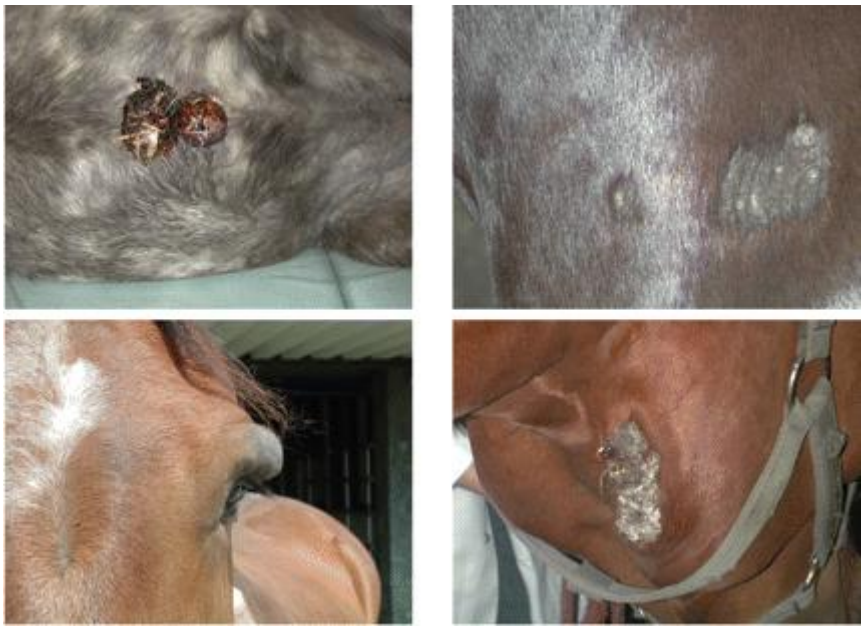
Setting up a PCR for the detection of Bovine Papilloma Virus in equine sarcoids



Bogaert et al., 2007

Research project

D. Smilde 3516210

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Supervisors: E.M. Broens & K.M.H.W. Verstappen
Department of Infectious Diseases & Immunology
Utrecht University, Faculty of Veterinary Medicine

Summary

Introduction. The Bovine Papilloma Virus (BPV) types 1 and 2 are thought to play a causal role in sarcoid pathogenesis. To support a clinical diagnosis of a sarcoid lesion PCR for detection of BPV DNA is performed.
Aim of the study. i) the set-up and validation of a PCR to detect BPV DNA in skin tissue samples and swabs derived from equine sarcoids ii) analysis of retrospective data to evaluate diagnostic value of the BPV PCR with respect to histologic examination.
Materials and methods. Primer- and probe concentrations were optimized using primer- and probe matrices. Two different DNA extraction methods were compared. Fifty-six samples (10 swabs and 46 skin tissue samples) were tested with the newly set-up PCR. Outcomes were compared with results from a reference laboratory (for 23 samples), histological outcomes (for 11 samples) or presumptions of BPV absence (for 22 healthy skin samples). Retrospective data was analyzed by classifying patients based on histologic and PCR test outcomes. Relative sensitivity, relative specificity, overall agreement and Cohen's kappa were calculated.
Results. The 300 nM primer concentration and 50 nM probe concentration were identified as optimal concentrations. The DNeasy® Blood & Tissue kit (Qiagen) was the least labor intensive method for DNA extraction of biopsies. The High Pure PCR Template Preparation kit (Roche diagnostics) yielded significantly lower Cp values for DNA extraction of swabs ($p=0.016$). For 22/23 samples outcomes of the newly set-up PCR agreed with the outcomes of the reference laboratory. For 8/11 samples outcomes of the newly set-up PCR agreed with histologic outcomes. In all 22 healthy skin samples no BPV was detected with the newly set-up PCR. Relative sensitivity of the PCR compared to histologic examination was 98.4%, relative specificity was 68.2%. Overall agreement was 90.4%, corresponding with a Cohen's kappa score of 0.729 (95% CI 0.518 – 0.940).
Conclusion. Validation of the PCR was successful for BPV detection in equine skin tissue samples. The PCR can be applied as diagnostic tool, though PCR outcomes must always be interpreted in combination with clinical data. Validation of the PCR for BPV detection in swabs was not yet finished.

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Introduction

Equine sarcoids

Equine sarcoids are non-metastasizing, yet locally invasive skin tumors. They represent the most common neoplasm of the horse, accounting for 12.5 – 67% of all equine neoplasms and 45 – 50% of all equine dermatological neoplasms (Baker and Leyland, 1975; Sullins et al., 1986; Teifke, 1994; Valentine, 2006). Sarcoids can occur anywhere on the body though the muzzle, ears, periocular regions, limbs, neck and traumatized sites seem to be places of predilection. Most patients are affected with one or several sarcoids, but horses can obtain up to hundreds of lesions (Nasir and Campo, 2008).

Sarcoids are classified according to their clinical appearance and behavior. Four singular forms are differentiated: occult, nodular, verrucous and fibroblastic sarcoids. Occult sarcoids are flat alopecic patches, sometimes with hyperkeratosis, mild scaling or small nodules on their surface. They are often quiescent neoplasms that evolve little over several years. Nodular sarcoids are firm, well defined masses with an intact epithelium. Verrucous sarcoids are lesions with a raised wart-like scaly appearance. Most of the nodular and verrucous sarcoids display moderate growth. Fibroblastic sarcoids are large, sometimes ulcerated neoplasms with a cauliflower-like appearance which often grow progressively and more aggressive. Sarcoids may also display characteristics of several types. These mixed sarcoids tend to become more aggressive as they transform towards the fibroblastic type. Occult, nodular and verrucous sarcoids usually remain stable for many years but are able to change suddenly into rapidly growing, mostly fibroblastic neoplasms, especially following trauma (Martens et al., 2000; Bogaert et al., 2007; Taylor and Haldorson, 2012).

Sarcoid presence decreases the value of affected animals because of aesthetical and functional aspects. In addition, treatment is challenging due to a high recurrence rate. Therefore sarcoids are of considerable veterinary importance.

