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A comparison of different vaccination schemes used in sheep combining inactivated bluetongue vaccines against serotypes 4 and 8

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ABSTRACT

Eight different vaccination schemes using four commercially available inactivated Bluetongue vaccines against serotypes 4 and 8 in three different combinations (setting 1–3) were tested under field conditions for their ability to generate a measurable immune response in sheep.

Animals of setting 1 (groups A–D) were simultaneously vaccinated using either individual injections at different locations (groups A & D) or double injection by a twin-syringe (groups B & C). For both application methods, a one-shot vaccination (groups C & D) was compared to a boosted vaccination (groups A & B). Sheep of setting 2 (groups E–G) were vaccinated in an alternating, boosted pattern at fortnightly intervals starting with serotype 4 (groups E & F) or vice versa (group G). Group H of setting 3 was vaccinated simultaneously and vaccines were injected individually as a one-shot application. Each group consisted of 30 sheep. The immunogenic response was tested in all sheep (n = 240) by ELISA (IDScreen®Bluetongue Competition), while serum neutralisation tests were performed in five to six sheep from each group (n = 45).

All vaccine combinations were well tolerated by all sheep. Of all vaccines and schemes described, the simultaneous double injected boosted vaccination of setting 1 (group B) yielded the highest median serotype-specific titres 26 weeks after the first vaccination (afv) and 100% seropositive animals (ELISA) one year afv. In setting 1, there were no relevant significant differences in the immunogenic response between simultaneously applied vaccines at different sites or at the same injection site. Importantly, a one-shot vaccination induced comparable immunogenicity to a boosted injection half a year afv. Low serotype-specific neutralising antibody levels were detected in settings 2 and 3 and are attributed to diverse factors which may have influenced the measured immunogenicity.

1. Introduction

Bluetongue (BT) is a notifiable disease of ruminants caused by the Bluetongue Virus (BTV), an RNA-Virus with currently 27 known serotypes [1–3] primarily transmitted by *Culicoides* spp. midges [4,5]. In 2006, BTV serotype 8 emerged in Central Europe including Germany, leading to over 60,000 infected animals, reported in 2006 and 2007 with case fatality rates of around 40% in sheep [6].

A compulsory vaccination programme using inactivated BTV-8 vaccines was thus established for all livestock ruminants in 2008/09, followed by voluntary vaccination in 2010/11. The virus was successfully eliminated, vaccinations were prohibited in 2011 and in 2012 Germany was declared free from BTV-8 [7], while in Southern European countries various serotypes continued to be active [8].

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In September 2015, serotype 8 re-emerged in France after five years of BTV-8-free status [9–11]. Almost simultaneously, the BTV-4 outbreak starting 2014 in Greece and spreading rapidly over the Balkan countries, reached Austria in November 2015 [8].

Due to the geographical proximity to these new outbreaks the risk of BTV being introduced to Germany was considered probable to high [12]. As the vaccinations between 2008 and 2011 had shown high efficacy in eradicating the disease [7,13] the Standing Committee on Veterinary Vaccination (StIKoVet) recommended the vaccination of susceptible animals [14]. With the amendment of the German Bluetongue Regulation in 2016 [15], voluntary vaccinations for BTV-4 and −8 using inactivated vaccines were no longer forbidden. BTV is known to generate only low to no cross-protection between serotypes [16]. No inactivated bivalent vaccine against BTV-4 and −8 was available in 2015/16. Vaccination therefore relied on a combination of two monovalent vaccines for these two serotypes. Although the presence of different BTV serotypes in the same geographic area resulted in simultaneous vaccinations with inactivated monovalent BTV vaccines in different European countries, there is no scientific report on how to combine two of these vaccines to achieve optimal protection.

This study was thus designed to compare different vaccination schemes and techniques using commercially available monovalent vaccines against BTV-4 and −8 from different companies to find the most effective immunisation scheme for sheep under field conditions with respect to safety, immunogenicity and saving in time.

This study was started in spring 2016 to compare simultaneous individual versus double injection (using a twin syringe) of two inactivated BTV-4 and BTV-8 vaccines and simultaneous one-shot versus boosted vaccination compared to a consecutive vaccination scheme. As vaccines were not continuously available in 2016, the consecutive vaccination group received a different vaccine combination (setting 2: Bluevac®4 and Bluevac®BTV8 (both CZ Veterinaria S.A., Porrino, Spain)) than the other groups (setting 1: Bluevac®4 and Zulvac® 8 Ovis (Zoetis Belgium S.A., Louvain-la-Neuve, Belgium)). Due to inconsistent serotype-specific antibody results of animals following vaccination using the consecutive protocol, we decided to rerun the consecutive scheme in the same and the reverse serotype order in summer 2017, using the same vaccines as in the previous experiment. In the meantime, an additional vaccine had become available (BTVPUR® AlSap 4 (Merial, Lyon, France)), which was licensed for a one-shot application. The one-shot experiment was therefore repeated using this vaccine in combination with Bluevac®BTV8 (setting 3).

Procedures on animals in this study were licensed by the federal state government of Upper Bavaria, Germany (for groups A-E, Regierung von Oberbayern Az. 55.2-1-54-2532.0-48-2016) and the federal state government of Lower Saxony, Germany (for groups F-H, Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Az. 33.8-42502-05-17A211) and were conducted in accordance with German animal welfare legislation and the EU Directive 2010/63/EU for animal experiments.

2. Material and methods

The three settings combined with the different application schemes resulted in eight different groups (groups A-H), each comprising 30 sheep. In total, 240 sheep were hence included in the study, kept on five different farms and representing a study under field conditions (Table 1). Virus strains and contents of the vaccines are summarised in Table 2.

2.1. Setting 1

In this setting, Bluevac®4 and Zulvac® 8 Ovis were used in four groups (A–D) to compare two modes of simultaneous application, and to compare a one-shot versus boosted vaccination scheme. Groups A and B derived from farm 1 and groups C and D from farm 2 and 3, respectively. All three commercial sheep farms were located in the federal state of Bavaria. They kept Merino Land Sheep on pasture, supplementing minerals during the summer and being housed in winter with a diet of silage, hay, concentrates and mineral supplements. The majority were female, between six

Table 1
Vaccination schemes. Application method and timing of injections of the vaccines Bluevac®4, Bluevac®BTV8 (both CZ Veterinaria S.A., Porrino, Spain), Zulvac® 8 Ovis (Zoetis Belgium S.A., Louvain-la-Neuve, Belgium) and BTVPUR® AlSap 4 (Merial, Lyon, France). Batch numbers of the individual vaccines used are given in brackets.

