is a small single-stranded RNA virus. The aim of this study is molecular detection of the aforementioned viruses and phylogenetic analysis of ABPV in Iran. Samples were taken from 89 suspected apiaries of four important beekeeping provinces (Mazandaran, Khorasan Razavi, Hormozgan, and Kurdistan). ABPV was detected in the samples with RT-PCR and sequencing was confirmed for all positive samples. In this research, we studied the phylogenetic analysis of ABPV. Nucleotide sequence analyses were used to identify ABPV and phylogenetic analysis was 94.5% to 98.9% identical to the reference strains. IR-APV-GMG-1 and IR-APV-GMG-3 showed maximum nucleotide sequence similarity to Hungary 1 (accession number: AF486072) from Hungary, but IR-APV-GMG-2 had no similarity to reference strains, forming a distinct group. The phylogenetic tree for the molecular relationship between the above viruses was drawn. This study of the phylogenetic analysis of ABPV indicates the presence of this virus and reveals its similarity to European-origin-related viruses.

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KEYWORDS
Acute bee paralysis virus; Iran; RT-PCR

Introduction

At least 32 viruses have been reported to be related to honey bees (*Apis mellifera* L.) (Allen & Ball, 1996; Bailey & Ball, 2013; Beaurepaire et al., 2020). The most important ones are sacbrood virus (SBV) and deformed wing virus (DWV), which belong to the genus Iflavirus of the Iflaviridae family, and Kashmir Bee Virus (KBV), Acute Bee Paralysis Virus (ABPV), and Black Queen Cell Virus (BQCV), which are assigned to the genus Cripavirus (family Dicistroviridae) (Mayo, 2002a). However, the chronic bee paralysis virus (CBPV) remains unclassified. ABPV with a global distribution was described to be the cause of unapparent infections of the honey bee for the first time (Bailey et al., 1963; Carpana et al., 1991; Faucon et al., 1992; Hung et al., 1995; Nordström et al., 1999; Topolska et al., 1995). The virus is also one of the causes of colony depopulation syndrome (CDS), especially in colonies that are infested with parasitic mites, Varroa destructor (Cox-Foster et al., 2007; Ellis et al., 2010). Furthermore, ABPV was reported to be an early cause of bee mortality in colonies from Germany (Ball & Allen, 1988), Yugoslavia (Ball & Mladjan, 1990), France (Faucon et al., 1992), and the United States of America (Hung et al., 1996). V. destructor plays a significant role in viral infection of bees as the Varroa mite is a possible vector for the virus (Ball & Allen, 1988; Bowen-Walker et al., 1999; Lim et al., 2020). The mite undermines bees and activates the viral infection, eventuating in clinical symptoms and severe losses in apiaries (Ball & Allen, 1988; Brødsgaard et al., 2000; Ilyasov et al., 2021; Ritter et al., 1984). In the United Kingdom, ABPV together with the slow paralysis virus was identified to be responsible for the rapid decrease and death of many colonies infested with the Varroa mite (Ball et al., 1997). The morphology and physiochemical of ABPV are similar to picorna viruses. It is a small (particle diameter 30 nm) single-stranded RNA virus that contains two open reading frames (ORFs) (Mayo, 2002b). ORF1 encodes the nonstructural proteins (RNA-dependent RNA polymerase, helicase, and protease), while ORF2 encodes the three major structural proteins (35, 33, and 24 kDa) and a minor protein (9.4 kDa) (Govan et al., 2000). Recently, a direct diagnosis of bee viral infections has been enabled using the polymerase chain reaction (PCR) (Bakonyi et al., 2002). PCR overcomes difficulties of other detection methods like cross-reactions and

