Evaluation of a quadrivalent inactivated vaccine for the protection of cattle against diseases due to common viral infections

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ABSTRACT

Efficacy of an inactivated quadrivalent vaccine containing infectious bovine rhinotracheitis (IBR) virus, parainfluenza type 3 (PI3) virus, bovine virus diarrhoea virus (BVDV) and bovine respiratory syncytial virus (BRSV) was assessed in naive bovine calves to evaluate short-term (4–18 weeks) and long-term (24–38 weeks) protection following the basic intramuscular vaccination regime of 2 inoculations a month apart. Vaccination was staggered between the long-term and the short-term groups by about 5 months so that both groups, along with a matched group of 6 unvaccinated (control) calves, could be challenged at the same time. Sequential challenges at intervals of 3–8 weeks were done in the order: IBR virus (intranasally, IN), PI3 virus (IN and intratracheally, IT), pestiviruses (IN) and BRSV (IN and IT). The IBR virus challenge produced febrile rhinotracheitis (FRT) in control calves but both the severity and the duration of FRT was significantly reduced in both vaccinated groups. The amount and the duration of IBR virus shed by the vaccinated groups was significantly reduced compared to the control group. Although PI3 virus, pooled pestivirus and BRSV challenges did not result in a noteworthy disease, challenge virus shedding (amount and duration) from the upper (all 3 viruses) and the lower (BRSV) respiratory tracts was significantly reduced in vaccinated groups. After pestivirus challenge, sera and leukocytes from all control calves were infectious for 6–9 days whereas virus was recovered only from leukocytes in vaccinated calves and only for 1.6–2.7 days. Thus a standard course of the quadrivalent vaccine afforded a significant protection against IBR virus, PI3 virus, BVDV and BRSV for at least 6 months.

Key words: BHV-1, bovine respiratory disease, BRSV, BVDV, efficacy, immunity, PI3 virus, virus neutralising antibody.

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INTRODUCTION

In those countries where the raising of cattle has a high economic priority, requiring intensive methods of animal husbandry, respiratory disease in young and adult cattle and infectious reproductive disorders in breeding animals are a major cause of economic loss. There are several viruses that commonly infect the bovine respiratory and/or reproductive tract. These comprise bovine herpesvirus 1 (BHV-1) commonly known as infectious bovine rhinotracheitis (IBR) virus, parainfluenza type 3 (PI3) virus, bovine respiratory syncytial virus (BRSV), adenovirus serotypes 1–6, reovirus serotypes 1–3, bovine virus diarrhoea virus (BVDV) and rhinoviruses[15]. Of these, however, IBR virus, PI3 virus, BVDV and BRSV are generally considered as important in the aetiology of bovine respiratory disease. IBR virus and BVDV are also significant causes of transplacental infections and reproductive losses. Experimentally, however, only IBR virus consistently produces febrile respiratory disease although natural infections by PI3 virus and BRSV are well-documented causes of severe respiratory disease in cattle[4,15,18]. BVDV is a common cause of abortions and infertility, and some strains of BVDV could also cause severe disease characterised by pyrexia, a marked thrombocytopaenia, profuse diarrhoea, dehydration and even deaths[3,6]. Hence the naturally occurring respiratory disease and bovine pneumonia are considered to be a multi-aetiology syndrome and the best understood interaction in the disease process is that between the primary IBR virus infection and secondary *Pasteurella* spp. infection in shipping fever[19]. PI3 virus and BVDV have also been implicated in pneumonic pasteurellosis[7,17]. It is conceivable that primary viral infections cause significant damage to ciliary epithelia, and thus to the mucus escalator, leaving a way open for secondary bacterial and/or mycoplasma infection of the lower respiratory tract[15]. Hence prevention of the primary viral infections through vaccination will significantly reduce the incidence of respiratory disease and the associated economic loss. This paper documents the quality of short and long-term protection afforded by a quadrivalent inactivated bovine vaccine containing IBR virus, PI3 virus, BRSV and BVDV. The experimental approach used is considered novel in that the efficacy against each of the 4 constituent viruses was assessed by sequential challenges. Also the vaccine is the 1st example of a polyvalent cattle vaccine containing BRSV antigen from a persistently infected, viable bovine cell line[12].

MATERIALS AND METHODS

Tissue culture methods

For (i) infectivity titrations, (ii) virus isolation from nasopharyngeal swabs (NS), buffy coat (BC), serum and lung washings (LW) and (iii) virus neutralising (VN) antibody titrations, bovine embryo lung (BEL) cells[14] were used between passages 20 and 40.

BEL cells were grown at 37 °C in minimum essential medium (MEM, Gibco BRL) supplemented with 10 % donor horse serum (HS, Sigma, Aldrich, Poole, Dorset, UK), 100 units/mℓ penicillin, 100 μg/mℓ streptomycin, 2 mM L-glutamine and 0.09 % sodium bicarbonate (HSGM). This medium containing 2 % HS (HSMM) was used for (i) taking nasal swabs and lung washings (ii) diluting samples for infectivity and antibody titrations as well as (iii) maintaining BEL cell monolayers during virus growth or isolation.

Infectivity titrations. For BHV-1, PI3 and BVDV NS infectivity titrations, using stored frozen specimens, serial 10-fold dilutions of virus were inoculated (200 μℓ/well and 4 wells/dilution) onto 1–3-day-old BEL cell monolayers in 96-well microtitration plates. The plates were incubated at 37 °C in an atmosphere of 5 % CO_2 in air. Cytopathic effects (CPE) were

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read 5–7 days later. However, for noncytopathic ruminant pestiviruses (NCRPVs) BEL cell monolayers were superinfected with cytopathic BVDV (CP-BVDV) strain C86 after 5 days incubation, at a multiplicity of infection of 0.1–0.01 and plates reincubated at 37 °C for 2–3 days. Monolayers completely or partially (at infectivity end point) free of CPE were scored as NCRPV infected as described before[13]. CPE-refractory wells were used to calculate the tissue culture median infective dose ($TCID_{50}$) by a standard method[16].

Virus isolation. BRSV isolations from NS and LW were performed piecemeal using fresh samples as they became available and BVDV isolations from BC and serum from stored frozen specimens. Isolation was on 1–3-day-old BEL cell monolayers in 24-well plates, inoculating 200 $\mu\ell$ of an undiluted sample per well and 1 well per sample. After inocula adsorption (1 hour, 37 °C) each well was re-fed with 800 $\mu\ell$ of HSMM and plates incubated at 37 °C in an atmosphere of 5 % CO_2 in air and CPE read 5 days later. All samples were re-passaged similarly on fresh monolayers of BEL cells using 200 $\mu\ell$ of each passage 1 sample. The 2nd- passage samples were read for CPE after 5 days incubation at 37 °C and results scored as definite findings.

Virus-neutralising antibody titrations. For VN antibody titrations, inactivated (56 °C, 30 minutes) sera were serially diluted in HSMM in 96-well microtitration plates followed by addition to each well of an equal volume (50 $\mu\ell$) of virus suspension in HSMM containing 100–200 $TCID_{50}$ of virus. Mixtures were incubated at 37 °C for 1 hour when 100 $\mu\ell$ of freshly dispersed suspension of 2–4 × 10^4 BEL cells in HSGM (see above) was added to each well and plates incubated at 37 °C as before. