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Original article

Quantitative PCR High-Resolution Melting (qPCR-HRM) curve analysis, a new approach to rapid detection and differentiation of bovine papillomavirus detected in equine sarcoids

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Abstract

The aim of the study was to evaluate a novel diagnostic scheme which combines quantitative PCR and High-Resolution Melting (qPCR-HRM) curve analysis for rapid differentiation based on E5 partial CDS of bovine papillomavirus type 1 or 2 (BPV-1 or BPV-2), and to perform a phylogenetic analysis of the complete CDS of the E5 gene of BPV detected in equine sarcoids. Samples of 38 skin lesions obtained from 27 horses were collected for molecular examinations. All lesions were clinically diagnosed as sarcoids, but results of histopathological examinations did not always corroborate the clinical diagnosis. Although all the samples were positive for the presence of BPV DNA, after qPCR-HRM analysis 6 (16%) specimens were recognized as BPV-1 “wild”, 24 (63%) as BPV-1 “European” and 8 (21%) as a “variant” of BPV E5 ORF partial CDS. Phylogenetic analysis based on nucleotide sequences of E2 ORF partial CDS and E5 ORF complete CDS was conducted on 7 specimens, whose sequences were published in GenBank and recognized as: 2PL (Accession Number – Acc. No. KC684939) – “variant” BPV-1, 7aPL (Acc. No. KC684940) – “European” BPV-1, 10PL (Acc. No. KC693480) – “variant” BPV-1, 16PL (Acc. No. KC693484) – “variant” BPV-2, 17PL (Acc. No. KC693481) – “variant” BPV-1, 20aPL (Acc. No. KC693482) – “European” BPV-1 and 20cPL (Acc. No. KC693483) – “wild” BPV-1. Amino acid (aa) sequences of E5 ORF complete CDS were also analyzed. The E5 variant of aa sequences found in isolate 10PL (protein identification – ID: AGM 20700) is a novel variant of E5 ORF complete CDS of BPV-1 detected in equine sarcoid in Poland.

Key words: BPV-1, BPV-2, equine sarcoids, E5 gene, PCR HRM, phylogenetic analysis

Table 1. Results of histopathological and qPCR HRM examinations.

| Isolate No. | Histopathological lesions Tissue type | qPCR HRM (E5 ORF partial CDS) | Total no |
|-----------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|----------|
| 11 20c, 22b, 23 27b 27a | Hyperkeratosis of epidermis Fibroblastic sarcoid Nodular hyperplasia of connective tissue | BPV-1 “wild” | 6 |
| 1, 4, 5, 6, 8, 9, 12, 13b, 14a, 15, 19, 20a, 20b, 21, 22a, 24a, 25a, 25b, 25c 13a 7a, 7b 26a 26b | Fibroblastic sarcoid Old granulation tissue Scar Hyperkeratosis of epidermis Epidermal cyst | BPV-1 “European” | 24 |
| 2, 10, 24b 3 14b 16 17 18 | Fibroblastic sarcoid Verrucose sarcoid Hyperkeratosis of epidermis Old granulation tissue Nodular hyperplasia of connective tissue Post-inflammatory lesions | BPV “variation” | 8 |

Introduction

Papillomaviruses (PVs) are a group of double-stranded DNA viruses associated with benign and malignant lesions of cutaneous and mucosal epithelia. They are classified in the *Papillomaviridae* family, which comprises 29 genera (Bernard et al. 2010). The number of recognized papillomavirus (PV) species and potential PV genera has been increasing throughout the past decade. To date, more than 150 different human papillomavirus (HPV) types have been identified, but only 13 bovine papillomavirus (BPV) types have been described (Lunardi et al. 2013). PVs are usually considered to be species specific and, even in experimental conditions, do not infect other species. However, BPV-1 and BPV-2 are accepted to be aetiological agents of equine sarcoids (Amtmann et al. 1980, Reid et al. 1994, Nasir and Reid 1999, Chambers et al. 2003a,b).

Equine sarcoids are the most common skin tumours in horses (Marti et al. 1993, Reid et al. 1994). They affect horses of all ages, breeds and hair coats without obvious sex predilection and are encountered worldwide. Sarcoids are divided into six different clinical types: verrucose, fibroblastic, occult, nodular, mixed and malignant (Knottenbelt and Kelly 2000, Knottenbelt 2005). Our previous studies also revealed the presence of fragments of E5 and L1 gene variants of BPV-1 in Poland (Szczerba-Turek et al. 2009, 2010, 2011).