Sarcoid pathogenesis

Bovine papillomavirus (BPV) causes papilloma formation in its natural host cattle. This virus is also known to infect equids, representing the only known cross-species papillomavirus infection; BPV types 1 and 2 are causally associated with equine sarcoid formation. This association was first postulated in the 1950's when scientists succeeded to provoke sarcoid-resembling lesions by inoculating horses intradermally with papilloma extracts derived from cattle (Olson and Cook, 1951; Ragland and Spencer, 1969). Numerous studies followed examining the role of BPV in sarcoid pathogenesis. BPV DNA was detected in up to 100% of examined sarcoids. BPV-1 predominated over BPV-2 (Teifke, 1994; Carr et al., 2001a; Martens et al., 2001b; Bogaert et al., 2007). The exact role of BPV in sarcoid pathogenesis is still unclear, but several pathogeneses have been suggested.

In cattle papillomas have a transient character, BPV virus particles are formed in the epithelium and the BPV life cycle is strictly linked to the differentiation process of infected epidermal cells (Campo, 1997). In horses sarcoids rarely regress. Studies of the 1980's and 1990's could not find viral particles and BPV DNA was solely detected in dermal cells of sarcoids (Amtmann et al., 1980; Lancaster, 1981; Lory et al., 1993; Teifke et al., 1994). Therefore it be can hypothesized that the unnatural equine cellular environment is suboptimal for BPV virion production, resulting in an abortive dermal infection that can favor progression to canker (Doorbar, 2006). However, recent studies detected BPV proteins and mRNA in sarcoid tissue, demonstrating that the virus is active and productive (Carr et al., 2001b; Bogaert et al., 2007). In addition, DNA-capsid complexes have been identified, which possibly represents production of virion precursors or intact virions (Brandt et al., 2008; Brandt et al., 2011).

Research examining the location of BPV in sarcoids succeeded to detect BPV in several epidermal samples. BPV was mainly detected in the epidermis of occult sarcoids and rarely in the epidermis of nodular, fibroblastic and 'advanced' sarcoids (Bogaert et al., 2010; Brandt et

al., 2011; Wobeser et al., 2012). Authors suggested that infection starts at the epidermis as in most papilloma infections in other hosts, but shifts towards the dermis once the sarcoid changes from less to more clinically aggressive (Bogaert et al., 2010; Wobeser et al., 2012).

Recent studies detected BPV DNA in a number of healthy skin samples (Bogaert et al., 2005; Bogaert et al., 2008; Wobeser et al., 2012), suggesting that infection alone is not sufficient for tumor production. Trauma might be necessary to activate the (latently) present BPV by inducing formation of growth factors which encourage proliferation of the infected cells (Campo et al., 1994). This could explain why sarcoids are often seen at traumatized sites. In addition, recurrence following surgical removal of sarcoid neoplasms could be due to activation of BPV in perilesional cells (Martens et al., 2001b). Besides trauma the genetic basis of the patient's immune system might be of influence. In horses an association between the ELA (equine leukocyte antigen) W13 allele and sarcoids was found (Brostrom et al., 1988). This could explain why most experimentally induced sarcoids regress; the inoculated horses might have an immune system that is competent of clearing BPV. Conversely, some of the inoculated animals developed a persistent or recurrent sarcoid. These patients might have a diverging immune system, incapable of destroying the virus (Olson and Cook, 1951; Ragland and Spencer, 1969; Hartl et al., 2011).

Diagnosing sarcoids

A presumptive diagnosis is based on clinical appearance. Differential diagnoses of the equine sarcoids depend on the sarcoid type and include dermatophytis, chronic skin rubbing, equine papillomatosis (warts), hyperkeratosis, equine sarcoidosis, squamous cell carcinoma, fibroma, fibrosarcoma, neurofibroma/neurosarcoma, melanoma and granulation tissue (Knottenbelt et al., 1995).

The diagnosis of sarcoids can be confirmed using histologic examination of a biopsy. At histological observation sarcoids show dermal proliferation of spindle-shaped fibroblasts forming whorls or herringbone patterns. Other alterations like epidermal hyperplasia, hyperkeratosis, rete peg formation (broad invaginations of epidermal cells into the dermis) and 'picket fence' (fibroblasts orientated perpendicular towards the basement membrane) are also commonly seen (Martens et al., 2000). However, histologic changes of sarcoids can be very subtle and diverse, leading to uncertain or ambiguous histologic outcomes.

In addition, a PCR test can be used to detect BPV DNA in biopsies or swabs. Detection of BPV DNA in combination with a clinical sarcoid appearance strongly indicates the diagnosis sarcoid.

Study objective

The aim of this study was to set-up and validate a real time PCR at Veterinary Microbiological Diagnostic Center of Utrecht University (UU VMDC) that detects BPV-1 and BPV-2 DNA in equine skin tissue samples and skin swabs. In addition, the diagnostic value of the BPV PCR compared to histologic examination was evaluated based on retrospective data.

Material and methods

PCR set-up

Primer- and probe matrix

To optimize primer concentrations several mixtures with a probe concentration of 250 nM and primer concentrations ranging from 300 to 900 nM were made based on a chessboard model (annex 1). These mixtures were used in Real Time Polymerase Chain Reaction (RT PCR). Isolated BPV-1 DNA provided by Ghent University served as a template. Probe concentration was optimized the same way using mixtures that contained the optimized primer concentrations and probe concentrations ranging from 50 to 250 nM (annex 1).

Real Time PCR description

DNA amplification reactions using the PCR were run on a LightCycler® 2.0 instrument (Roche diagnostics, Almere, the Netherlands) at Veterinary Microbiological Diagnostic Center of Utrecht University (VMDC UU). Reactions were performed in a 20 µL reaction volume consisting of PCR grade H_2O, primers, hydrolysis probes and the LightCycler® TaqMan Mastermix (Roche diagnostics, Almere, the Netherlands). For BPV DNA amplification the primers and probes targeting the E1 region of both BPV-1 and BPV-2 were used as described in the article of Bogaert et al. (2010). Primer- and probe sequences are listed in table 1.

The PCR program consisted of enzyme activation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 10 sec, primer annealing at 58 °C for 20 sec and polymerase extension at 72 °C for 20 sec. The program ended with a cooling step at 40 °C for 10 sec. Fluorescence was monitored continuously during the run and Cp (crossing point) values were calculated by the software after the PCR as the cycle at which the signal exceeded a threshold of background noise.

Table 1. Sequences of oligonucleotides used for PCR reactions in this study.

