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Safety and immunogenicity of BPV-1 L1 virus-like particles in a dose-escalation vaccination trial in horses

E. K. HAINISCH, **S. BRANDT**[*,†], **S. SHAFTI-KERAMAT**[‡], **R. VAN DEN HOVEN**[§], and **R. KIRNBAUER**[‡]

[†]Large Animal Surgery and Orthopaedics, Equine Biotechnology Unit, Equine Clinic, University of Veterinary Medicine, Vienna, Austria.

[§]Large Animal Surgery and Orthopaedics, Equine Internal Medicine, Equine Clinic, University of Veterinary Medicine, Vienna, Austria.

[‡]Laboratory of Viral Oncology (LVO), Division of Immunology, Allergy and Infectious Diseases (DIAID), Department of Dermatology, Medical University Vienna, Austria.

Summary

Reasons for performing study—Infection with bovine papillomaviruses *types 1* and *2* (BPV-1, BPV-2) can lead to the development of therapy-resistant skin tumours termed sarcoids and possibly other skin diseases in equids. Although sarcoids seriously compromise the welfare of affected animals and cause considerable economic losses, no prophylactic vaccine is available to prevent this common disease. In several animal species and man, immunisation with papillomavirus-like particles (VLP) has been shown to protect efficiently from papillomaviral infection.

Hypothesis—BPV-1 L1 VLPs may constitute a safe and highly immunogenic vaccine candidate for protection of horses against BPV-1/-2-induced disease.

Methods—Three groups of 4 horses each received 50, 100 or 150 μg of BPV-1 L1 VLPs, respectively, on Days 0, 28 and 168. Three control horses received adjuvant only. Horses were monitored on a daily basis for one week after each immunisation and then in 2 week intervals. Sera were collected immediately before, 2 weeks after each vaccination and one and 2 years after the final boost and analysed by pseudovirion neutralisation assay.

Results—None of the horses showed adverse reactions upon vaccination apart from mild and transient swelling in 2 individuals. Irrespective of the VLP dose, all VLP-immunised horses had developed a BPV-1-neutralising antibody titre of ≥1600 plaque forming units (pfu)/ml 2 weeks after the third vaccination. Eight of 10 trial horses still available for follow-up had neutralising antibody titres ≥1600 pfu/ml one year and ≥800 pfu/ml 2 years after the last immunisation.

Conclusion—Intramuscular BPV-1 L1 VLP vaccination in horses is safe and results in a long-lasting antibody response against BPV-1. Neutralisation titres were induced at levels that correlate with protection in experimental animals and man.

Potential relevance—BPV-1 L1 VLPs constitute a promising vaccine candidate for prevention of BPV-1/-2-induced disease in equids.

[*]Corresponding author sabine.brandt@vetmeduni.ac.at;.

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Keywords

horse; bovine papillomavirus; sarcoid; vaccine; virus-like particles

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Introduction

Bovine papillomaviruses (BPV) are small nonenveloped viruses that consist of an icosahedral capsid harbouring a circular double-stranded DNA genome. The latter comprises an early (E) and a late (L) region coding for 6 functional (E1, E2, E4–E7) and 2 capsid proteins (L1, L2), respectively, and a long control region (LCR) required for replication and transcription of the viral genome (Campo 2006a). In cattle, BPV infection mainly results in the development of epithelial lesions. In immunocompetent animals, these lesions usually regress spontaneously. In some individuals, however, and particularly upon ingestion of immunosuppressants contained in bracken fern, lesions may progress to malignant carcinomas (Campo 2006b; Borzacchiello and Roperto 2008).

In addition to disease induced in cattle, BPVs of *types 1* and *2* (BPV-1; BPV-2) contribute to the onset and progression of nonmetastasising yet locally aggressive skin tumours termed sarcoids (Nasir and Campo 2008). With a prevalence of 2–11.5% (Sullins *et al*. 1986; Studer *et al*. 2007), sarcoids represent the most common neoplasm in horses. Due to their location, extension and tendency to recur in a more progressive form upon therapeutical intervention or accidental trauma, sarcoids compromise the welfare of affected animals and lead to considerable economic losses (Scott and Miller 2003). This is even more so as no effective prophylaxis is available thus far.