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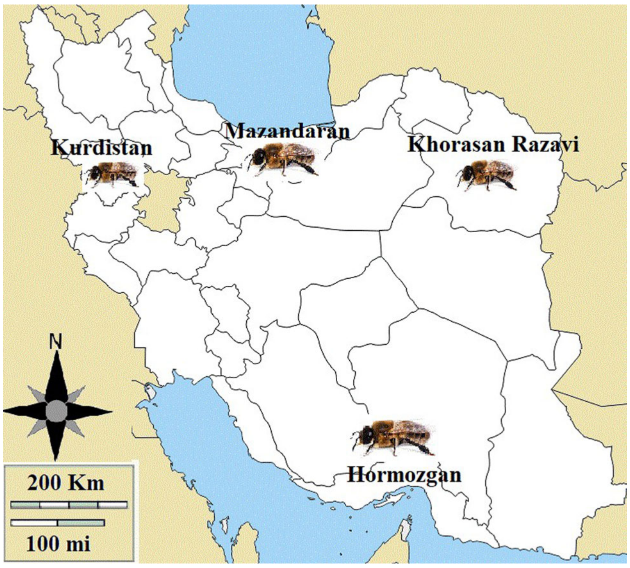

Figure 1. The location of sampling regions. The North: Mazandaran, the South: Hormozgan, the East: Khorasan Razavi, and the West: Kurdistan.

provides an opportunity to identify and characterize honey bee viruses, even in circumstances in which the titer of these viruses is low (Benjeddou et al., 2001; Grabensteiner et al., 2001; Hung & Shimanuki, 1999, 2000). In this research, we used RT-PCR as a molecular method for the detection of ABPV in the samples. The nucleotide sequences of the ABPV RNA-dependent RNA polymerase gene region of different Iranian isolates were also compared and the genetic relationship between the ABPV strains of various geographic regions was determined.

Materials and methods

Study area and sample collection

Four different geographical regions of Iran in which the beekeeping industry has expanded were selected [Mazandaran (north), Hormozgan (south), Khorasan Razavi (east), Kurdistan (west)] (Figure 1, Table 1). Beekeeping industries have been originated from the aforementioned provinces. The samples were taken from colonies with symptoms of depopulation, sudden collapse, paralysis, or dark color. Eighty-nine samples were randomly collected between October 2015 to September 2016, of which 23 were from Mazandaran, 20 from Hormogan, 23 from Khorasan Razavi, and 23 from Kurdistan apiaries. From each apiary, 250 adult dead worker bees were sent to the virology laboratory. All of the specimens were pulled and stored at −20 °C until analysis onset.

Extraction of RNA and synthesis of cDNA

Sterile diethylpyrocarbonate (DEPC) treated water was used for the homogenization of the samples in ceramic mortars. After centrifugation at 20,000 g for 1 min, 140 μl of supernatant was used for the RNA extraction (Berényi et al., 2006). Viral RNA was obtained using a silica-based CinnaPure RNA extraction kit (Sinaclone, Iran) according to manufacturer instructions. After adding 1 μl (0.2 μg) of random hexamer primer (SinaClone, Iran) to 5 μl of extracted RNA, the mixture was heated at 65 °C for 5 minutes to synthesize cDNA. Fourteen μl of cDNA master mix containing 7.25 μl of DEPC-treated water (SinaClone, Iran), 2 μl of dNTP mix (SinaClone, Iran), 0.25 μl of Ribo Lock RNase Inhibitor (Thermo Fisher Scientific, USA), 0.5 μl of Revert Aid Reverse Transcriptase (Thermo Fisher Scientific, USA), and 4 μl of 5X RT reaction buffer was added to each tube, resulting in a final volume of 20 μl. Then, the mixture was incubated at 25 °C for 5 min, 42 °C for 60 min, 95 °C for 5 min, and 4 °C for 1 min. Finally, the cDNA was stored at −20 °C until use.

Table 1. The geographical features of the sampling sites.

Province name	Geographical location	Area
Mazandaran	36.5656°N 53.0588°E	23,833 km^2
Hormozgan	27.1884°N 56.2768°E	70,697 km^2
Khorasan Razavi	36.2980°N 59.6057°E	118,884 km^2
Kurdistan	35.3113°N 46.9960°E	29,137 km^2

RT-PCR for ABPV detection

Table 2 lists primers used to detect ABPV by RT-PCR. The PCR condition for amplification was 95 °C for 5 min, 35 cycles of 95 °C for 30 s (denaturation), 50 °C for 30 s (annealing), and 72 °C for 30 s (polymerization), followed by 72 °C for 10 min. The products were sent to Bioneer Company, Korea to be sequenced for confirmation.