Primer/Probe	Primer/probe sequence (5'- 3')
BPV-1 and BPV-2 E1 forward primer	AATCGGGTGAGCAACCTTT
BPV-1 and BPV-2 E1 reverse primer	TGCTGTCTCCATCCTCTTCA
BPV-1 probe	FAM-CGTCAATCAGGTCTAAACGCCC-BHQ1
BPV-2 probe	YY-TCAACCAGGTCTAAGCGCCC-BHQ1
pUC19 forward primer	GAGACGGTCACAGCTTGTCT
pUC19 reverse primer	TGATGCGGTATTTTCTCCTT
pUC19 probe	YY-CGGCATCAGAGCAGATTGTA-BHQ1

DNA extraction methods

Validation of the BPV PCR and comparison of two DNA extraction methods ran alongside each other. Collected skin tissue samples were split in half with a maximum of 25 mg per piece and DNA was purified in two different ways:

1) Tissue was suspended and pulverized using 500 µl tissue lysis buffer (from the High Pure PCR Template Preparation kit, Roche diagnostics, Almere, the Netherlands) and the MagNA lyser instrument (Roche diagnostics, Almere, the Netherlands). The MagNA lyser was set to 6500 rpm for 1 min and ran up to four cycles. In between runs the samples were cooled. Subsequently, 200 µl supernatant was used for DNA purification on a High Pure PCR Template Preparation kit (Roche diagnostics, Almere, the Netherlands) (method R_{skin}) according to the manufacturer's instructions, resulting in a 200 µL elution volume.

2) DNA extraction was done using the DNeasy® Blood & Tissue kit (Qiagen, Venlo, the Netherlands) (method Q_{skin}) following manufacturer's instructions. This method included an overnight tissue lysis. The total incubated volume of 200 µl was used for purification resulting in a 200 µl elution volume.

Swabs were suspended in 1 ml FE buffer (0.15 M NaCl, 1 mM EDTA). Per swab 200 µL suspended fluid was purified using the High Pure PCR Template Preparation kit (Roche diagnostics, Almere, the Netherlands) (method R_{swab}) and another 200 µL suspended fluid was processed with the DNeasy® Blood & Tissue kit (Qiagen, Venlo, the Netherlands) (method Q_{swab}) according to manufacturer's instructions. For both methods the elution volume was 50 µL.

PCR validation

In total 56 samples (46 skin tissue samples and 10 swabs) for validation were provided by Ghent University (GU), Utrecht University (UU) and a private horse clinic (Lingehoeve Diergeneeskunde, Lienden, The Netherlands). Skin tissue samples were stored at -20°C under dry conditions. One swab provided by GU was stored at -20°C under dry conditions for 3 days. The other swabs were stored at 4 °C for a maximum of 24 hours and were processed subsequently.

All 56 samples were tested with the newly set-up PCR at UU. Linearized pUC19 DNA was added to each sample as internal amplification control. Of each sample two DNA extracts were generated using extraction methods Q_{skin} or Q_{swab} and R_{skin} or R_{swab}. For each DNA extract two reactions were run with the PCR: one to amplify pUC19 DNA and another to amplify BPV-1 and BPV-2 DNA. pUC19 forward primer, reverse primer and probe were used in reaction concentrations of respectively 600 nM, 300 nM and 125 nM (concentrations optimized in earlier experiments of UU). BPV-1 and BPV-2 primer- and probe concentrations were used as optimized in this study. BPV-1 and BPV-2 DNA amplifications were discriminated by viewing two different channels of the Roche LightCycler® 2.0 instrument and examining which measured wavelength yielded the highest fluorescence signal. In each PCR run testing sample DNA extracts a negative extraction control was included. Isolated BPV-1 and BPV-2 DNA provided by GU were used as positive controls and were diluted 1:100 and 1:10.000, respectively.

For 23 samples (22 skin tissue samples and 1 swab, referred to as validation samples) outcomes were compared with outcomes of the PCR at GU (annex 2, 4). For 11 samples (2 skin tissue samples and 9 swabs, referred to as diagnostic samples) outcomes were compared with results from histological examination (annex 3, 4). The remaining 22 samples had been derived from 11 horses without any clinical signs of sarcoids (2 samples per horse, 1 derived from the neck and 1 from the inguinal area) and were presumed to be negative; PCR outcomes were compared with this presumption.

Evaluation of diagnostic value BPV PCR and histological examination

Retrospective data was evaluated of 83 patients affected with skin lesions that attended the equine clinic of UU between December 2007 and May 2012. Patients were included if sarcoid tissue of the patient had been tested with the PCR of GU and had been examined histologically.

Of all patients PCR and histology test outcomes were collected. Histology outcomes were subdivided in two groups: sarcoid or presumptive sarcoid histology (I) and no sarcoid histology (II). Samples with the histologic outcome 'sarcoid', 'spindle-celled sarcoma of low malignancy', 'spindle-celled sarcoma of high malignancy', 'sarcoid cannot be excluded', 'sarcoid or perivascular dermatitis' or 'suspicious of sarcoid' were assigned to group I. If the histologic description did not include any of the aforementioned terms or if the terms 'sarcoid excluded' or 'no evidence for sarcoid' were used the sample was assigned to group II. Regarding the conventional BPV-PCR of GU there were only two possible outcomes: BPV detected or no BPV detected.

Patients were classified based on PCR and histology outcomes (table 3). Relative sensitivity and specificity of the PCR compared to histologic examination were determined and overall agreement and Cohen's kappa were calculated.

Statistical analysis

Cp values obtained with real time PCR on skin tissue samples extracted with method R_{skin} were compared with Cp values obtained with real time PCR on skin tissue samples extracted with method Q_{skin}. For this comparison a two tailed paired sampled t-test was used. The same comparison was made for swab extracts generated with methods R_{swab} and Q_{swab}.

Cp values obtained with real time PCR on positive controls, negative extraction controls and internal controls were summarized by calculating the mean (μ) and standard deviation (σ) using SPSS 20. Cut-off values were defined as $\mu+2\sigma$.

To analyze retrospective data Cohen´s kappa (κ) was calculated using Win Episcope 2.0. $\kappa = 0$ suggest that the agreement is no better than the agreement which would be acquired by chance. $\kappa = 1$implies perfect agreement. Kappa is considered:

- Poor if $\kappa \leq 0.20$
- Fair if $0.21 \leq \kappa \leq 0.40$
- Moderate if $0.41 \leq \kappa \leq 0.60$
- Substantial if $0.61 \leq \kappa \leq 0.80$
- Good if $\kappa > 0.80$ (Petrie and Watson, 2006)

Results

PCR set-up

Primer- and probe matrix

The primer matrix resulted in Cp values ranging from 27.18 to 28.14 cycles. On average, lowest Cp values were generated by the mixture containing 900 nM forward primer and 600 nM reverse primer. Highest Cp values were generated by the mixture containing 300 nM forward primer and 900 nM reverse primer.