Bovine papillomas are the result of a productive BPV infection, with high numbers of virions being assembled in the squamous epidermal layer and released (Campo 2006b). In equids, BPV infection is currently thought to be abortive, with virus solely residing in fibroblast in an episomal form (Amtmann *et al*. 1980; Lancaster 1981). However, there is increasing evidence towards sarcoids also harbouring and spreading infectious virions. Voss (1969) succeeded in transferring sarcoids from naturally affected to healthy horses by inoculating scarified skin with minced sarcoid suspension or cell free sarcoid extract supernatant. The possibility that infection of horses may be achieved by viral genome transduction has been refuted by Robl *et al*. (1972) who showed that tumour development can be experimentally induced by intracranial inoculation of hamsters with wild-type, but not with heat-denatured BPV-1 virion. In addition, we have recently shown that intradermal inoculation of foals with BPV-1 virion, but not with naked BPV-1 genome or sarcoid cells containing viral episomes, leads to pseudo-sarcoid formation (Hainisch *et al*. 2010). Using immunocapture PCR, we have detected BPV-1 genome-associated L1 capsomeres in a subset of sarcoids (Brandt *et al*. 2008). Moreover, we have recently demonstrated the presence of BPV-1 DNA and protein in sarcoid epidermis (Brandt *et al*. 2011a). Finally, co-stabling of sarcoid-affected with healthy donkeys has been shown to result in sarcoid development in the latter, thus providing strong evidence for a natural disease transmission from equid to equid (Nasir and Campo 2008) effected by *de novo* infection with virion.

In Europe, sarcoids are mainly caused by BPV-1, with BPV-2 being detected in only ~10% of lesions (Otten *et al*. 1993; Chambers *et al*. 2003). However, in the western USA, BPV-2 is responsible for 62% of sarcoids (Carr *et al*. 2001). BPV-1 and BPV-2 share a genetic similarity of 87%. *In vitro*, we have recently demonstrated that immunisation of rabbits with BPV-1 L1 VLPs induces neutralising antibodies to BPV-1 that also cross-neutralise BPV-2. Similarly, anti BPV-2 VLP sera raised in rabbits were shown to neutralise BPV-2 virions

and to cross-neutralise BPV-1. By this, we could demonstrate that BPV-1 and BPV-2 are related serotypes (Shafti-Keramat *et al*. 2009).

Neutralising antibodies are considered the main protection factor against experimental and natural infection in animals and man (Breitburd *et al*. 1995; Paavonen *et al*. 2007). Natural immunity against BPV-1 and -2 in equids appears to be poor. Sarcoid-affected horses examined in this regard revealed no measurable BPV-1 L1 antibodies (Ashrafi *et al*. 2008; Mattil-Fritz *et al*. 2008). This circumstance may help to explain why sarcoids usually present as persistent lesions (Chambers *et al*. 2003). BPV may escape from immune surveillance because of its paramount localisation in cutaneous cells (O'Brien and Campo 2002), yet also by its capacity to inhibit MHC class I-mediated antigen presentation via its major oncoprotein E5 (Marchetti *et al*. 2009).

In the early 1990s, the discovery was made that recombinant papillomavirus major capsid protein L1 spontaneously self-assembles into virus-like particles (VLPs) (Kirnbauer *et al*. 1992; Hagensee *et al*. 1993; Rose *et al*. 1993). Papillomavirus-like particles are morphologically and immunologically very similar to the ordered 72 pentamer array of L1 major capsid protein that (together with 12 L2 minor capsid protein monomers) forms the outer shell of the authentic virion. This discovery led the way to VLP-based prophylactic vaccines against PV infection and PV-associated tumours (Kirnbauer 1996). VLPs do not contain viral DNA and are, therefore, nonpathogenic. However, they resemble authentic virions in their ability to induce high titres of type-specific neutralising antibodies, the prerequisite for effective prophylactic vaccination (Zinkernagel 2003; Schiller 2007). VLP vaccination was successful in preventing infection with cottontail rabbit papillomavirus (CRPV) in domestic rabbits (Breitburd *et al*. 1995), canine oral papillomavirus (COPV) in dogs (Suzich *et al*. 1995) and BPV *type 4* (BPV-4) in cattle (Kirnbauer *et al*. 1996). Bivalent and tetravalent HPV VLP (HPV-6, -11, -16, -18) vaccines (Villa *et al*. 2005) have been made commercially available worldwide.

