DETECTION OF HOP VIRUSES AND VIROIDS BY RT-PCR IN THE CZECH REPUBLIC

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The hop plant, *Humulus lupulus* L., is a dioecious perennial species, and only female cones are used for beer brewing. Hop as a perennial and vegetative-propagated crop is endangered by viruses and viroids. The most important viruses are: Apple mosaic virus (ApMV) from the genus Ilarvirus, Hop mosaic virus (HMV) and Hop latent virus (HLV) from the genus Carlavirus. Hop latent viroid (HLVd) from the group Pospiviroid is the most frequent viroid found out in hops. DAS-ELISA, Dot-blot molecular hybridization and RT-PCR have been used for control of infection hop plant status (PATZAK et al. 2001; SEIGNER et al. 2014; ZIEGLER et al. 2014). Nowadays, the use of real time PCR systems have enabled to precise quantification of viruses and viroids (ZIEGLER et al. 2014; WINKOWSKÁ et al. 2016; PAPAYIANNIS 2014).
In our study, we developed real-time quantitative RT-PCR (RT qRT-PCR) specific primers for detection of ApMV, HMV, HLV and HLVd RNA molecules in hop plants (Table 1). The results of virus infection plant status were comparable with DAS-ELISA results (Table 2). We confirmed that HMV antibodies could cross-react with HLV coat protein. Reference genes, (GAPDH, DRH1) were used for relative virus quantification (MATOUŠEK et al. 2012). Viroid quantification was absolute to known concentration levels in tissues obtained by Dot-blot molecular hybridization (Table 3). We proved that this one-step RT qRT-PCR has been the valuable tool for molecular detection of virus and viroid pathogens within hop plants.

MATERIALS AND METHODS

MegAlign module (LASERGENE system v. 7.1, DNAStar, Madison, WI, USA) was used for multiple alignments of ApMV (GRIMOVÁ et al. 2013), HpMV (POKE et al. 2010) and HLV (ZIEGLER et al. 2014) sequences to find conservative regions of coat proteins (Figure 1). RealTimeDesign software (LGC Biosearch Technologies, Petaluma, CA, USA) was used for design of real time PCR primers in these regions as in sequence of HLVd (X07397). Therefore, qRT-PCR primers for HLV hadn't found we used PrimerSelect module (LASERGENE system v. 7.1, DNAStar, Madison, WI, USA) for their design. All primer sequences were shown in Table 1.
For real-time quantitative RT-PCR (RT qRT-PCR), total RNAs, from the young leaves of infected and health plants from world hop collection of Hop Research Institute Co.Ltd., Žatec, were isolated from 100 mg of samples (FW) using Concert™Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). Ten nanogram of total RNA were used for RT qRT-PCR by QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) according to company protocol. All amplifications were carried out on Real-time PCR cycler CFX Connect (Bio-Rad Laboratories, Hercules, CA, USA), started by reverse transcription at 50°C for 30 min, followed by initial denaturation/Taq activation step (95°C for 15 min), PCR for 45 cycles (94°C for 15 s, 50-58°C for 25 s, 72°C for 20 s) and melting curve analysis (from 65°C to 95°C) (Figure 2). Annealing temperatures of primers were presented in Table 1. Reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and DEAD-box RNA helicase 1 (DRH1) (MALOUKH et al. 2009; MATOUŠEK et al. 2012) were used for relative quantification by "Delta-delta method" (PFAFFL 2001). The calibration curves were used in relative (ApMV, HMV, HLV) and absolute (HLVd) quantifications (Figure 3, 4) based on known concentrations of diluted standards cloned in plasmid pSC-A-amp/kann (Agilent Technologies, Santa Clara, CA, USA).
DAS-ELISA for ApMV and HMV (SEIGNER et al. 2014) were used for control of plant samples virus infection status (Figure 5) according to company protocols (Agdia, Elkhart, IN, USA; Bioreba AG, Reinach, Switzerland; Leowe Biochemica GmbH, Saurlach, FRG). Dot-blot molecular hybridization with 32P[dCTP]-labelled cDNA HLVd probe was used for control of plant samples viroid infection status (MATOUŠEK et al. 1994).

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Table 1. Primer sequences for individual hop viruses and viroids. Ta – PCR annealing temperature, Tm – melting temperature of PCR product

Primers	Organism	Primer sequence (5'–3')	Ta (°C)	Product size (bp)	Tm (°C)
ApMV2	Apple mosaic virus	F: GCYCCTCCAAACACAACTYYTG R: CCAGCGGCGAAATTCGTCTTA	58	80	75
HMV1	Hop mosaic virus	F: GCGCAGTGCTGTTCAAATCAC R: ACGCAGCAGTGARTCAAGAG	58	77	81
HLVa	Hop latent virus	F: TCCCGGTGCATCTCTTCCTCATA R: TCTCACGGCCAACTGCTCATAACC	50	512	78
HLVb	Hop latent virus	F: GTGCCACCACGAGTTGTAGCC R: CCGTCCTGGTTGGCTGTCC	50	519	78
HLVd1	Hop latent viroid	F: CACTACGTGACTTACCTGTATGGTG R: GCCTCGCTCGAGTAGGTTTC	59	72	79,5

Figure 1. Aligment of ApMV coat protein sequences from hop (Grimová et al., 2013) by MegAlign module