The probe matrix resulted in Cp values ranging from 27.03 to 27.86 cycles. On average, lowest Cp values and lowest fluorescence values were generated by the mixture containing 50 nM probe. Highest Cp values and fluorescence values were generated by the mixture containing 250 nM probe. Fluorescence curves of the probe matrix are shown in fig 1.

The concentrations 300 nM for the forward as well as the reverse primer and 50 nM for the probe were selected as optimal concentrations for the BPV PCR.

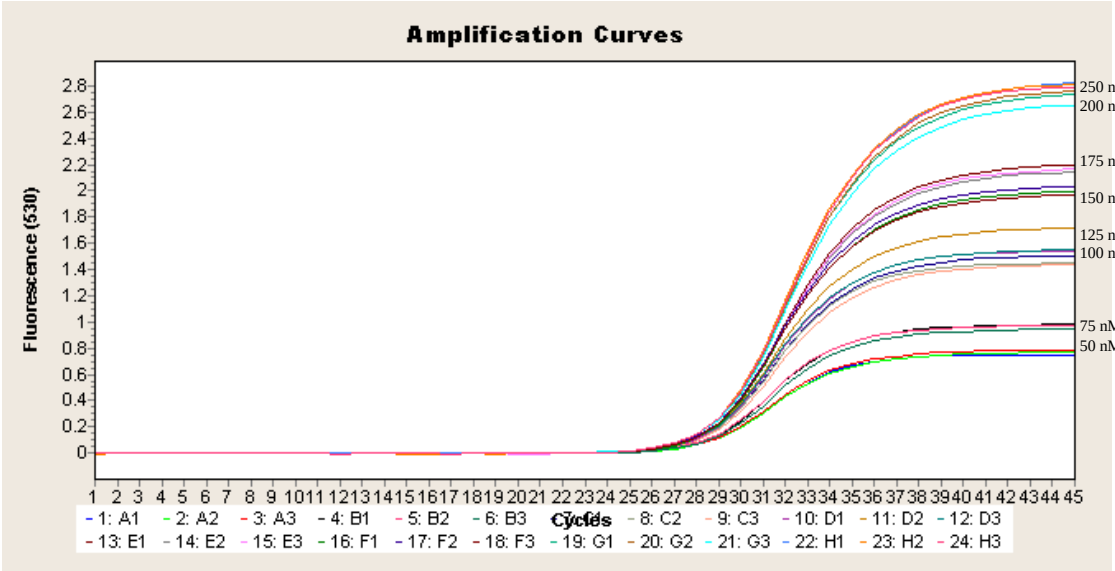

Fig. 1. Fluorescence curves of the probe matrix.

Detection of pUC19, BPV-1 and BPV-2 DNA

Fluorescence signals of the pUC19 and BPV-2 signals resembled, though the pUC19 amplification showed a slightly different curve form. In channel 1 (530 nm) the BPV-1 probe yielded an extensively higher fluorescence value than the BPV-2 and pUC19 probe. In channel 2 (560 nm) the pUC19 yielded a higher fluorescence signal than BPV-1, which in turn yielded a higher fluorescence signal than BPV-2 (fig. 2 and 3). In channel 2 the BPV-1 signal was lowered compared to the signal in channel 1 while that of BPV-2 was enhanced, resulting in a reduced difference between the fluorescence values of BPV-1 and BPV-2.

These results show that BPV and pUC19 amplifications need to be run in separate reactions in order to distinguish pUC19 and BPV-2 amplifications. BPV-1 and BPV-2 DNA signals can be discriminated by viewing channel 1 and 2 and examining in which channel the fluorescence signal is enhanced.

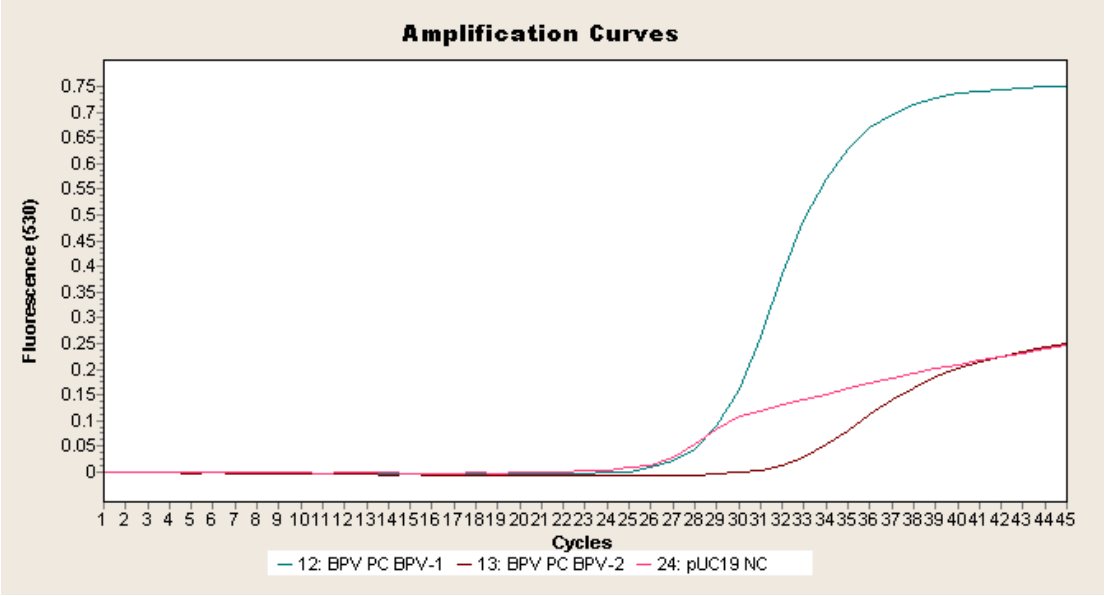

Fig. 2. Fluorescence curves of BPV-1, BPV-2 and pUC19 DNA amplifications generated by the newly set-up PCR. Fluorescence values are measured in channel 1 (530 nm).