However, VLPs were not successful in inducing regression of pre-existing tumours in cattle and human patients, thus lacking therapeutic efficacy (Schiller 2007). Chimeric BPV-1 L1 VLPs expressing E7 oncoprotein (CVLP) were assessed as a possible therapeutic agent in sarcoid-bearing horses (Mattil-Fritz *et al*. 2008) and donkeys (Ashrafi *et al*. 2008), but immunisation did not lead to a significant reduction in the number of sarcoids.

Based on the circumstance that equine sarcoids develop upon naturally acquired BPV-1/-2 infection, we hypothesised that BPV-1 VLPs are safe and immunogenic in horses and thus have the potential to protect equids from this common tumour disease. We addressed this assumption in a dose escalation trial in horses.

Materials and methods

The animal trial was approved by the institutional ethics committee, the Advisory Committee for Animal Experiments (§12 of Law for Animals experiments, Tierversuchsgesetz - TVG) and the Austrian Federal Ministry for Science and Research (animal experiment license No. BMBWK-68.205/ 0022-BrGT72007).

Animals

Twenty-four horses belonging to the Veterinary University of Vienna were clinically examined as to general health and the presence of BPV-associated malignancies. Full thickness punch biopsies were obtained from the skin of the neck and their DNA subjected to PCR for BPV-1/-2 DNA as described earlier (Brandt *et al*. 2008). Blood was taken and serum tested in a BPV-1 pseudovirion neutralisation assay as described below. From 23/24

animals that had scored negative by all tests, 8 were excluded on the grounds of various health problems. The remaining 15 horses were enrolled in the study and assigned to 4 different groups (Table 1).

Generation of BPV-1 L1 VLPs

To generate the VLPs, Sf9 cells were infected with BPV-1 L1 recombinant baculovirus, and high molecular weight complexes purified on density gradients. Aliquots were tested for VLP content by SDS-PAGE, Coomassie staining and transmission electron microscopy (TEM). Aliquots were then frozen and stored for further use at −24°C (Kirnbauer *et al*. 1992, 1993, 1996; Kirnbauer 1996).

Immunisation and monitoring

Vaccines were prepared freshly on the day of vaccination, with 250 μg $Al(OH)_3$ per dose as adjuvant (Lindblad 2004). Phosphate buffered saline (PBS) was added to a final volume of 1.5 ml per dose. Three groups of 4 horses each (*Groups 2–4*), were immunised with doses of 50, 100 or 150 μg of BPV-1 VLPs, respectively, by deep intramuscular injection into the left side of the neck. Three horses formed the control group (*Group 1*) and received adjuvant in PBS. The horses were vaccinated 3 times, i.e. on Day 0, after 4 weeks and after 6 months. Horses were monitored on a daily basis for one week after each immunisation and then in 2 weeks intervals. Serum for neutralisation assay was collected immediately before and 14 days after each vaccination, as well as one and 2 years after the last boost.

Determining neutralising antibody titres

Neutralising antibody titres were determined by BPV-1 pseudovirion (PsV) neutralisation assay. PsVs are synthetic virus-like particles containing a plasmid vector encoding a reporter gene. Upon *in vitro* infection of permissive cell lines by PsVs, the plasmid is replicated to high copy numbers, which in turn leads to high-level expression of the reporter gene. Under the presence of PsV-neutralising serum, the expression of the reporter gene is correspondingly reduced, and thus inversely proportional to the amount of neutralising serum antibody (Pastrana *et al*. 2004). The BPV-1 PsVs used in this assay were produced as described by Buck *et al*. (2004, 2008) and contained a plasmid coding for secreted placental alkaline phosphatase (SEAP). BPV-1 PsVs were incubated in triplicates with 2-fold serial dilution of horse sera (1:50 to 1:25,600), plated on 293 TT cells for 48 h and alkaline phosphatase secreted into the cell culture medium was measured as mean ± s.d. optical density (OD) (Schiller 1997, 1998; Pastrana *et al*. 2004)

In order to be able to determine neutralising serum antibody titres and monitor inter-assay variability, the following controls have been included: 293TT cells in growth medium (background control), PsV-infected 293TT cells without antiserum (no serum control used as reference; maximal AP secretion of 100%), 293TT cells treated with PsV preincubated with neutralising monoclonal anti-BPV-1 L1 antibody 5B6 (positive control) and cells treated with PsV preincubated with unspecific monoclonal anti-HPV-16 antibody V5 (negative control). Serum dilutions causing at least a 50% reduction in SEAP in comparison to the no serum control were considered as being neutralising.