Setting	Group	Farm	Injection	Week 0	Week 2	Week 4	Week 6
1	**A**	1	Simultaneous individual injection	2 mL Bluevac®4 (160900) 2 mL Zulvac® 8 Ovis (149320/00)		2 mL Bluevac®4 (160900) 2 mL Zulvac® 8 Ovis (149320/00)	
	B	1	Simultaneous double injection	2 mL Bluevac®4 (160900) 2 mL Zulvac® 8 Ovis (149320/00)		2 mL Bluevac®4 (160900) 2 mL Zulvac® 8 Ovis (149320/00)	
	C	2	Simultaneous double injection	2 mL Bluevac®4 (160900) 2 mL Zulvac® 8 Ovis (149320/00)			
	D	3	Simultaneous individual injection	2 mL Bluevac®4 (160900) 2 mL Zulvac® 8 Ovis (149320/00)			
2	**E**	4	Consecutive individual injection	2 mL Bluevac®4 (162426)	2 mL Bluevac®BTV8 (162427)	2 mL Bluevac®4 (162426)	2 mL Bluevac®BTV8 (162427)
	F	5	Consecutive individual injection	2 mL Bluevac®4 (171559)	2 mL Bluevac®BTV8 (162428)	2 mL Bluevac®4 (171559)	2 mL Bluevac®BTV8 (162428)
	G	5	Consecutive individual injection	2 mL Bluevac®BTV8 (162428)	2 mL Bluevac®4 (171559)	2 mL Bluevac®BTV8 (162428)	2 mL Bluevac®4 (171559)
3	**H**	5	Simultaneous individual injection	1 mL BTVPUR® AlSap 4 (L443854) 2 mL Bluevac®BTV8 (171559)			

Table 2
Details of the vaccines used in this study: virus strains, virus content, adjuvants, preservatives and recommended application in accordance with the manufacturers' instructions. $CCID_{50}$ = 50% cell culture infectious dose.

Vaccine	Virus strain	dose and applica-tion	amount of virus per dose	Aluminium-hydroxide per Dose	Saponin per Dose	Thiomersal per Dose
Bluevac®4 (CZ Veterinaria S.A., Porrino, Spain)	BTV-4/SPA-1/2004	2 mL s.c., boosted	Per Dose $\geq 10^{6.8}$ $CCID_{50}$ (equivalent to titre prior to inactivation (log_{10}))	4.15 mg	0.1 mg	0.2 mg
Bluevac®BTV8 (CZ Veterinaria S.A., Porrino, Spain)	BTV-8/BEL-2006	2 mL, s.c., boosted	Per mL $\geq 10^{6.5}$ $CCID_{50}$ (equivalent to titre prior to inactivation (log_{10}))	12 mg	0.1 mg	0.2 mg
Zulvac 8 Ovis (Zoetis Belgium S.A., Louvain-la-Neuve, Belgium)	BTV-8/BEL2006/02	2 mL, s.c., boosted	Per Dose RP ≥ 1 (Relative Potency by a mice potency test compared to a reference vaccine that was shown efficacious in sheep)	4 mg	0.4 mg	0.2 mg
BTVPUR AlSap 4 (Merial, Lyon, France)	BTV-4/CORS	1 mL, s.c., one-shot	Per mL and Dose ≥ 1.86 log_{10} Pixel/mL antigen content (VP2) in the immunoassay	2.7 mg	30 haemo-lytic units	

months and eleven years of age and with unknown pregnancy status at the time of vaccination.

All sheep in groups A and B (n = 60) were examined for local reactions (e.g. erythema, swelling, heat, pain or wool loss) for five days after the first vaccination (afv) and after the booster vaccination. The rectal temperature of these animals was measured every evening on the same days (Veterinary-Thermometer SC 12, Scala Electronic GmbH, Stahnsdorf, Germany) as well as the environmental temperature (TechnoLine WS 9180, TechnoTrade Import-Export GmbH, Berlin-Wildau).

Groups A and B were booster vaccinated four weeks afv whereas groups C and D received a one-shot vaccination (Table 1). In groups A and D, the two vaccines were injected individually on each side of the neck using an HSW VET-MATIC (Henke Sass Wolf, Tuttlingen, Germany). For groups B and C, a Socorex®287 twin-syringe (Socorex Isba S.A., Eclubens, Switzerland) was used, which allows double injection of two distinct liquids 28 mm apart. In all animals, the vaccines were administered subcutaneously in the neck with 1.6×15 mm needles (Acufirm, Dreieich, Germany) which were disinfected with isopropanol (Dr. Schumacher GmbH, Malsfeld, Germany) after each injection.

Blood samples were collected by jugular venipuncture prior to vaccination and 2, 4, 8, 16, 26 weeks afv and before re-vaccination in the following year (42–59 weeks afv) referred to as 52 weeks afv below.

As all farms in this vaccine group were commercial farms, animal numbers per group declined during the study due to some animals being culled, sold or slaughtered (Table 4).

2.2. Setting 2

Groups E-G were vaccinated using Bluevac®4 and Bluevac®BTV8. These three groups were housed in two research facilities. Group E consisted of Alpine sheep, kept in the federal state of Bavaria (farm 4), housed with access to a small exercise pasture. They received hay and a mineral supplement, plus concentrates in late pregnancy and during lactation. All included 30 animals were female, between eight months and six years of age and five ewes were pregnant at the time of vaccination. Groups F and G were located in the federal state of Lower Saxony (farm 5). All participating sheep were German Black-Headed Mutton, female and between two and seven years old. They were kept indoors, fed silage and concentrates, supplemented with minerals. At the time of vaccination, over 90% were pregnant.

All groups were vaccinated using consecutive boosted schemes at fortnightly intervals, groups E and F starting with BTV-4 followed by BTV-8 vaccination and group G in the reverse serotype order (BTV-8 followed by BTV-4) (Table 1).

The animals in group E were injected subcutaneously on the lateral thoracic wall using an HSW VET-MATIC with 1.6x40 mm needles (Easy-Lance 16G, WDT, Garbsen, Germany) and needles were exchanged after approximately every eighth sheep. Blood samples were collected by jugular venipuncture prior to vaccination and 2, 4, 8, 16 and 26 weeks afv.