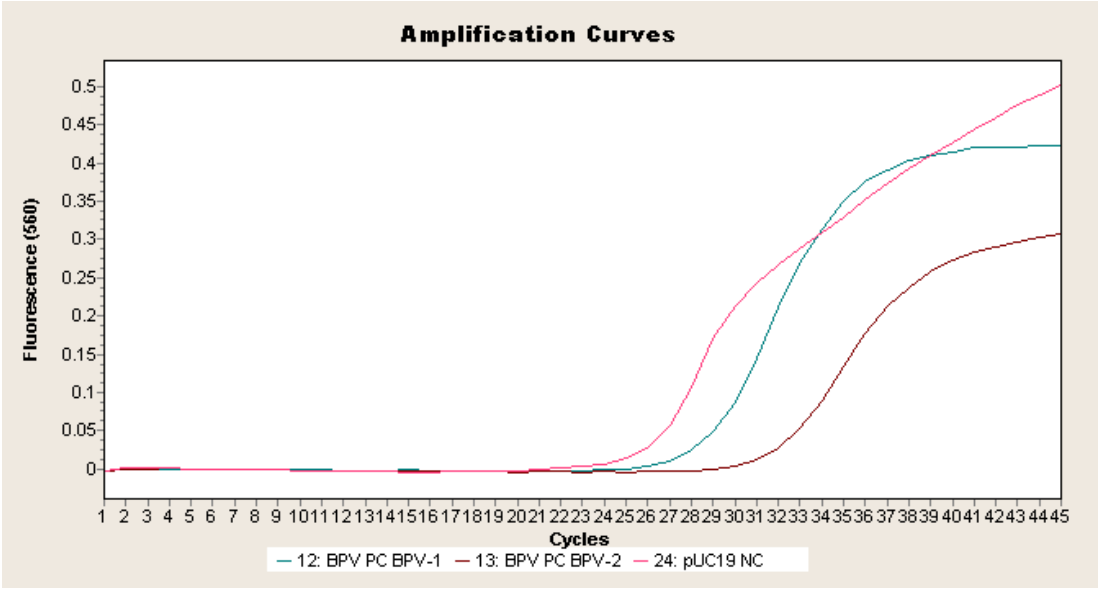

Fig. 3. Fluorescence curves of BPV-1, BPV-2 and pUC19 DNA amplifications generated by the newly set-up PCR. Fluorescence values are measured in channel 2 (560 nm).

Comparison of DNA extraction methods

Skin tissue sample lysis using the MagNa Lyser (method R_{skin}) took approximately 45 minutes per sample batch. A batch included a maximum of 14 samples. For 31 out of 46 samples the process resulted in incomplete tissue lysis. The protocol of method R_{skin} included 6 centrifugation steps. DNA extraction and the PCR were completed within one day.

Following the protocol of method Q_{skin} preparation of the sample tissue before lysis took approximately one minute per sample. The protocol included an overnight incubation step and 4 centrifugation steps. DNA extraction and the PCR were completed within two days.

BPV DNA was amplified in 14 out of 46 skin tissue samples. Extraction of skin tissue samples using both extraction methods lead to the same qualitative PCR results (BPV detection or no BPV detection) for each sample. Cp values obtained with real time PCR on

skin tissue samples extracted with method R_{skin} did not differ statistically from Cp values obtained with real time PCR on skin tissue samples extracted with method Q_{skin} ($p = 0.925$). Real time PCR on extractions of method Q_{skin} resulted in a mean Cp value of 16.90 cycles. Real time PCR on extractions generated with method R_{skin} resulted in a mean Cp value of 16.96 cycles.

Method Q_{skin} was selected as most efficient DNA extraction method for skin tissue samples. Cut-off value for pUC19 Cp values obtained with PCR on skin tissue samples extracted with method Q_{skin} was set at 32.92 cycles. Cut-off value for pUC19 Cp values obtained with PCR on negative extraction controls generated using method Q_{skin} was set at 31.80 cycles. Cut-off values of the positive controls BPV-1 and BPV-2 DNA were set at 31.64 and 30.97 cycles, respectively. Corresponding means and standard deviations are shown in table 2.

Swab DNA extraction protocols were comparable in implementation apart from the 6 centrifugation steps included in the protocol of method R_{swab} versus the 4 centrifugation steps included in the protocol of method Q_{swab}.

For 8 out of 10 swabs BPV DNA was detected in extracts produced with method R_{swab} as well as method Q_{swab}. For one swab BPV was only detected in the extract generated with method Q_{swab}. Regarding the first 8 swabs method Q_{swab} generated a mean Cp value of 29.41 cycles and method R_{swab} generated a mean Cp value of 28.03 cycles. Cp values obtained with real time PCR on swabs extracted with method R_{swab} were significantly lower than Cp values obtained with real time PCR on swabs extracted with method Q_{swab} ($p = 0.016$).

Method R_{swab} was selected as most efficient DNA extraction method for swab samples.

Table 2. Means and standard deviations of Cp values obtained with real time PCR on internal controls added to skin tissue samples, negative extraction controls (skin tissue extraction protocol) and positive controls

Sample type	Extraction method	n	Amplification target DNA	μ	σ
Skin tissue[1]	Method Q_{skin}	22	pUC19	30.32	1.30
NEC skin tissue extraction	Method Q_{skin}	6	pUC19	29.14	1.33
Positive control BPV-1 (1:100 dilution)	n/a	17	BPV-1	28.08	1.78
Positive control BPV-2 (1:10.000 dilution)	n/a	17	BPV-2	30.41	0.28

[1]Cp values obtained by real time PCR on the 22 skin tissue validation samples.

PCR validation

Outcomes of the PCR at Ghent University and the newly set-up PCR at Utrecht University agreed for 95.7% (22/23) (annex 2). All skin tissue samples derived from healthy horses (n = 22) tested negative for BPV in our PCR. From the diagnostic samples, 73% (8/11) of the PCR results agreed with the histologic diagnosis.

In total, BPV DNA was detected in 24 of the 56 samples using the newly set-up PCR. BPV-2 DNA was detected in 3 samples; the other 21 samples contained BPV-1 DNA.

Evaluation of diagnostic value BPV PCR and histological examination

Table 3 shows the classification of the 83 patients based on retrospective data. Relative sensitivity of the PCR compared to histologic examination was 98.4%, relative specificity was 68.2%. The overall agreement was 90.4%, corresponding with an unweighted Cohen's kappa score of 0.729 (95% CI: 0.518 - 0.940). This kappa score is considered substantial.