Bioinformatics and phylogenetic analysis

An AccuPrep® PCR Purification Kit (Bioneer Co., Korea) was used for the purification of the PCR products. Sequencing was performed with the primers (both directions) used after the PCR (Bioneer Co., Korea). The neighbor-joining method and MEGA 7.0.21 software were used for the construction of the phylogenetic tree. The nucleotide sequences of the RNA-dependent RNA polymerase (RdRp) gene were compared with several RdRp sequences from GenBank. ABPV sequences were aligned and compared with reference strains and the obtained sequence was submitted to the NCBI GenBank database. Table 3 indicates the country of origin, strain name, and GenBank accession number of the ABPV strains.

Results

ABPV was detected using RT-PCR in the samples of two provinces. Of the 89 apiaries examined, three of

Table 2. Primes used for the detection of ABPV.

Primer	Primer sequence (5′–3′)	Length of product (bp)	Gene	Reference
ABPV-F	TGAGAACACCTGTAATGTGG	452	RNA-dependent RNA polymerase (RdRp)	(Tentcheva et al., 2004)
ABPV-R	ACCAGAGGGTTGACTGTGTG			

Table 3. Data for reference ABPV isolates used in this study.

Isolate (strain)	Country	Gen Bank (accession number)
R8/2008	Slovenia	HQ877400
R4/2007	Slovenia	HQ877402
M99/2010	Slovenia	HQ877404
Hungary 1	Hungary	AF486072
DP2	United Kingdom	DQ434978
AV2	United Kingdom	DQ434990
JH3	United Kingdom	DQ434984

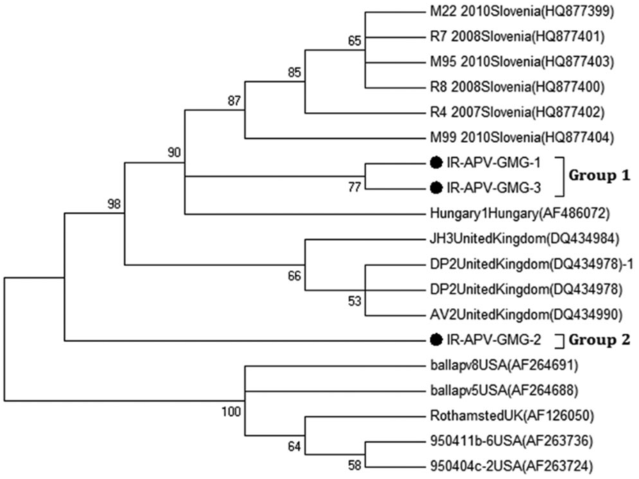

Figure 2. Phylogenetic tree derived from the RdRp. Phylogenetic tree indicating the genetic relationship between Iranian ABPV (IR-APV-GMG-1, IR-APV-GMG-3, IR-APV-GMG-2) samples based on the RdRp gene according to reference strain HQ877404, AF486072, DQ434984 from the Slovenia, Hungary, and United Kingdom respectively.

Table 4. Percent identity of partial nucleotide sequences of the nonstructural polyprotein (RdRp) genes of some Iranian ABPVs to those of ABPV reference strains.

	R4/2007	M99/2010	Hungary 1	JH3	DP2	AV2	R8/2008	IR-APV-GMG-1	IR-APV-GMG-3
R4/2007	100	98.339	97.502	93.918	94.236	93.938	99.453	97.492	97.200
M99/2010	98.339	100	97.496	93.646	93.964	93.667	98.334	97.486	97.194
Hungary 1	97.502	97.496	100	95.148	95.454	95.163	97.494	98.902	98.622
JH3	93.918	93.646	95.148	100	99.727	99.453	93.297	94.537	94.223
DP2	94.236	93.964	95.454	99.727	100	99.728	93.619	94.849	94.537
AV2	93.938	93.667	95.163	99.453	99.728	100	93.319	94.555	94.241
R8/2008	99.453	98.334	97.494	93.297	93.619	93.319	100	97.484	97.191
IR-APV-GMG-1	97.492	97.486	98.902	94.537	94.849	94.555	97.484	100	99.727
IR-APV-GMG-3	97.200	97.194	98.622	94.223	94.537	94.241	97.191	99.727	100