On farm 5, animals (groups F and G) were injected subcutaneously on the lateral thoracic wall using an HSW VET-MATIC with 1.1×25 mm needles (CuraVet, WDT, Garbsen, Germany) which were exchanged for every sheep and injection, respectively.

Blood samples were collected by puncturing the Vena cava [17] prior to vaccination and 2, 4, 6, 8 and 16 weeks afv.

2.3. Setting 3

Group H was vaccinated with Bluevac®BTV8 and BTVPUR® AlSap 4 as simultaneous individual injections and a one-shot vaccination scheme (as recommended by the manufacturer for

BTVPUR® AlSap 4, while a booster vaccination was recommended for Bluevac®BTV8 (Table 2)). These 30 sheep belonged to farm 5, and husbandry conditions as well as procedures were identical to groups F and G.

All animals in all groups were clinically healthy before vaccination and were monitored daily for any adverse effects of the vaccination and clinical signs of BT.

The centrifuged (2300× g for 5 min) and decanted serum samples were stored at −20 °C until testing.

To ensure no BTV was circulating or approaching from recent cases in France, EDTA blood samples collected before the first vaccinations were tested by the State Veterinary Investigation Centre Aulendorf (STUA Aulendorf, Germany) for detection of BTV RNA using the VIROTYPE® BTV Plus RT-PCR Kit (Qiagen®, Hilden, Germany) following the instruction manual. All tested samples were negative (data not shown). Germany was BTV-free by declaration throughout the study period and first BTV-8 cases were detected in December 2018 [18].

2.4. ELISA

All serum samples were tested for pan-BTV antibodies using a commercial competitive ELISA (ID Screen® Bluetongue Competition assay, IDvet, Grabels, France) in accordance with the manufacturer's instructions at the Clinic for Swine and Small Ruminants of the University of Veterinary Medicine Hannover, Germany. Results are expressed as percentage negativity (PN) compared to the negative control. Results were classified as positive (PN ≤ 50), inconclusive (50 < PN < 60) or negative (PN ≥ 60) in accordance with the cut-offs provided by the manufacturer (ELISApos/neg). This competitive ELISA is validated [19] and showed a measured sensitivity of 100% ($CI_{95\%}$: 99.49% – 100%, n = 754 (cattle, sheep, goats)) and a measured specificity of 100% ($CI_{95\%}$: 99.84% – 100%, n = 2461 (cattle, sheep, goats)) (personal communication K. Klewer-Fromentin, IDvet, Grabels, France).

2.5. Serum neutralisation test (SNT)

Serotype-specific neutralising antibody (nAb) titres against BTV-4 and BTV-8 were determined in serum samples taken up until 26 weeks afv. Five to six animals were chosen arbitrarily of each group for these tests (n = 6 for groups A-D, H; n = 5 for groups E-G). These tests were carried out at the Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Isle of Riems, Germany.

Heat inactivated serum samples were diluted five-fold in cell medium, RPMI 1640 (Biochrom, Berlin, Germany) containing 2% foetal calf serum, L-Glutamin (2 mM; Biochrom, Berlin) and Penicillin/Streptomycin (100 IU/mL; Biochrom, Berlin). Subsequently, sera were serially diluted two-fold. Of each dilution 50 μL were mixed in quadruplicate with 50 μL cell medium containing 1×10^2 $CCID_{50}$ BTV-8 or BTV-4, respectively. The sera were co-incubated with virus for 3 h at 37 °C and afterwards added to almost confluent layers of Madin-Darby-Bovine-Kidney (MDBK) cells in 96 well flat bottom microtiter plates. After incubation for 2–3 days at 37 °C, the monolayer was scored for cytopathic effect. The Neutralising Dose 50 (ND_{50}) is given as the reciprocal of the highest serum dilution that caused virus neutralisation in 50% of the tested quadruplicates. ND_{50} were calculated according to the method developed by Spearman and Kärber [20] and adjusted to the actual volume used in the assays (100 μL). SNTs with inconsistent results were performed twice and statistics were calculated with means. There was no opportunity to correlate the nAbs to actual protective efficacy and hence no threshold values for the SNT were defined. As robust inhibitory activity up to a dilution of 1:56.6 was observed, we consider titres below a level of 7.4 as weak or negative.

To ensure only initially naïve animals were included in the evaluation of the immunogenic results, we raised the manufacturers cut-off in the ELISA to PN > 80 and excluded all animals with ELISA results below this threshold or positive SNT results ($ND_{50} \geq 7.4$) from further analyses.

2.6. Statistics

Statistical analyses were carried out using the statistical software SAS® version 9.4 (SAS Institute Inc., Cary, NC, USA) with the SAS Enterprise Guide 7.1 Client.

Data were checked for normal distribution using the Kolmogorov-Smirnov test and visual assessment of the q-q plots. In the case of right-skewed distributed results (PN-ELISA), data were log transformed prior to the analysis (logELISA).

The results were tested for statistically significant differences between groups within each setting: in setting 1, groups A-D were compared separately pairwise for each measured time point. In setting 2, groups E-G were compared accordingly (Table 3).

In addition, within setting 1, the groups were combined according to their respective scheme and differences were tested pairwise. Groups A and D (individual injection) were compared to groups B and C (double injection) accordingly for potential differences associated with the application method. Likewise, groups C and D (one-shot vaccination) were compared to groups A and B (boosted vaccination). Setting 3 was compared to group D of setting 1 because the same scheme was applied in these two groups (simultaneous individually injected one-shot vaccination) using different vaccines.

For groups A and B, the differences in body temperature for successive days per animal were calculated and averages thereof per measured time and group. Significant differences between these averages were tested using the *t*-test.

The ELISApos/neg results were tested for statistically significant differences using Fisher's exact test with adjusted p-values (Bonferroni). Seroprevalences were calculated by counting inconclusive results as positive.

Differences in logELISA were tested with the two-way ANOVA for independent and repeated measurements in terms of interactions and the post hoc Tukey test for multiple pairwise comparisons, taking into account the experiment-wise error rate.

BTV-4 and BTV-8 nAb results were calculated separately with the two-way ANOVA for independent and repeated measurements in terms of interactions and the post hoc Tukey test. Proc "Mixed" was used for calculating the linear models. Differences were considered to be statistically significant when $p < 0.05$.