Table 3. Classification of patients based on of retrospective data

	(presumptive) sarcoid histology	No sarcoid histology	Total
BPV detected	60	7	67
BPV not detected	1	15	16
Total	61	22	83

Discussion

PCR set-up

In this study lowest primer- and probe concentrations were chosen to be implemented in the PCR. This choice was made in view of implementation of the internal control; the used primer and probe concentrations limit BPV DNA amplification and yield low fluorescence values, thereby lowering interference with the fluorescence signal of the internal control. Differences in Cp values generated by the variety of used mixtures were too small to be of influence on selection of primer- and probe concentrations.

The pUC19 probe as well as the BPV-2 probe was labeled with the YakimaYellow label, which made fluorescence signals of pUC19 and BPV-2 amplifications undistinguishable. Therefore BPV and pUC19 DNA amplifications were run separately. Ideally, different probe labels would be used to be able to combine these reactions in a single analysis. However, implementation of a new probe exceeded the scope of this project.

Due to limitations of the used PCR instrument fluorescence signals of the BPV-1 FAM-labeled probe and the YakimaYellow probe severely interfered. Therefore BPV-1 and BPV-2 DNA amplifications were discriminated by viewing channel 1 and 2 and examining in which channel the fluorescence signal was enhanced.

DNA extraction methods were evaluated based on factors like extraction efficiency, test duration and labor intensity. As to biopsies the extraction methods did not generate significantly different Cp values for BPV DNA amplification indicating that extraction efficiency is similar. It must be noted that in method R_{skin} only 200 µl supernatant of the 500 µl suspension volume was used for DNA extraction, while method Q_{skin} used the total 200 µl suspension volume. Use of the total suspension volume in method R_{skin} would possibly lower Cp values with 1.3 (= $^2\log(2.5)$) cycli. Nevertheless, both extraction methods yielded very low Cp values and are therefore considered as sufficiently efficient. Furthermore, differences were found for test duration and labor intensity: method Q_{skin} generated test outcomes within two days but was less labor intensive compared to method R_{skin} which generated test outcomes within one day. Sarcoids are not life-threatening, hence a several days waiting time for test outcomes is acceptable. Labor intensity raises the costs and efforts, which is important in view of the application of the PCR as a diagnostic tool. Therefore labor intensity outweighs test duration, resulting in recommendation of method Q_{skin} for DNA extraction of skin tissue samples at UU VMDC. However, it was not tested whether enough tissue was lysed after one disruption cycle in the MagNA lyser which would make method R_{skin} less labor intensive.

Regarding swabs, DNA extraction methods hardly differed in test duration or labor intensity, while remarkable differences were found for extraction efficiency. In case of one sample method Q_{swab} yielded an extract in which BPV DNA could be detected, while BPV could not be detected in the extract of the same swab generated by method R_{swab}. However, the Cp value generated by PCR on the Q_{swab} method extract was considerably high (39.43), indicating that very little BPV DNA was present in the sample. Therefore it can be assumed that the qualitative difference in outcomes is due to accidental absence of BPV DNA in the solution volume processed with method R_{swab} rather than due to a difference in efficiency of extraction methods. Furthermore, Cp values generated with method R_{swab} were significantly lower than those generated with method Q_{swab}, although it must be noted that the significant difference was based on a relatively small sample amount. Nevertheless, method R_{swab} is recommended for DNA extraction of swabs at UU VMDC. Cut-off value for pUC19 Cp values obtained with PCR on swabs was not calculated because the group of validation samples included only one swab.

PCR validation

One out of 23 validation samples yielded an incongruent test outcome: the PCR at GU detected BPV in this sample, while BPV was not detected with PCR at UU. This sample was derived from healthy skin of the fetlock (data not shown). Therefore the PCR outcome of UU is likely to be correct. The outcome is in accordance with the 22 healthy skin samples in which no BPV DNA was found in this study. On the other hand, the outcome of PCR at GU is in accordance with several other studies that detected BPV in healthy skin (Bogaert et al., 2005; Bogaert et al., 2008; Wobeser et al., 2012). In conclusion, both PCR outcomes of GU and UU are plausible.

For 3 out of 11 diagnostic samples test outcomes of the PCR were incongruent with histological diagnosis. These discrepancies could possibly be due to a suboptimal sensitivity of histologic examination for histologic changes of sarcoids can be very subtle and diverse (Martens et al., 2000) or due to a suboptimal diagnostic specificity of the PCR for several studies have showed that BPV DNA can also be detected in healthy and inflamed skin using PCR (Bogaert et al., 2005; Yuan et al., 2007; Bogaert et al., 2008; Wobeser et al., 2012). Discrepancies could also be caused by suboptimal sensitivity of the swab sampling method, as the study of Martens et al. (2001) showed an overall qualitative diagnostic sensitivity of 88% for swabs compared to clinical or histological diagnosis (Martens et al., 2001a).

In a study of Martens et al. (2001) was postulated that the diagnostic sensitivity of swabs is influenced by the clinical sarcoid type; BPV DNA was the least detected in sarcoids of the occult type while a 100% diagnostic sensitivity was obtained for ulcerated types (Martens et al., 2001a). Therefore exclusive application of the swab sampling method to ulcerated sarcoid types might enhance diagnostic sensitivity of swabbing. Further research is necessary to define the diagnostic sensitivity of swabs and its relation to sarcoid types.

In addition, the thin needle aspiration biopsy method might also be suitable for sarcoid sampling. This method is less invasive than full-thickness biopsies but, even so, collects cells of the dermis as well as the epidermis. Invasiveness is important, not only because it diminishes the patient's welfare but also because trauma is thought to activate sarcoid growth (Taylor and Haldorson, 2012).

BPV-1 and BPV-2 are the two types of BPV that are commonly associated with equine sarcoids. In this study, BPV was detected in 24 samples, which were derived from a total of 18 lesions (annex 2 and 3). BPV was classified as BPV-2 in 3 of the samples. Two of these BPV-2-harboring samples were derived from the same lesion. To summarize, 11% (2/18) of the lesions in which BPV DNA was detected harbored the BPV-2 type. This corresponds with percentages of BPV-2 positive horses as published in other European studies, ranging from 5-27% (Angelos et al., 1991; Otten et al., 1993; Martens et al., 2001b; Bogaert et al., 2010).