The aim of the present study was to evaluate a novel diagnostic scheme which combines quantitative PCR and High-Resolution Melting (qPCR-HRM) curve analysis for rapid differentiation based on E5

partial CDS of bovine papillomavirus type 1 or 2 (BPV-1 or BPV-2), and to perform a phylogenetic analysis of the complete CDS of the E5 gene of BPV detected in equine sarcoids.

Materials and Methods

Samples

The qPCR-HRM curve analysis was carried out using 38 DNA samples isolated from skin lesions from 27 horses, clinically diagnosed as sarcoids. The results of histopathological examinations are presented in Table 1. The PCR and phylogenetic analysis study were conducted using 7 DNA samples isolated from skin lesions from 6 horses. All sample collection procedures were performed in compliance with international ethical standards for animal welfare.

Gene mutations by qPCR HRM curve analysis

The qPCR-HRM curve analysis was performed using a Corbett Rotor-Gene™ 6000 instrument (Corbett Life Science, Australia) and Type-it® HRM™ PCR kit (Qiagen, Germany). The qPCR-HRM was carried out using E₅L₂up/E₅L₂lo primer sets published by Teifke et al. (1994). The primer set in standard PCR amplified a 242 base pair (bp) fragment of the E5 gene of BPV-1, at nucleotide positions 3760 – 4003 (accession number (Acc. No) – X02346), and a 245 bp fragment of the E5, E25 genes of BPV-2, at positions 3760 – 4006 (Acc. No. M20219). The 25 µl PCR

mixture consisted of 12.5 µl 2x HRM PCR Master Mix, 7.75 µl RNase-free water, 1.75 µl primer mix (final concentration 0.7 µM of each) and 3 µl DNA (60 ng/reaction). A negative control containing all reagents minus DNA was included in each run. The following PCR cycle profile was used: initial PCR activation step: 5 min at 95°C, 40 cycles: denaturation 10s at 95°C, annealing 30s at 55°C, extension 10s at 72°C. The melt analysis was performed once amplification was completed by ramping the temperature from 65°C to 95°C, increasing 0.1° each step with continuous acquisition of fluorescence. The automated genotype calling software (Corbett Life Science, version 1.7) was used to determine the genotypes of individual lines. For the HRM analysis, the fluorescence versus temperature graphs were normalized to 100 to allow all the curves to be compared, thus having the same starting and ending fluorescent signal level. The raw data graph was used to adjust the regions of normalization. The procedure was done according to the protocol provided by the supplier (Corbett Life Science).

PCR procedure

The purpose of the PCR was to amplify the E5 gene complete CDS of BPV-1 or BPV-2. The PCR was carried out using primer sets BPVE5F/BPVE5R published by Chambers et al. (2003b). The reactions were performed using a HotStarTaq Plus PCR kit (Qiagen, Germany). The 20 µl PCR mixture consisted of 4.8 µl of RNase-free water, 10 µl of HotStarTaq Plus Master Mix, 2 µl of CoralLoad, 0.1 µl of BPVE5F primer (final concentration 0.5 µM), 0.1 µl of BPVE5R (a final concentration of 0.5 µM) and 3 µl of matrix (DNA isolated earlier). Cloned BPV-1 was used as a positive control. Ultrapure water was used as negative control for the PCR. The molecular weight of the products obtained was determined on the basis of a GeneRuler™ 100 bp DNA Ladder molecular weight marker (Fermentas, Lithuania). The primer set amplified 603 bp of BPV-1, at nucleotide positions 3661 – 4263 (Acc. No. X02346), and 601 bp fragment of BPV-2, at positions 3662 – 4262 (Acc. No. M20219). The amplicons were purified using the Clean-up purification kit (A&A Biotechnology, Poland) according to the manufacturer's recommendations. Purified amplicons were independently sequenced (Genomed S.A., Poland) in both directions.

Phylogenetic analysis

Sequence data from the specimens were compared to the nucleotide sequence of previously identi-

fied BPVs using BLASTN version 2.2.18. (Altschul et al. 1997). A multiple sequence alignment was carried out using ClustalW (Larkin et al. 2007) and Muscle (Edgar 2004) algorithms incorporated in the freeware Computational Evolutionary Biology package MEGA version 5.2.1. (Tamura et al. 2011). The identification of the nucleotide and aa sequences was determined using BioEdit v.7.2.0 software.