Figure 2. Differentiated melting curves for ApMV and HLVd

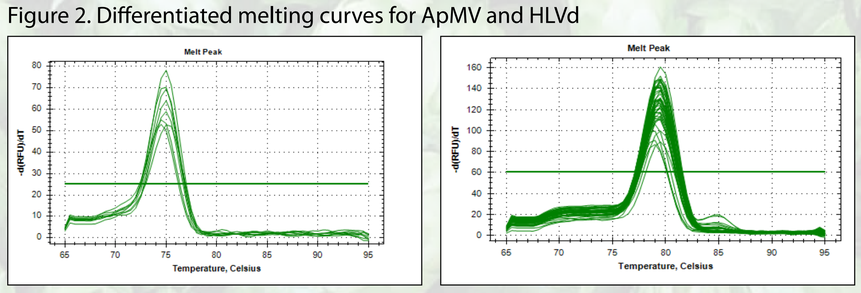

Figure 3. Calibration curves for quantification of ApMV and HLVd

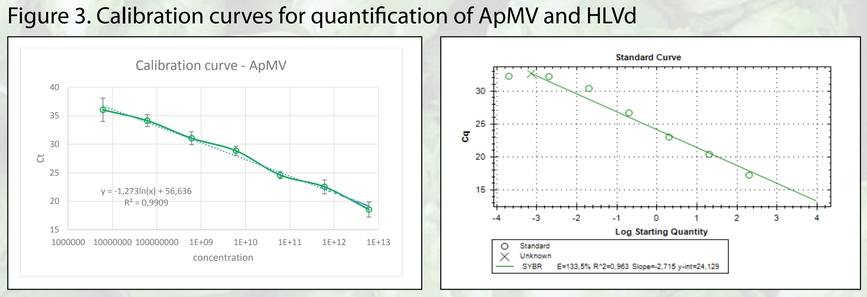

Figure 4. Amplification curves for ApMV and HLVd callibration standards

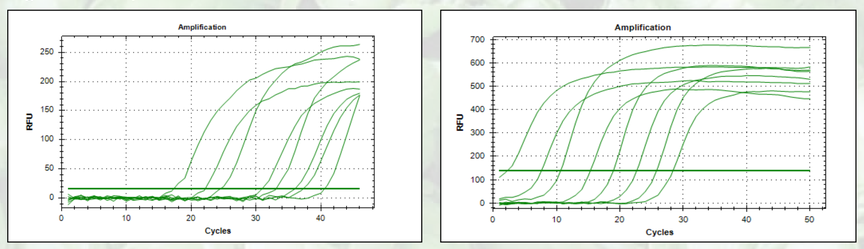

Table 2. Results of viruses quantification by ELISA (extension value) and RT qRT-PCR (relative quantification to reference genes) methods. Values are average of three repetitions.

Samples	ELISA ApMV	ELISA HMV	ApMV	HMV	HLVa	HLVb
ApMV 2	0,494		73,562	0,001	205,996	47,837
ApMV 6	0,914		402,233	0,040	0,088	0,016
ApMV 9	1,748		60,998	0,000	30,281	45,183
ApMV 10	0,508		96,680	1,088	60,132	93,870
ApMV 14	0,041	0,046	0,246	0,003	0,133	0,015
ApMV 16	0,024	0,047	0,088	0,006	0,038	0,013
ApMV 20	0,032	0,048	0,177	0,036	0,332	0,254
HMV 2		0,276	21,571	0,004	25,135	33,748
HMV 3		0,725	1825,992	35,621	10,891	2,153
HMV 5	0,45	0,306	46,264	0,009	16,033	0,881
HMV 6	0,174	0,67	14,927	0,037	59,695	68,907
HMV 11	0,057	0,047	0,166	0,018	1,177	0,044
HMV 16		0,047	2,909	0,030	0,005	0,002
Cal 1			87,224	686,445	85,115	17,321
Cal 2			5,560	142,815	14,822	1,504
Cal 3			1,332	15,541	1,364	0,105
Cal 4			0,069	1,838	0,114	0,016
Cal 5			0,015	0,461	0,019	0,005
Cal 6			0,002	0,030	0,003	0,002
Blank	0	0	0,000	0,014	0,002	0,001

Infection status: positive; plus/minus; negative

Table 3. Results of HLVd quantification by molecular hybridization and RT qRT-PCR methods. Values are average of two repetitions in pg/g of FW.

Samples	Mol. Hybrid.	RT qRT-PCR
1	0,1608	0,5497
2	0,0238	0,0028
3	0,1314	0,0732
4	0,0268	0,0025
5	0,1546	0,0622
6	0,1004	0,5392
7	0,0224	0,0041
8	0,0244	0,0035
9	0,1292	0,7523
10	0,1857	0,1711
11	0,0241	0,0253
12	0,0223	0,0035
13	0,0228	0,0006
14	0,0196	0,0040
15	0,0253	0,0115
16	0,0236	0,0110
Cal 1	21,3461	20,2675
Cal 2	1,9930	2,7275
Cal 3	0,2222	0,5930
Cal 4	0,0481	0,1446
Cal 5	0,0241	0,0211
Cal 6	0,0199	0,0060
Blank	0,0000	0,0008

Infection status: strong; middle; weak; negative

Figure 5. DAS-ELISA

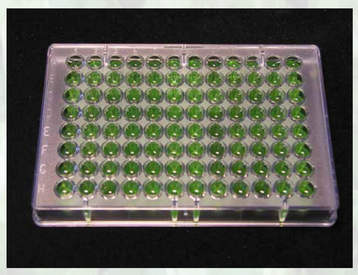
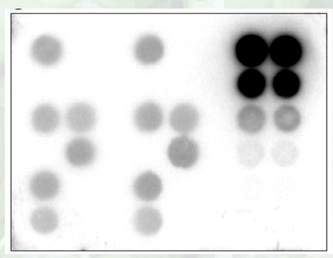
Figure 6. Molecular hybridization with radioactive labelled HLVd probe (Matoušek et al., 1995). Samples 1-16 and callibration standards (Cal 1-6) in two repetitions.