3. Results

Other than a slight increase in body temperature in group B, no side-effects of vaccination (e. g. erythema, swelling, wool loss, hyperallergic reaction, abortion, nervous syndromes) were observed in the animals. None of the animals showed any clinical signs of BT.

Eighteen animals were ELISA positive in pre-vaccination samples according to the manufacturer's cut-off (PN ≥ 60) and four sheep showed inconclusive pre-vaccination results (50 < PN < 60). In addition to these another eleven animals were excluded from further analyses, eight of which showed pre-vaccination ELISA results of 60 ≤ PN ≤ 80, while three had positive BTV-8 SNT results (ND_{50} 9.6, 18 and 56.6, respectively) (Fig. 1).

Table 3
Significant differences ($p \leq 0.05$) within settings in terms of ELISA and serum neutralisation results. Significant differences are indicated by superscript letters.

Group	Laboratory and *statistical* method	Week 0	Week 2	Week 4	Week 6	Week 8	Week 16	Week 26
Setting 1								
Individual (A and D) versus double (B and C) injection	SNT BTV-4 *Two-Way ANOVA, post hoc Tukey test*			0.0284				0.0317
	SNT BTV-8 *Two-Way ANOVA, post hoc Tukey test*	0.0351					0.0206	
One-shot (C and D) versus boosted vaccination (A and B)	ELISA pos/neg *Fishers exact test, Bonferroni adjustion:* ***a** * 0.05/6 = **0.0083***			0.0241		0.0251		
	SNT BTV-4 *two-way ANOVA. post hoc Tukey test*					<0.0001	0.0062	
	SNT BTV-8 *Two-Way ANOVA, post hoc Tukey test*			0.0189				
Setting 2								
Pairwise groups E-G	ELISA pos/neg *Fishers exact test, Bonferroni adjustion:* ***a** * 0.05/9 = **0.0056***		0.0243^{FG}					
	SNT BTV-4 *Two-Way ANOVA, post hoc Tukey test*	0.0458^{EF}	0.0011^{EF} 0.0097^{FG}	0.0115^{EF}	0.0050^{FG}			
	SNT BTV-8 *Two-Way ANOVA, post hoc Tukey test*	0.0119^{EF}						
Setting 1 and 3								
	SNT BTV-8 *Two-Way ANOVA, post hoc Tukey test*					0.0052	0.0040	

3.1. Setting 1

Adverse effects: Of the animals in groups A and B, no reactions on the injection sites were detected. Due to relatively high mean body temperatures at the beginning of the study (group A 40.22 ± 0.48 °C and group B 39.86 ± 0.54 °C, environmental temperature 26 °C, respectively) only temperature increases between successive days were evaluated statistically.

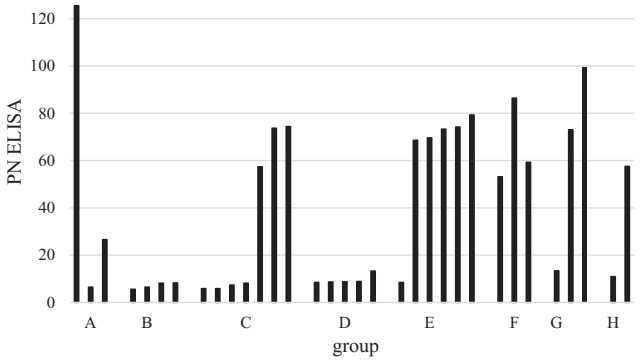

Fig. 1. Seropositive animals at day 0 as measured by percentage negativity (PN) ELISA ≤ 80. The three animals > 80 (group A, F and G) were positive in serum neutralisation for BTV-8.

The highest average increase in body temperature was measured in group B on days 28/29 (0.88 ± 0.61 °C), ie., between the day of booster injection and the following day (environmental temperature 17 °C and 15 °C, respectively). Proximate increases were between days 32/33 in group A (0.76 ± 0.34 °C) and between the same days in group B (0.71 ± 0.37 °C), environmental temperatures 17 °C and 23 °C, respectively.

Significantly higher increases in body temperatures in group B compared to group A were found after the first as well as the booster vaccination, i. e., between days 0/1 ($p = 0.0071$) and between days 28/29 ($p = 0.0001$).

Immunogenicity: Groups A-C reached 100% seroprevalence two weeks afv according to ELISA, representing a rapid seroconversion (Table 4). Groups B and C showed the highest consistency of seroconversion with 100% seroprevalence for four consecutive measured times (weeks 8, 16, 26 and 52). Significant differences were found in the logELISA results, showing lower antibody activity in group D at weeks 2, 4, 8, 16 and 26 afv (Fig. 2a). However, no significant differences were found when considering the ELISApos/neg results (Table 3).

Group B showed the highest overall BTV-4 nAb titres by 8 weeks afv (median ND_{50} 48,3), resulting in significant differences

Table 4
pan-BTV seroprevalence according to ELISA (ID Screen® Bluetongue Competition assay, IDvet, Grabels, France). Results given as seropositive animals/total animals in specific group and resulting percentage (in brackets).

Setting	Group	Week 0	Week 2	Week 4	Week 6	Week 8	Week 16	Week 26	Week 52
1	**A**	0/27 (0%)	27/27 (100%)	27/27 (100%)		26/26 (100%)	25/25 (100%)	20/21 (95%)	18/19 (95%)
	B	0/26 (0%)	25/25 (100%)	24/25 (96%)		23/23 (100%)	21/21 (100%)	16/16 (100%)	9/9 (100%)
	C	0/23 (0%)	22/22 (100%)	21/22 (96%)		23/23 (100%)	23/23 (100%)	21/21 (100%)	21/21 (100%)
	D	0/25 (0%)	23/25 (92%)	18/24 (75%)		19/24 (79%)	18/22 (82%)	16/17 (94%)	10/10 (100%)
2	**E**	0/24 (0%)	21/24 (88%)	24/24 (100%)		24/24 (100%)	22/24 (92%)	17/19 (89%)	
	F	0/27 (0)%	26/27 (96%)	27/27 (100%)	27/27 (100%)	27/27 (100%)	26/26 (100%)		
	G	0/27 (0%)	19/27 (70%)	26/27 (96%)	25/27 (93%)	26/26 (100%)	23/25 (92%)		
3	**H**	0/28 (0%)	25/28 (89%)	26/28 (93%)	19/28 (68%)	20/28 (71%)	22/28 (79%)		