Phylogenetic analysis was carried out with aa sequences of E5 protein of BPV types using the Maximum Likelihood method with WAG+G as an aa substitution model (date not shown).

Nucleotide sequence accession numbers

The nucleotide sequences analyzed in this study are available in the GenBank database under accession numbers KC684939 – KC684940 and KC693480 – KC693484. All of the other representative PVs sequences used in this study for phylogenetic comparison were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>).

Results

The qPCR-HRM results were in high concordance with the sequencing results described previously (Szczerba-Turek et al. 2011). HRM analysis showed that 6 (16%), 24 (63%) and 8 (21%) specimens were identified as BPV-1 – “wild”, BPV-1 – “European” and BPV “variation”, respectively (Table 1). HRM normalized curve of E5 ORF partial CDS from genomic DNA extracted from equine sarcoids is presented in Fig. 1.

Phylogenetic analysis based on nucleotide sequences of E2, E5 ORF of BPV-1 (584 bp) and E25, E5 ORF of BPV-2 (582 bp) is presented in Fig. 2. Isolates 2PL, 7aPL, 10PL, 17PL in comparison with BPV-1 (Acc. No. X02346) showed a 98.3% sequence identity, isolate 20aPL 98.4% and isolate 20cPL 99.8% sequence identity. Isolate 16PL in comparison with BPV-2 (Acc. No. M20219) showed a 98.9% sequence identity. Isolates 2PL, 7aPL in comparison with BPV-1 EqSar1 (Acc. No. JX678969) showed a 99.1% sequence identity and respectively isolates 10PL, 17PL – 98.8%, isolate 20aPL – 99.3% and 20cPL – 98.6% homology. All nucleotide substitutions in nucleotide sequences from own BPV-1 isolates are presented in Table 2, in comparison with the nucleotide sequence of BPV-1 (Acc. No. X02346). The E5 ORF in BPV-1 (Acc. No. X02346) are coded in the position 3878- 4012. In this region isolates: 2PL, 7aPL, 10PL, 17PL, 20aPL showed two of the same nucleot-

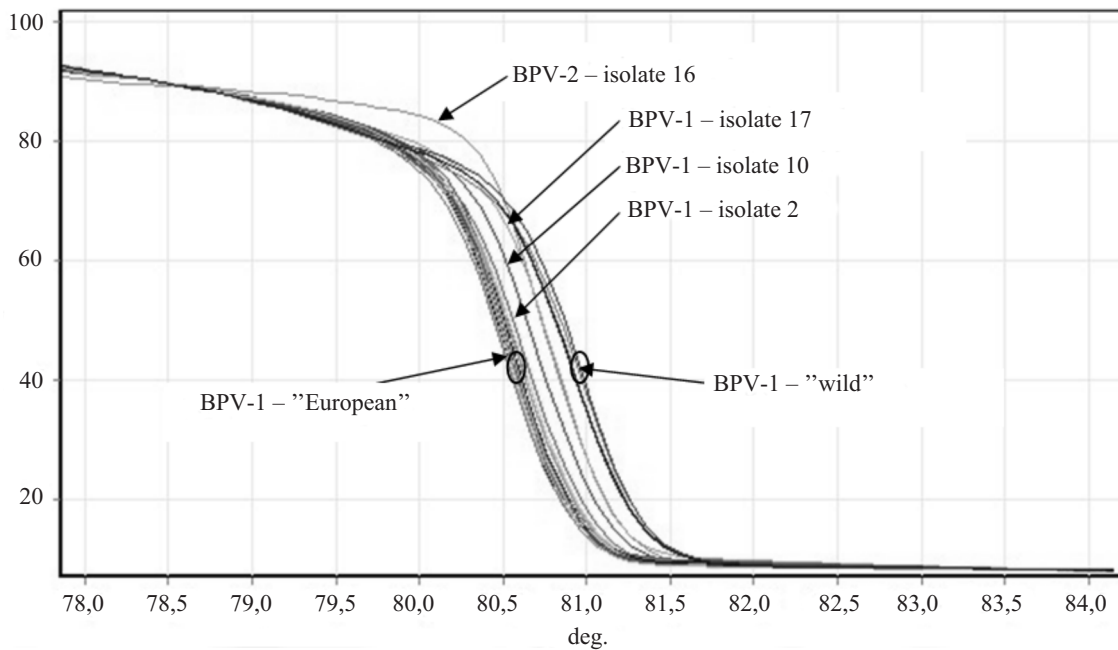


Fig. 1. HRM normalized curve of E5 ORF partial CDS from genomic DNA extracted from equine sarcoids.