them (3.37%), were infected with the ABPV (Kurdistan:2, Hormozgan:1). All positive samples were confirmed by sequencing and three ABPV isolates were identified among all the collected samples. Phylogenetic analysis based on the RNA-dependent RNA polymerase nucleotide sequences showed no significant difference between Iranian isolates (Figure 2). IR-APV-GMG-1 (accession number: OK170031) and IR-APV-GMG-3 (accession number: OK170033) from Kurdistan showed maximum nucleotide sequence similarity to Hungary 1 (accession number: AF486072) from Hungary (Table 4). IR-APV-GMG-2 (accession number: OK170032) from Hormozgan organized a separate group.

Discussion

ABPV is one of the bee viruses causing paralysis and economic losses in the beekeeping industry. Since ABPV has a worldwide distribution, the genomic analysis of further isolates from other continents may increase our knowledge of the virus diversity. RT-PCR is one of the molecular techniques for the detection of RNA viruses. Our knowledge of the molecular properties and genome organizations of this virus is the result of some studies (Bakonyi et al., 2002; Govan et al., 2000). Bee viral diseases still suffer from the scarcity of information on taxonomy and serotype classification (Bakonyi et al., 2002; Chen & Siede, 2007; Evans & Hung, 2000; Gisder & Genersch, 2015). The relationships between and within different species can be exactly and reliably assessed by molecular comparison of various isolates because it directly detects the changes of the genetic information in the course of evolution and employs the data for statistical evaluation. Moreover, molecular phylogenetic should be considered for clarification of the relationships between bee viruses as a whole. In Iran, the presence of ABPV RNA was demonstrated in 5.62% of unhealthy apiaries (Moharrami & Modirrousta, 2016). CBPV was molecularly detected by Modirrousta and Moharrami for the first time in Iran (Modirrousta & Moharrami, 2015). Also, the presence of three honey bee viruses—SBV, BQCV, and CBPV—has been reported from Semnan province apiaries in Iran (Moharrami et al., 2012). Our team also evaluated the presence of six honey bee viruses in bee samples collected from sick colonies using molecular techniques (Ghorani et al., 2017a, 2017b). In the present study, 89 samples were collected from sick colonies of Iranian apiaries were molecularly evaluated and the presence of ABPV was confirmed. The specimens were sent to the laboratory for the diagnosis of viral infections in affected colonies. We evaluated the molecular presence of ABPV in the samples and study the phylogenetic analysis of ABPV. This study is part of the honey bee viral infections assessment program in Iran, where an unusual loss in the adult bee population and significant honey bee mortality during the study period were recorded. Three (3.37%) out of the 89 apiaries examined were infected by the ABPV. RT-PCR assays employing primer designed for the amplification of fragments covering the RdRp gene region of the ABPV were developed. Due to the relatively conserved region of the genome, this study as the first one, focused on the nonstructural polyprotein (RdRp) to determine the primary relationship of Iranian viruses to reference strains. The phylogenetic tree indicated the molecular relationship between the viruses of different geographic regions and reference strains. The isolates were 94.5% to 98.9% identical to the reference strains (HQ877404: Slovenia, HQ877402: Slovenia, AF486072: Hungary) (Table 4). According to Figure 2, the Iranian ABPV was divided into two groups: Group 1: IR-APV-GMG-1 and IR-APV-GMG-3 showed maximum nucleotide sequence similarity to Hungary 1 (accession number: AF486072). Group 2: IR-APV-GMG-2 formed a new group (Group 2), since it was not similar to other reference strains, belonging to a second distinct cluster. Similarities between IR-APV-GMG-1 and IR-APV-GMG-3 in Group 1 with reference strains may refer to importing the beekeeping supplies, bee colonies, and queens from Eastern Europe countries. This is only a hypothesis because according to the Iranian Veterinary Organization Office, beekeeping supplies were not imported officially borders. Iran is a large country with a wide border with different uncontrolled countries, therefore, veterinary offices are needed to control the borders to control bee virus entrance.

Disclosure statement

No potential competing interests were reported by the authors.

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