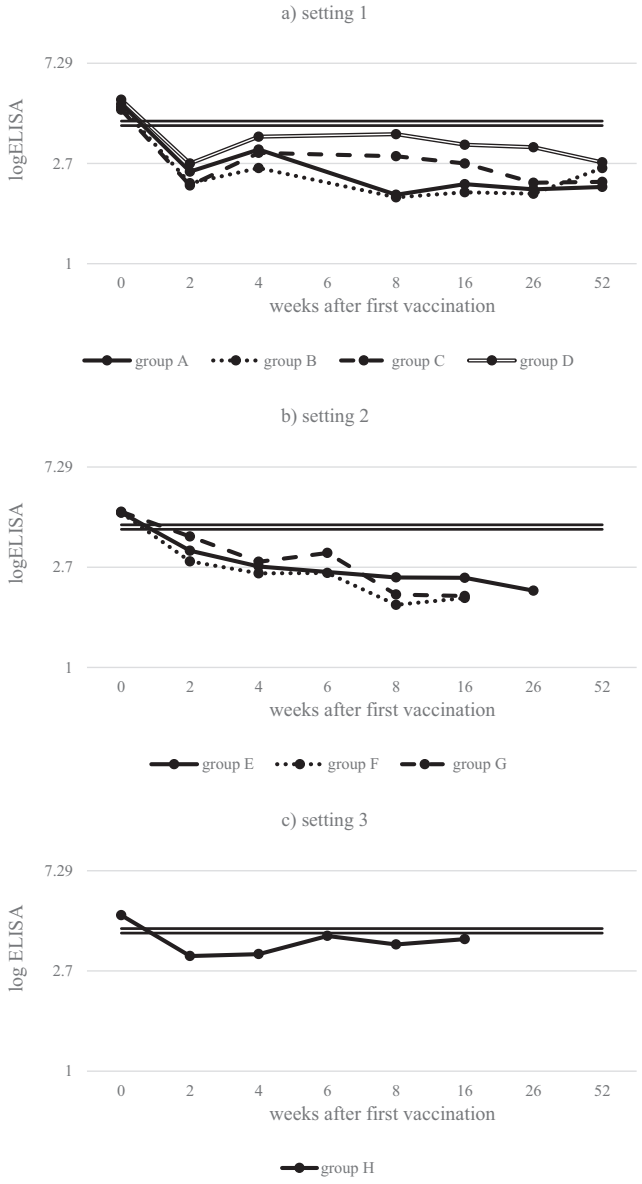

Fig. 2. Medians of log transformed percentage negativity results as measured by ELISA for setting 1, setting 2 and setting 3. The black double line indicates the log transformed threshold of the ELISA (≥4.11 = negative, 4.11–3.93 = inconclusive, ≤3.93 = positive).

to groups C and D ($p = 0.0121^{BC}$, $p = 0.0046^{BD}$), which at this time-point reached a median ND_{50} of 14.35 and 10.15, respectively (Tables 3 and 5, Fig. 3a).

Pairwise comparison of groups within setting 1 showed significant differences in BTV-8 nAb responses between group A (median ND_{50} 13) and group C (median ND_{50} 38.3) at 4 weeks afv ($p = 0.0023$) (Tables 3 and 6, Fig. 4a). One animal in experimental group A did not develop any detectable BTV-8 nAb at all.

Individual versus double injection: double injection (groups B and C) resulted in significantly higher levels of pan-BTV antibody activity measured by logELISA in comparison to individual injection (groups A and D): week 2 ($p < 0.0001$), week 4 ($p = 0.0003$), week 8 ($p = 0.0107$), week 16 ($p = 0.0496$) and week 26 ($p = 0.0186$), respectively. However, no significant differences were found when considering the ELISApos/neg results (Table 3). Significant differences in BTV-4 nAb response were seen at week 4 ($p = 0.0284$) and week 26 afv ($p = 0.0317$) (Tables 3 and 5, Fig. 3a). In BTV-8 nAb response a significant difference was seen before vaccinations ($p = 0.0351$) and 16 weeks afv ($p = 0.0206$) (Tables 3 and 6, Fig. 4a).

One-shot versus boosted vaccination: boosted vaccination (groups A and B) led to significantly higher levels of pan-BTV antibodies measured by logELISA, when compared to one-shot application (groups C and D) in week 4 ($p = 0.0005$), week 8 ($p < 0.0001$), week 16 ($p < 0.0001$) and week 26 ($p = 0.006$), respectively. In general, ELISA results of the one-shot groups were more heterogenous than the results of the boosted groups. However, no significant differences could be shown when taking into account the ELISApos/neg results (Table 3). There was a marked booster effect in BTV-4 nAb titres resulting in significantly higher results for boosted groups (groups A and B) in week 8 ($p < 0.0001$) and week 16 ($p = 0.0062$) afv. BTV-4 nAb titres in groups A and B then dropped to the level of the one-shot groups by week 26 afv (Fig. 3a and Table 5). For BTV-8 nAb the booster effect was not significant compared to the one-shot groups C and D. These groups had significantly higher BTV-8 titres in week 4 afv ($p = 0.0189$), which was immediately before the booster injection (Fig. 4a and Tables 3 and 6).

3.2. Setting 2

Group F showed 100% ELISA-positive animals after the first (BTV-4) and second (BTV-8) injection (week 4) up until week 16 afv, whereas group E with the identical vaccination scheme only fully seroconverted on two of these occasions (week 4 and 8). Group G, which was vaccinated in reverse serotype order, only

Table 5
BTV-4 neutralising antibodies (nAb) as measured by serum neutralisation, results are expressed as Neutralising Dose 50. Total of tested animals (n) given in brackets after minimum (min) and maximum (max).