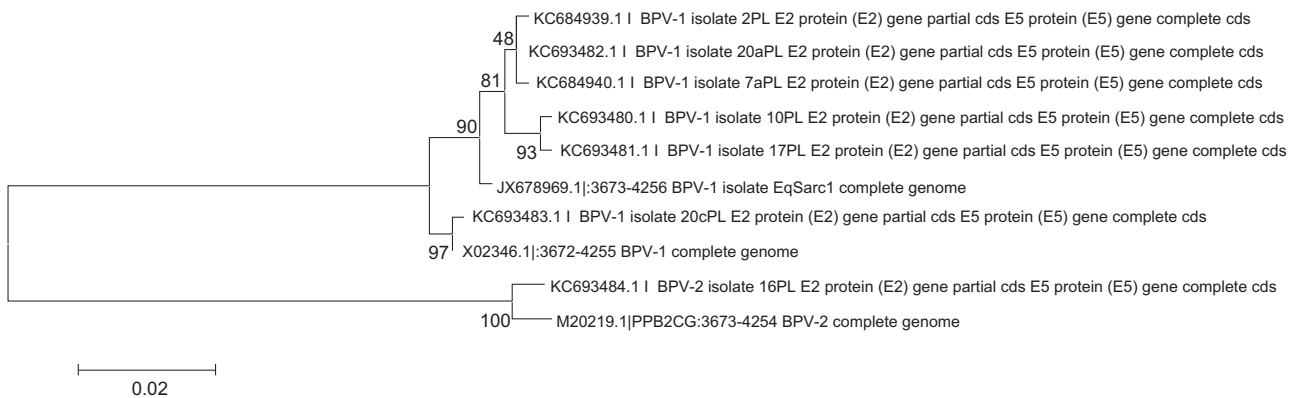


Fig. 2. Evolutionary relationships of 10 taxa of the E2 partial CDS E5 complete CDS of BPV 1 and 2. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-1214.2713) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search was (were) obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2167)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Non-coding. All positions containing gaps and missing data were eliminated. There were a total of 578 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

ide substitutions (G3920T and A3937G) (Table 2). These substitutions are characteristic for the prototype sequence BPV-1 subtype III isolated in Switzerland (sequence available from the GenBank database, Acc. No. AY232259) (Chambers et al. 2003a,b, Yuan et al. 2007a,b). In addition, isolates 10PL and 17PL showed one extra transversion C3929G, T3919G, respectively (Table 2). The G3920T transversion alter the aa sequence, from alanine to serine at residue 15 (isolate 2PL, 7aPL, 10PL, 17PL 20aPL) and the

transversion C3929G in isolate 10PL, alter the aa sequence, from leucine to valine at residue 18 (Fig. 3). The other changes were silent. The E5 variant found in isolate 10PL is a novel variant of E5 ORF complete CDS detected in equine sarcoid in Poland. In isolate 16PL, six nucleotide substitutions were detected, four transversion C3686G, C3687G, T3723A, T3856A and two transition G4099A and C4150T. All of these substitutions were outside the E5 ORF-coding region (BPV-2-M20219 E5 CDS 3882-4016).

| | | 10 | 20 | 30 | 40 | |
|-------------|--------------------|-----------|-------------|-----------|------------|---------|
| NP_056742.1 | [BPV-1] | MPNLWFLFL | GLVAAMQLLL | LLFLLFFLV | YWDHFECSCT | GLPF 44 |
| AAA66838.1 | [BPV-2] | | | | | 44 |
| AFV52371.1 | E5 [BPV-1] EqSarc1 | | | | | 44 |
| AAF69965.1 | [BPV-1] Swiss III | |S..... | | | 44 |
| AGM20696.1 | [BPV-1- 2PL] | |S..... | | | 44 |
| AGM20698.1 | [BPV-1 7aPL] | |S..... | | | 44 |
| AGM20700.1 | [BPV-1 10PL] | |S..V.. | | | 44 |
| AGM20702.1 | [BPV-1 17PL] | |S..... | | | 44 |
| AGM20704.1 | [BPV-1 20aPL] | |S..... | | | 44 |
| AGM20706.1 | [BPV-1 20cPL] | | | | | 44 |
| AGM20708.1 | [BPV-2 16PL] | | | | | 44 |

Fig. 3. E5 ORF complete CDS amino acid (aa) BPV-1 and BPV-2 sequence variation.