Results

Apart from mild transient swelling in 2 horses on Day 10 after the second immunisation no adverse reactions, i.e. changes in body temperature or inflammation/swelling at the injection sites were recorded. All BPV-1 VLP-vaccinated horses seroconverted after the second shot, with detectable neutralising antibody titres ≥50 (data not shown). Two weeks after the third immunisation, all VLP-vaccinated horses had developed neutralising antibody titres ranging

from 1600–12,800 plaque forming units (pfu)/ml (Fig 1a). As expected, none of the immune sera obtained from control horses (*Horses 1*, *2*, *3*) were neutralising at the lowest dilution 1:50 (data not shown).

One year after the third vaccination, *Horses 1*, *6* and *13* were unavailable for follow-up due to reasons unrelated to this trial. Sera from 9 of 10 still available VLP-immunised horses revealed neutralising antibody titres ranging from 800–12,800 pfu/ml, while serum from *Horse 10* had lost its neutralising capacity (Fig 1b). Two years after the third immunisation, sera from 8 of 10 horses still revealed neutralising antibody titres ranging from 800–3200 pfu/ml. As, expected serum of *Horse 10* tested negative and serum of *Horse 11* now revealed titres <400 pfu/ml (Fig 1c). The 2 remaining control horses scored negative in this assay, as anticipated (data not shown).

Discussion

Immunisation with PV VLPs has been shown to be well tolerated in equids (Ashrafi *et al*. 2008; Mattil-Fritz *et al*. 2008), rabbits (Breitburd *et al*. 1995), dogs (Suzich *et al*. 1995), cattle (Kirnbauer *et al*. 1996) and man (Villa *et al*. 2005). The fact that vaccination with BPV-1 VLPs produced no significant adverse reactions in our study is encouraging for its intended use as a prophylactic vaccine. Mattil-Fritz *et al*. (2008) have conducted a dose-escalation trial in 12 sarcoid-affected horses to determine the therapeutic potential of chimaeric virus-like particles (CVLPs of BPV-1 L1-E7). Using the described protocol herein, Mattil-Fritz *et al*. (2008) analysed 1:1000 diluted horse sera collected 3 weeks after the second immunisation (Day 21) for their ability to neutralise infectious BPV-1 L1/L2 pseudovirions. In agreement with the current data, seroconversion could be achieved in 11 out of 12 animals. Both reports also agree in that different CVLP doses (40–400 μg) induce comparable anti-L1 IgG levels (Mattil-Fritz *et al*. 2008; Fig 1). Whereas the work of Mattil-Fritz *et al*. (2008) otherwise focuses on the therapeutic effect of CVLPs, the current study was designed to specifically address the duration and stability of BPV-1 L1 VLPs as potential prophylactic vaccine candidate. Accordingly, presence of VLP-induced neutralising antibodies was monitored over a period of 2.5 years and antibody levels determined by titration at several points in time, i.e. before and 2 weeks after each of 3 immunisations and one and 2 years after the final boost.

Neutralising antibodies raised by PV VLP vaccination are protective and usually type-restricted. For example, rabbits vaccinated with CRPV VLP produced a significantly lower number of skin lesions after experimental infection with CRPV virions and none developed cancer. In contrast, immunisation of rabbits with BPV-1 VLP could not prevent CRPV infection and subsequent papilloma formation (Breitburd *et al*. 1995). Vaccination of dogs with COPV VLPs resulted in complete protection against COPV-induced oral mucosal papillomas (Suzich *et al*. 1995). Similarly, 13 of 15 BPV-4 VLP-immunised calves did not develop oral papillomas upon experimental infection, whereas disease developed in 9 of 10 control animals (Kirnbauer *et al*. 1996). In young women immunised with a quadrivalent VLP vaccine, the combined incidence of persistent infection with or disease induced by HPV-6, -11, -16 or -18 decreased by 90% in comparison to the placebo group (Villa *et al*. 2005).

High titres of neutralising antibody were obtained in all of the vaccinated horses after the third vaccination. No correlation between antibody titres and administered antigen dose has been observed, indicating that the lowest dose of 50 μg/BPV1 VLP may suffice to achieve maximum immune responses. This agrees with the observation that PV VLPs are highly immunogenic even at small doses and without the use of adjuvant (Schiller 2007).