Evaluation of diagnostic value BPV PCR and histological examination

Patients with a presumptive sarcoid histology were merged with the sarcoid histology group for the reason that all presumptive sarcoid histology outcomes were open to or tended towards a sarcoid diagnosis. In- or exclusion of patients with a presumptive histology hardly influenced calculations: relative sensitivity would be 0.3% lower and relative specificity would remain unaltered when patients with a presumptive sarcoid histology would be excluded from these calculations (data not shown).

Relative sensitivity approached 100%, showing that BPV was detected in nearly 100% of the lesions with a sarcoid or uncertain histology. Relative specificity was considerably lower, showing that BPV was detected in a substantial amount of lesions with a histologic diagnosis other than sarcoid. This lower relative specificity could be due to a suboptimal diagnostic sensitivity of histologic examination or due to a suboptimal diagnostic specificity of the PCR.

The overall agreement and Cohen's kappa show that value of the PCR as diagnostic tool for sarcoids is substantially comparable with that of histology.

Conclusion

In the newly set-up PCR two reactions are run per sample, one for BPV DNA amplification and one for pUC19 DNA amplification. For BPV DNA amplification the 300 nM primer concentration and 50 nM probe concentration are identified as optimal concentrations for running the PCR in the used settings. BPV-1 and BPV-2 types are distinguished by examining the effect of measuring different wavelengths on the fluorescence signal. The DNeasy® Blood & Tissue kit (Qiagen) is recommended for DNA extraction of skin tissue samples and the High Pure PCR Template Preparation kit (Roche diagnostics) is recommended for DNA extraction of swabs at the UU VMDC.

Validation showed a high agreement of results from the PCR at UU compared to results of the PCR at GU, histological outcomes and presumptions. Discrepancies were possibly due to a limited diagnostic sensitivity of histology, a limited diagnostic specificity of the PCR and/or limited sensitivity of the swab sampling method. The analysis of retrospective data showed that diagnostic value of PCR is substantially comparable with that of histologic examination.

These outcomes validate application of the newly set-up PCR as diagnostic tool for testing skin tissue samples. PCR outcomes must be interpreted using the clinical diagnosis of the tested lesion, as the PCR solely identifies BPV DNA and does not characterize the lesion.

Further research is needed to finish validation of the newly set-up PCR for BPV DNA detection in swabs, examine the diagnostic sensitivity of swabs and its linkage with sarcoid types, test the application of thin needle aspiration biopsies as a sampling method and form an advice concerning use of sampling methods.

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Annex 1: Primer and probe matrices

Table 4. Primer matrix, consisting of 9 solutions with different forward and reverse primer concentrations.

Component	A	B	C	D	E	F	G	H	I
Forward primer (nM)	300	300	300	600	600	600	900	900	900
Reversed primer (nM)	300	600	900	300	600	900	300	600	900
Probe (nM)	250	250	250	250	250	250	250	250	250
PCR-grade water (uL)	37.2	34.8	32.4	34.8	32.4	30.0	32.4	30.0	27.6
Forward primer (10μM) (uL)	2.4	2.4	2.4	4.8	4.8	4.8	7.2	7.2	7.2
Reversed primer (10μM) (uL)	2.4	4.8	7.2	2.4	4.8	7.2	2.4	4.8	7.2
Probe(10μM) (uL)	2	2	2	2	2	2	2	2	2
Mastermix (5x conc.) (uL)	16	16	16	16	16	16	16	16	16
Isolated BPV DNA (uL)	20	20	20	20	20	20	20	20	20

Table 5. Probe matrix, consisting of 8 solutions with optimized forward and reverse primer concentrations and different probe concentrations.

Component	A	B	C	D	E	F	G	H
Forward primer (nM)	300	300	300	300	300	300	300	300
Reversed primer (nM)	300	300	300	300	300	300	300	300
Probe (nM)	50	75	100	125	150	175	200	250
PCR-grade water (uL)	38.8	38.6	38.4	38.2	38.0	37.8	37.6	37.2
Forward primer (10μM) (uL)	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Reversed primer (10μM) (uL)	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Probe(10μM) (uL)	0.4	0.6	0.8	1.0	1.2	1.4	1.6	2.0
Mastermix (5x conc.) (uL)	16	16	16	16	16	16	16	16
Isolated BPV DNA (uL)	20	20	20	20	20	20	20	20

Annex 2: Validation samples

Samples U1A – U17A were provided by the equine clinic for surgery of Utrecht University (the Netherlands). Samples G1A – G7 were provided by the clinic for surgery and anesthesiology of domestic animals of Ghent University (Belgium). Samples derived from the same lesion are numbered the same but are marked with a different addition (A or B). Several associated samples are listed in annex 3.

Table 6. Qualitative outcomes (BPV detection or no BPV detection) generated by the PCRs at UU and GU and Cp values generated by the PCR at UU.

Sample	Sample method	PCR Ghent[1]	PCR Utrecht[1]	Cp Qiagen[2]	Cp Roche[3]
U1A	Biopsy	+	+	19.12[4]	16.56[4]
U2	Biopsy	-	-	-	-
U3	Biopsy	+	-	-	-
U4	Biopsy	+	+	16.17[4]	14.98[4]
U5	Biopsy	-	-	-	-
U6A	Biopsy	+	+	17.93	17.28
U7A	Biopsy	+	+	16.06	20.00
U8	Biopsy	+	+	16.66	19.07
U9	Biopsy	-	-	-	-
U10B	Biopsy	+	+	16.71	19.34
U12B	Biopsy	+	+	13.86	16.06
U14	Biopsy	+	+	19.07	16.64
U15	Biopsy	-	-	-	-
U16	Biopsy	-	-	-	-
U17A	Biopsy	+	+	19.62	18.49
G1A	Biopsy	+	+	17.27	15.16
G1B	Swab	+	+	23.08	20.19
G2	Biopsy	+	+	14.83	12.89
G3	Biopsy	+	+	15.00	17.04
G4	Biopsy	-	-	-	-
G5	Biopsy	-	-	-	-
G6	Biopsy	+	+	16.02	14.44
G7	Biopsy	+	+	15.42	14.56

[1] The symbol + denotes that BPV DNA was detected. The symbol – denotes that BPV DNA was not detected.
[2] Cp values obtained with the newly set-up real time PCR on DNA extracts generated with the DNeasy® Blood & Tissue kit (Qiagen diagnostics, Venlo, the Netherlands).
[3] Cp values obtained with the newly set-up real time PCR on DNA extracts generated with the High Pure PCR Template Preparation kit (Roche diagnostics, Almere, the Netherlands).
[4] BPV-2 DNA.