BTV-4 nAb		Setting 1				Setting 2			Setting 3
		A	B	C	D	E	F	G	H
Week 0	*Median*	0.1	2.55	0.1	4.2	0.1	5.3	2.2	1.15
	Min-max *(n)*	0.1–0.5 (6)	0.1–7.1 (6)	0.1–4.2 (6)	0.1–5 (6)	0.1 (5)	0.1–7 (5)	1.5–7.1 (5)	0.1–3 (6)
Week 2	*Median*					0.1	11.9	2.8	7.6
	Min-max *(n)*					0.1 (5)	5.2–27.1 (5)	1.5–5.5 (5)	4.2–10.9 (6)
Week 4	*Median*	5.9	5.9	13.4	7.15	0.1	14.4	2.6	6.75
	Min-max *(n)*	4.2–8.3 (6)	5–20 (6)	5.9–33.6 (6)	4.2–8.4 (6)	0.1–4.2 (5)	6–23.9 (5)	2.2–11.9 (5)	2.6–14.2 (6)
Week 6							33.6	12.3	7.25
	Min-max *(n)*					8.4	17–53.6 (5)	0.1–25.9 (5)	4.6–13 (6)
Week 8	*Median*	40	48.3	14.35	10.15	9.2	22.8	40	6.4
	Min-max *(n)*	4.2–56.6 (6)	23.8–56.6 (6)	7.1–23.8 (6)	5–33.6 (6)	2–40 (5)	21.2–52.1 (5)	11.3–52.1 (5)	2.2–10.6 (6)
Week 16	*Median*	45.1	30.95	16.8	13	5.9	21.8	24.1	6.2
	Min-max *(n)*	0.1–56.6 (6)	23.8–56.6 (6)	7.1–40 (6)	10–23.8 (6)	0.1–16.8 (5)	7.1–42.4 (5)	10–48.3 (5)	0.1–14.4 (6)
Week 26	*Median*	14.35	28.3	23.8	5.9	20			
	Min-max *(n)*	0.1–40 (6)	23.8–56.6 (5)	16.8–33.6 (6)	0.1–20 (3)	7.1–40 (5)			

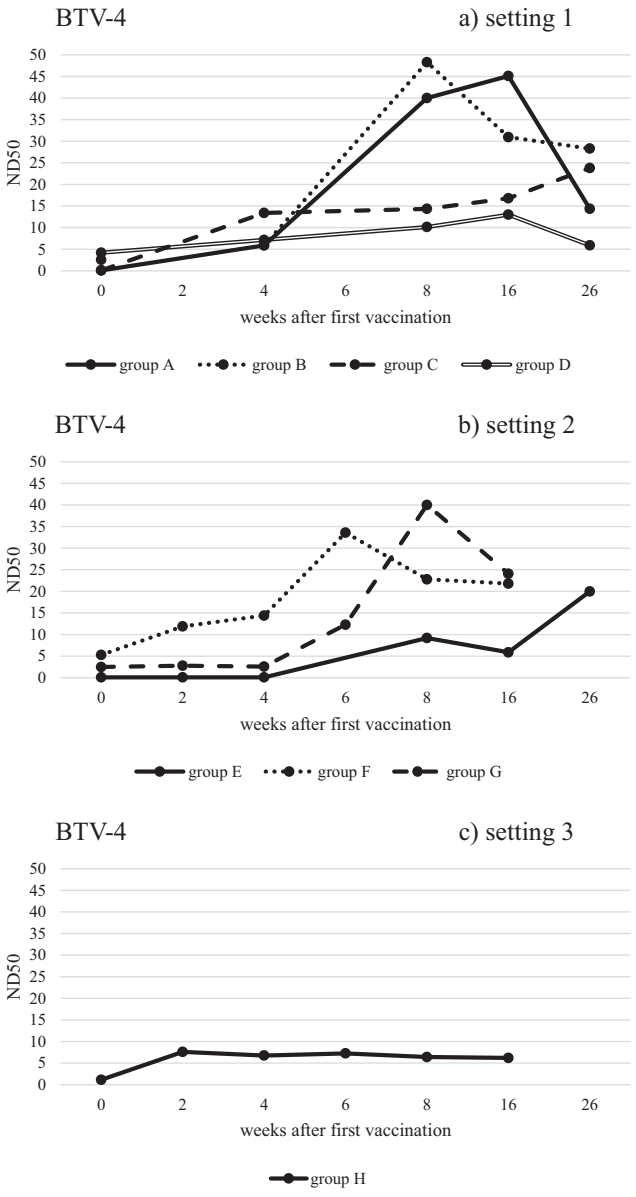

Fig. 3. Medians of BTV-4 neutralising antibodies as measured by serum neutralisation for setting 1, setting 2 and setting 3 given in Neutralising Dose 50 (ND50).

showed 100% seroconversion at week 8 afv (Table 4). LogELISA revealed significantly different results between groups E and F at week 8 ($p = 0.0054$) and week 16 (0.0131) afv, but no significant differences were observed when assessing ELISApos/neg results.

BTV-4 nAb titres varied widely between groups, showing significantly higher titres in group F before vaccinations and in weeks 2, 4 and 6 afv (Fig. 2b, Tables 3 and 5). Setting 2 showed low to no BTV-8 nAb response. Group F showed few cytopathic effects in SNT before vaccinations (Table 6) resulting in the overall highest median ND_{50} after vaccination at week 2 (ND_{50} 4.2, Table 6, Fig. 4b). The only significant differences in BTV-8 nAb within groups E-G were seen before vaccinations between group E and F ($p = 0.0119$). Two animals in group E did not show any detectable BTV-8 nAb at all.

3.3. Setting 3

Group H did not seroconvert completely and showed the lowest percentage of ELISA-positive animals in comparison to the other groups (Table 4). BTV-4 and BTV-8 nAb responses were low with highest median ND_{50} in week 2 afv reaching 7.6 (BTV-4) and 4.85 (BTV-8), respectively (Figs. 3c and 4c, Tables 5 and 6).

Compared to group D of setting 1 with a simultaneously and individually injected one-shot scheme, significant differences were seen in BTV-8 nAb in week 8 and week 16 afv (week 8 $p = 0.0052$, week 16 $p = 0.0040$), respectively (Table 3) due to higher BTV-8 nAbs in group D (Table 6).

4. Discussion

All combinations of two inactivated BTV vaccines were well tolerated by the sheep in this study with no clinical side effects other than a moderate increase in body temperature immediately after the initial and booster vaccination. We did not observe any other adverse effects following simultaneous vaccinations due to the double amount of aluminium hydroxide as adjuvant which showed diverse side effects in previous studies [21,22].

Regarding possible adverse effects of the vaccination, an increase in body temperature was to be expected according to previous findings [23–27]. Astonishingly, the increase was significantly higher in the simultaneous, double injected group B compared to the simultaneous but individually injected group A. This could have been caused by an interaction of adjuvants when the two vaccines were injected in close proximity using a twin syringe as opposed to injection at distinct sites. Relatively high mean

Table 6
BTV-8 neutralising antibodies (nAb) as measured by serum neutralisation, results are expressed as Neutralising Dose 50. Total of tested animals (n) given in brackets after minimum (min) and maximum (max).