One year after the third immunisation, high titres of neutralising antibodies were still detectable in VLP-immunised horses, except in one 20-year-old mare. It has been shown that older horses have an impaired immune response to vaccinations (Fermaglich and Horohov 2002), although *Horse 15* (age 30 years; neutralising antibody titre of 800 pfu/ml 2 years after the last immunisation) does not respect this rule. As with in young human subjects, longer lasting antibody titres may be expected in younger horses (Villa *et al*. 2005).

The minimal antibody titres required for VLP vaccination-mediated protection against PV infection have not yet been determined for man or animals. This is mainly due to the high prophylactic efficacy of VLP-based vaccines. Although a substantial number of vaccinates have become sero-negative to HPV-18 in one vaccine trial at end-of-study, protection against HPV-18 induced disease remained high, indicating protection through an anamnestic response following exposure (or re-challenge by vaccination) (Joura *et al*. 2008).

Antibody responses to VLP vaccination are mainly type-restricted, yet cross-neutralising immune responses have been observed that confer partial cross-protection to closely related HPV types in human vaccine trials (Roden *et al*. 1996; Pastrana *et al*. 2005; Villa *et al*. 2005). BPV-1 and BPV-2 are closely related genotypes. Their respective L1 capsid protein sequences have homology of 92%, which is comparable to that between L1s of closely related HPV types that share several cross-neutralisation epitopes. We have recently re-evaluated the serological relationship of BPV-1 and BPV-2. Using BPV-1 and BPV-2 neutralisation assays, we have demonstrated that VLP vaccination with either type was able to induce neutralising antibodies to both the homologous and the heterologous type (Shafti-Keramat *et al*. 2009). This established that BPV-1 and BPV-2 are closely related serotypes and indicates that vaccination with BPV-1 L1 VLP can protect against infections with homologous BPV-1 and heterologous BPV-2.

Bovine papillomavirus *type 1* DNA has also been detected in some cases of equine inflammatory skin disease (Yuan *et al*. 2007) and was consistently found in lesions, intact skin and blood of horses affected by hoof canker (Brandt *et al*. 2011b). These findings indicate that BPV-1 may also be involved in the pathogenesis of other equine skin diseases. In this case, a prophylactic BPV-1 vaccine may have an even broader protective significance.

In the present study, we were able to demonstrate that BPV-1 VLPs in horses are safe and highly immunogenic in the vast majority of horses. In addition, we provide further evidence that low VLP doses (≤50 μg) may be sufficient to induce protection. In man, a 3 dose regimen of quadrivalent HPV vaccine has been shown to induce stable neutralising anti-HPV antibody levels for at least 5 years and a robust immune memory (Olsson *et al*. 2007). This finding and high neutralising antibody titres still present in most horse sera 2 years after the third immunisation are indicative for a long-lasting effect of vaccination. A virus challenge study aiming at determining the prophylactic potential of BPV-1 VLPs in equids is currently in preparation.