Table 2. Nucleotide changes in own BPV-1 specimens examined in position 3672 – 4255 in comparison with the BPV-1 genome.

| Isolate no. | Point mutation – transition | Point mutation – transversion |
|--------------|---------------------------------------------------------|-----------------------------------|
| | Position in BPV-1 Acc. No. X02346 | Position in BPV-1 Acc. No. X02346 |
| 2PL | C3701T, C3853T, T3857C, A3937G, G4035A, T4044C, G4050A, | G3920T, T4066G, T4093A |
| 7aPL | C3701T, C3853T, A3937G, G4035A, T4044C, G4050A, C4215T | G3920T, T4066G, T4093A |
| 10PL | C3701T, C3853T, A3937G, G4035A, C4147T, C4215T | G3920T, C3929G, T4066G, T4093A |
| 17PL | C3701T, C3853T, A3937G, G4035A, C4147T, C4215T | T3919G, G3920T, T4066G, T4093A |
| 20aPL | C3701T, C3853T, A3937G, G4035A, T4044C, G4050A | G3920T, T4066G, T4093A |
| 20cPL | A4022G | |

Discussion

The present results confirm that equine sarcoids are closely related to BPV, especially BPV-1 and need a three level diagnosis: clinical, histopathological and molecular. After clinical diagnosis, all examined specimens were recognized as sarcoids, the histopathological results were different but after molecular examination the BPV DNA in all samples was detected. Positive molecular results after negative histopathology examinations were observed previously by Brandt et al. (2008) and Wobeser et al. (2012).

The qPCR-HRM analysis based on nucleotide sequences approximately 250 bp seems to be a good tool for rapid identification of E5 ORF virus variants. The results were closely correlated with those previously described (Szczerba-Turek et al. 2011). The point mutation in HRM analysis exists in 3763-4002 in comparison with BPV-1 (X02346) – described as a “wild”. At this region, isolates described as “European” had only 3 nucleotide substitutions C3853T, G3920T and A3937G and sequences recognized as BPV

“variation” had 4 nucleotide substitutions at this region (Table 2). The substitutions in BPV-1 “European” were described previously by Chambers et al. (2003a,b) and Yuan et al. (2007a,b). Isolate 16 recognized as BPV-2 in this region had only one substitution T3856A. For this number of point mutations, the qPCR-HRM curve analysis is a good tool for rapid recognition of this BPV E5 gene variation. All of these results were confirmed in phylogenetic analysis of E5 ORF complete CDS (Table 2). Only four isolates: 3, 14b, 18, 24b were recognized not exactly the same as previously (Szczerba-Turek et al. 2011). In the present study, they were recognized as a BPV “variation” of E5 ORF partial CDS, while previously, after phylogenetic analysis of E5 ORF partial CDS isolates, 3 and 18 were recognized as a “European” BPV-1 (phylogenetic group D) and isolates 14b and 24b were recognized as a “wild” BPV-1 (phylogenetic group A1) (Szczerba-Turek et al. 2011). The phylogenetic analysis of these short nucleotide sequences (250 bp) was conducted for all specimens examined and the results of HRM analysis may

suggest the first selection for future sequencing. The HRM analysis confirmed that isolates 2, 10, 16 and 17 were a variation of nucleotide sequences of E5 ORF partial CDS as shown in Fig. 1.

Phylogenetic analysis of nucleotide sequences showed that all sequenced isolates had new sequence variations at a chosen fragment of BPV DNA. Although point mutations detected in the viral gene in this study were often in the encoding region or were silent, only in isolate 10PL did they change the aa sequence and this isolate is a novel variant of E5 ORF complete CDS of BPV-1 (protein ID: AGM 20700) detected in equine sarcoid in Poland.

In summary, data collected in the present study provide important information on the main BPV types/variants associated with equine sarcoids in Poland. The qPCR-HRM curve analysis of E5 ORF partial CDS represents a simple, rapid technique for initial selection of BPV isolates. The results obtained in this study confirm that different variations of E5 partial CDS of BPV can be detected in equine sarcoids (Chambers et al. 2003a,b, Yuan et al. 2007a,b, Szczerba-Turek et al. 2009, 2011).

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