BTV-8 nAb		Setting 1				Setting 2			Setting 3
		A	B	C	D	E	F	G	H
Week 0	*Median*	0.1	0.1	0.1	4.2	0.1	6.7	2.8	2.6
	Min-max *(n)*	0.1–7.1 (6)	0.1 (6)	0.1–5 (6)	0.1–5 (6)	0.1 (5)	0.7–7.3 (5)	1.5–5.1 (5)	0.1–5.5 (6)
Week 2	*Median*					0.1	4.2	3.4	4.85
	Min-max *(n)*					0.1 (5)	0.1–5.6 (5)	1.5–6.3 (5)	2.2–12.6 (6)
Week 4	*Median*	13	16.8	38.3	20	0.1	3.6	2.2	1.15
	Min-max *(n)*	0.1–20 (6)	14.1–28.3 (6)	7.1–56.6 (6)	11.9–33.6 (6)	0.1 (5)	2.6–6 (5)	0.1–8.4 (5)	0.1–18.4 (6)
Week 6	*Median*						4	3	2.6
	Min-max *(n)*						4–7.5 (5)	2.6–9.9 (5)	0.1–16.8 (6)
Week 8	*Median*	40	48.3	36.8	30	0.1	2.2	3	2.2
	Min-max *(n)*	0.1–56.6 (6)	28.3–56.6 (6)	23.8–40.1 (6)	10–47.6 (6)	0.1–8.4 (5)	0.1–5.1 (5)	0.1–5.6 (5)	0.1–8.5 (6)
Week 16	*Median*	32.2	47.6	40	28.3	0.1	2.2	0.1	0.1
	Min-max *(n)*	0.1–56.6 (6)	28.3–56.6 (6)	30–56.6 (6)	14.1–40 (6)	0.1 (5)	0.1–8 (5)	0.1–5.6 (5)	0.1–7.5 (6)
Week 26	*Median*	34.15	40	34.15	47.6	0.1			
	Min-max *(n)*	0.1–56.6 (6)	14.1–56.6 (5)	20–56.6 (6)	8.4–47.6 (3)	0.1–7.1 (5)			

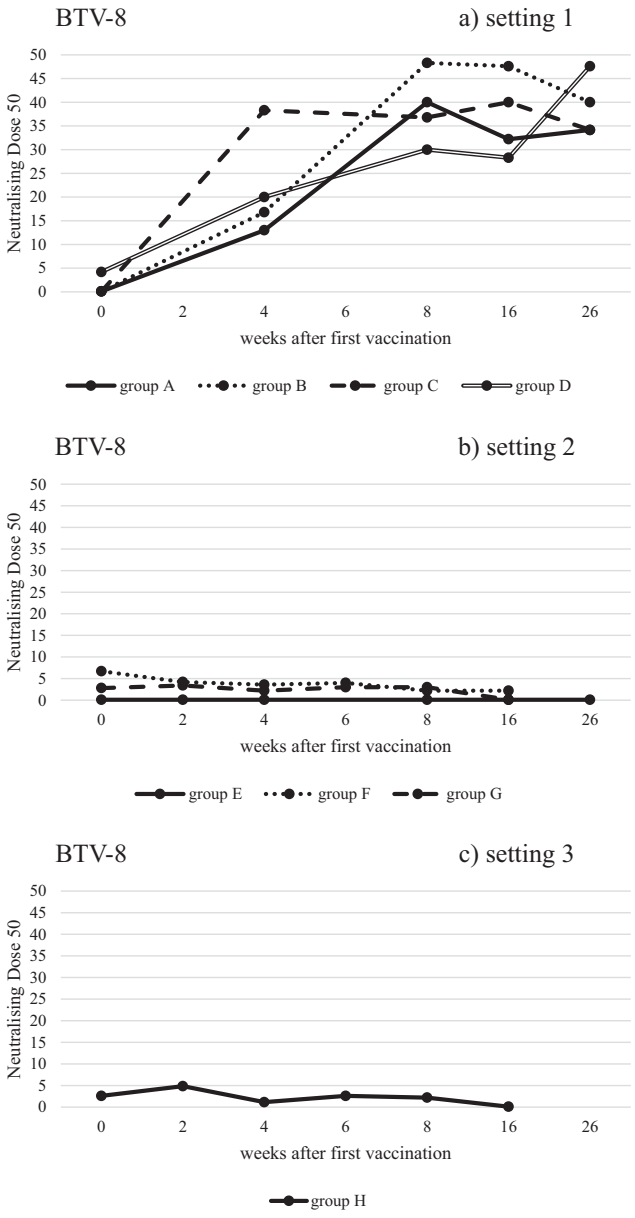

Fig. 4. Medians of BTV-8 neutralising antibodies as measured by serum neutralisation for setting 1, setting 2 and setting 3, given in Neutralising Dose 50 (ND50).

pre-vaccination body temperatures were measured in all animals in groups A and B (mean body temperature 40.04 °C, n = 60). The physiological body temperature of adult sheep is reported to vary between 38°–40 °C [28]. The animals did not show any signs of illness and the relatively high body temperatures were attributed to high environmental temperatures [28]. Exercise [29] and stress [29–31] during gathering as well as the circadian rhythm [32] may also have had an impact on higher body temperatures.

The lack of an unvaccinated control group and the small number of animals limits the value of the temperature data.

Inactivated vaccines are commonly thought to stimulate an immune response dominated by CD4 + Th2 cells which in their turn act as stimulators for Ab-producing B cells [33]. Umeshappa et al. proved stimulation of the cellular immune response after vaccination with inactivated BTV [34], so both arms of the immune system are assumed to participate in the protective effect of vaccination with inactivated BTV. On the cellular side of the immune reaction, cytotoxic T-cells (CTLs) are considered responsible for immediate and cross-serotype protection [35,36]. On the humoral side of the immune reaction, pan-BTV species-specific, non-neutralising Abs against the inner capsid protein VP7 are produced. They are used for diagnostic purposes (ELISA) but their role in protective immunity is unclear [37]. Serotype-specific nAb are directed against outer capsid proteins VP2 and to a smaller extent against VP5 [16]. As virus challenge was beyond the scope of this study it remains unclear whether the low serotype-specific nAb titres in setting 2 and 3 would reach protective levels in case of infection. In addition, because no investigation of the cellular immune response was undertaken. Previous studies also showed very low nAb, which increased markedly after virus challenge and prevented viraemia and clinical disease [26,38].