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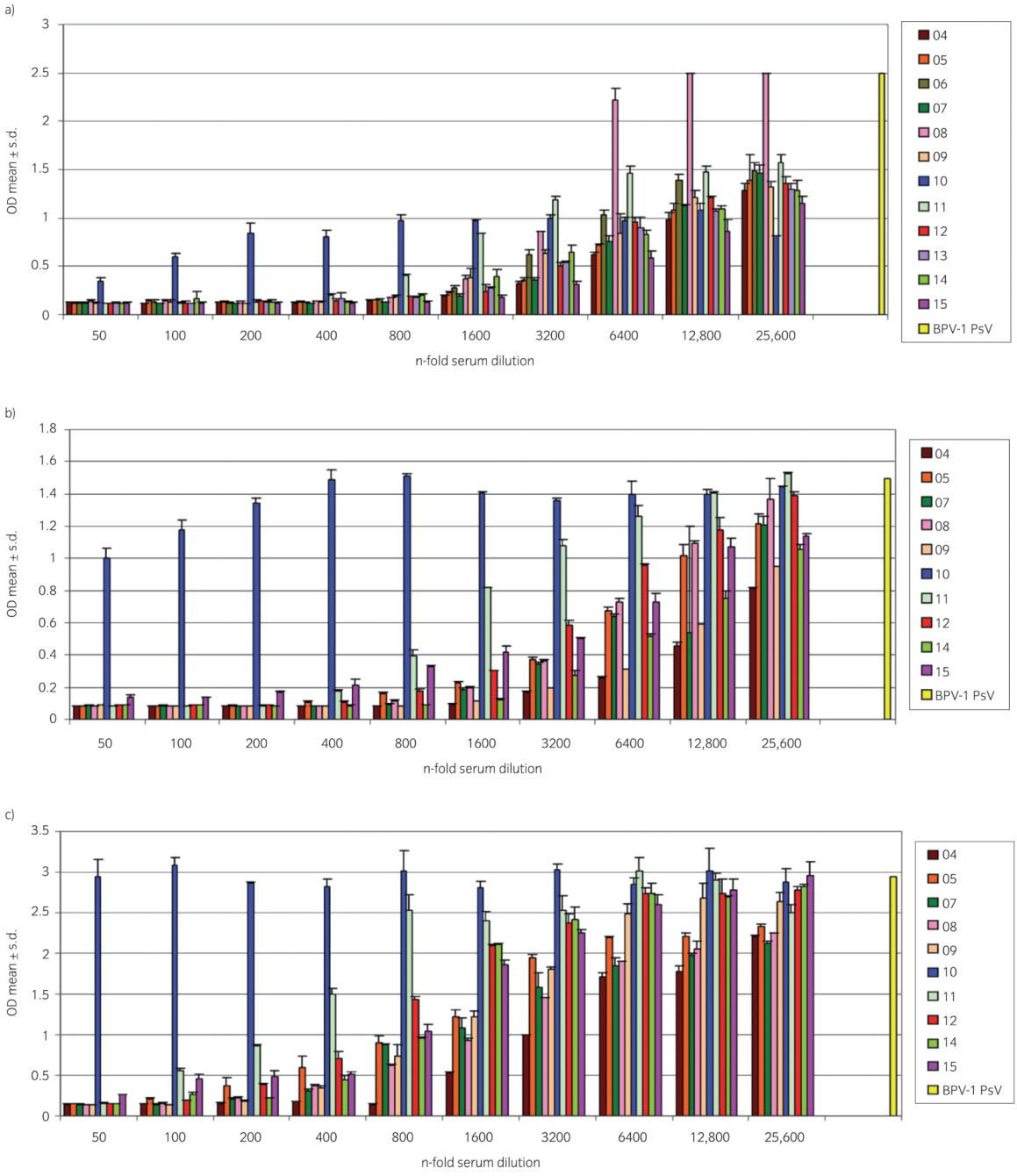

Figure 1. VLP-induced neutralising serum antibody titres 2 weeks, one year and 2 years after the third immunisation.

BPV-1 pseudovirions (PsV) were incubated with serial dilutions of horse serum and then plated on 293 TT cells. Release of SEAP into culture medium was measured as mean ± s.d. OD and inversely correlates with PsV neutralisation. Sera were deemed neutralising when causing a SEAP reduction of at least 50% in comparison to the no serum control (BPV-1 PsV; 100%). a) Irrespective of the VLP dose, all sera revealed neutralising antibody titres ≥1600 pfu/ml. b) One year after the third immunisation, sera of all but one horse (*Horse 10*) showed neutralising antibody titres ≥800 pfu/ml. Unfortunately, *Horses 6* and *13* were not

available for long-term follow-up. c) 2 years after the third immunisation, all sera showed neutralising titres ≥800 pfu/ml apart from sera of *Horses 10* and *11*.

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Table 1

Immunisation scheme

Group	VLP antigen dose	*Horse*	Age	Sex	Breed
1	Adjuvant only	*1*	24	G	WB
	($Al(OH)_3$, 250 μg)	*2*	8	M	SB
		3	18	G	SB
2	50 μg	*4*	7	M	SB
		5	7	G	SB
		6	17	G	WB
		7	19	G	TB
3	100 μg	*8*	17	G	WB
		9	18	G	WB
		10	20	M	WB
		11	20	M	WB
4	150 μg	*12*	4	G	SB
		13	4	G	SB
		14	19	G	WB
		15	30	G	WB

Incremental doses of antigen as indicated were injected deep intramuscularly into the left side of the neck (*Groups 2*, *3*, *4*). Three control horses (*Group 1*) received alum hydroxide adjuvant only. Age, gender and breed are indicated (G: gelding, M: mare; SB: Standardbred trotter, TB: Thoroughbred, WB: Warmblood).