Annex 3: Diagnostic samples

Samples U1B – U19 were provided by the equine clinic for surgery of Utrecht University (the Netherlands). Sample L12 was provided by the private horse clinic (Lingehoeve Diergeneeskunde, Lienden, The Netherlands).

Samples derived from the same lesion are numbered the same but are marked with a different addition (A or B). Associated samples are listed in annex 2.

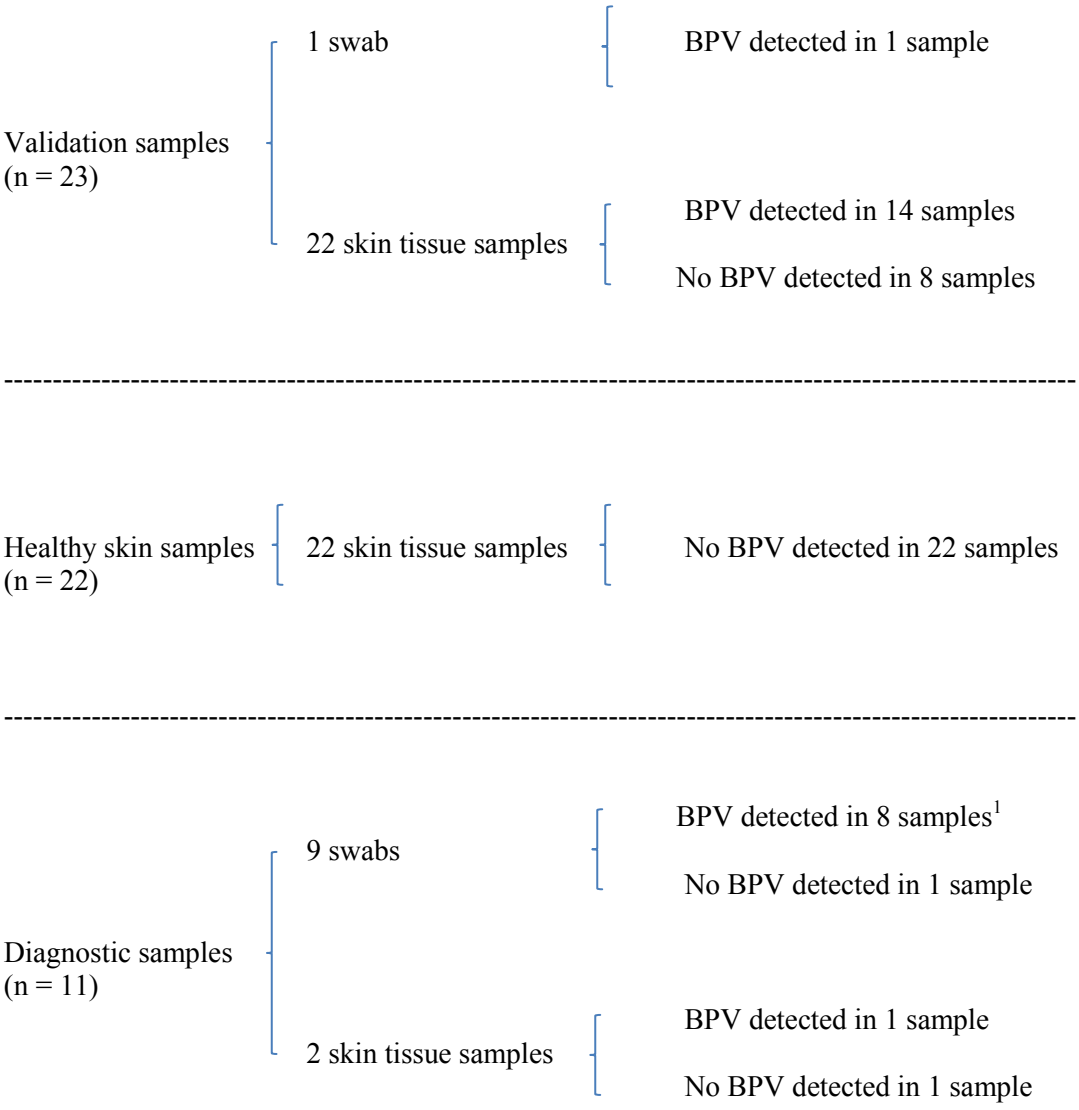
Table 7. Outcomes of the PCR at UU and histologic diagnoses. Red marking indicates incongruent outcomes.

Sample	Sample method	Histologic diagnosis	Cp Qiagen[1]	Cp Roche[2]
U1B	swab	Sarcoid	24.59[3]	21.94[3]
U6B	swab	spindle-celled sarcoma of low malignancy	36.74	34.90
U7B	swab	spindle-celled sarcoma of low malignancy	30.78	30.77
U10A	swab	Sarcoid	29.73	30.02
U11	swab	Sarcoid	26.65	25.61
U12A	swab	Sarcoid	-	-
U13	biopsy	Papilloma	-	-
U17B	swab	Chronic lymphocytic dermatitis with mild eosinophilic component[4]	39.43	-
U18	swab	spindle-celled sarcoma of low malignancy	34.42	33.88
U19	swab	Sarcoid	29.27	26.96
L12	biopsy	Chronic lymphocytic dermatitis with mild eosinophilic component[4]	19.85	21.90

[1] Cp values obtained with the newly set-up real time PCR on DNA extracts generated with the DNeasy® Blood & Tissue kit (Qiagen diagnostics, Venlo, the Netherlands).
[2] Cp values obtained with the newly set-up real time PCR on DNA extracts generated with the High Pure PCR Template Preparation kit (Roche diagnostics, Almere, the Netherlands).
[3] BPV-2 DNA.
[4] Clinical diagnosis: sarcoid.

Annex 4: Overview of samples

Compositions of the 3 sample groups are schematically displayed and BPV-1 or BPV-2 detection in these samples using the newly set-up PCR at UU are presented. In all but one case[1] DNA purification of the samples using two DNA extraction methods led to the same qualitative (BPV detection or no BPV detection) PCR outcome.



[1] In one swab BPV was detected after DNA extraction using the DNeasy® Blood & Tissue kit (Qiagen diagnostics, Venlo, the Netherlands), while BPV could not be detected after extraction using the High Pure PCR Template Preparation kit (Roche diagnostics, Almere, the Netherlands).