Within setting 1 the one-shot immunisation resulted in more heterogeneous ELISA-activities than boosted vaccination, supporting a previous study by Wäckerlin et al. [39]. As the majority of samples still achieved positive ELISA results, this led to no significant differences between one-shot and boosted groups when considering the ELISApos/neg-results. The weakest ELISA responses within setting 1 were seen in the double injected one-shot group D, emphasizing the recommendation of boosted injections. On the other hand, all animals in both one-shot groups within setting 1 were seropositive by ELISA one year afv, similar to the boosted group B. If pan-BTV seropositivity correlates with protection from viraemia, animals could be protected for one year after a one-shot vaccination with the vaccine combination of this setting (Bluevac®4, Zulvac® 8 Ovis), even though this scheme was not in accordance with the manufacturers' instructions. This could be of interest in flocks which are not accurately booster vaccinated, e.g. after vaccine shortages. However, this aspect needs further research and cannot be transferred to other vaccine combinations. Still, the licence of Bluevac®4 has since been changed in 2018, now recommending an annual one-shot injection.

Animals of setting 2 showed slower seroconversion according to ELISA results, nevertheless reaching 96–100% seropositive results 4 weeks after one vaccination. Several potential causes may be responsible for the significant differences in logELISA results between group E and F with the identical vaccination scheme. These are discussed later as potential causes for further differences in the serotype-specific nAb response.

Whereas even low nAb titres are considered to protect from clinical infections [40,41], we occasionally observed no cytopathic effect in the lowest serum dilution in unvaccinated animals at week 0. This nominally corresponds to a ND_{50} of 7, we therefore consider results up to this level as weak or negative. This was the case for BTV-4 as well as BTV-8, even causing significant differences between groups before vaccinations (Table 3). As BTV-4 has never been reported in Germany, we attribute these pre-vaccination titres to a non-specific serum effect rather than representing nAbs.

Of the initial groups A-E, only group E did not show a BTV-8 nAb response. We therefore considered the possibility that an initial vaccination with one serotype impairs the immune response to a consecutive vaccination with a different serotype according to the original antigenic sin hypothesis [42]. This possibility was ruled out by including the additional groups F and G in the study, repeating the consecutive vaccination of BTV-4 followed by BTV-8 in group F and vaccinating group G in the reverse serotype order (BTV-8 followed by BTV-4). In all instances, a good response to BTV-4 but only very low BTV-8 nAb titres were observed.

According to Poirier et al. [43] several factors may influence the efficacy of vaccination. These are primary factors like injection technique (angle of injection, needle length, anatomic site), vaccination interval and number of vaccine doses, adjuvants and differences between batches, whereas the animal's body condition, potential pregnancy, health condition, breed, sex and age are con-

sidered secondary factors. Several of these factors may have influenced the differences in measured immunogenicity in this study. As the study was conducted under field conditions, different veterinarians performed injections, resulting in different injection techniques as described in material and methods. Shawn et al. showed that vaccine efficacy in humans depended on whether the vaccine was more likely to be injected into subcutaneous, muscular or adipose tissue [44]. Paucity of antigen-presenting cells (in the case of BTV most importantly dendritic cells [45,46]) in adipose tissue are assumed responsible for lower immune responses.

In addition, differences in the vaccines might also have played a role. Protective epitopes could have been destroyed during inactivation, some vaccines may have contained more or less amount of antigen, or damage could have incurred during improper delivery or storage [33]. More likely, differences in the amount and proportion of saponin and aluminium hydroxide in the different vaccines (Table 2) may have stimulated the immune response in different manners. When comparing groups D and H, both individually injected as one-shot with the same BTV-4 vaccine (Bluevac®4) but different BTV-8 vaccines, a significantly higher BTV-8 nAb response induced by Zulvac® 8 compared to Bluevac®BTV8 was seen. If this observation can be attributed to the different vaccines alone or to other factors, which might have additionally influenced nAb-titres, cannot be clarified within this study design. Batch-to-batch variation [33] must also be taken into account for the significant differences between groups E and F, vaccinated using an identical scheme but different vaccine lots.

Secondary factors also varied between groups, e. g. body condition, general health condition, stress and pregnancy rate, which are all known to act as factors influencing the immune system [33,47]. Genetic factors may have played a more important role as various breeds were involved in the study. Merino Sheep are known to be more susceptible to BTV as for example Dorset Poll [48].

In total, three animals remained entirely seronegative for BTV-8. Inappropriate application is unlikely, as the respective animals were vaccinated repeatedly and by experienced veterinary surgeons. Underlying health problems may hinder an adequate immune response [33,38,39], but none of the animals showed signs of clinical disease. Presumably these animals are so-called "non-responders" which do not develop a nAb response after vaccination with certain vaccines [33,49,50]. No vaccination leads to 100% protection and focus should be laid on flock or population immunity [33].

It should be noted that only five to six animals per group were tested for their serotype-specific antibody response representing a small sample size (17–20% of each group) and therefore posing a limitation to this study. Regardless of a one-shot or boosted injection, our results do not exclude the possibility that the duration of immunity may extend beyond one year as previously shown [41,51,52]. Further research is required to establish the duration of immunity and the level of protection following challenge, especially on a flock or population basis.

5. Conclusion

This study shows that the combination of two inactivated BTV-vaccines against different serotypes was well tolerated by all sheep (n = 240). Of all vaccines and schemes described in this study, the simultaneous double injected boosted vaccination with the combination of Bluevac®4 and Zulvac® 8 Ovis (group B) yielded the highest median serotype-specific nAb titres 26 weeks afv and 100% seropositive animals one year afv as measured by pan-BTV ELISA. In setting 1, there were no relevant significant differences in immunogenic response between the vaccines applied simultaneously at different or at the same injection site. Importantly, a one-shot vaccination induced comparable immunogenicity to a boosted injection half a year afv. The use of a one-shot application in combination with the twin syringe technique allows a time- and cost-effective vaccination of large animal numbers and thereby will enhance farmers' compliance and participation in voluntary vaccination programmes. Low nAb levels were detected in settings 2 and 3 and are attributed to diverse factors which may have influenced the measured immunogenicity.

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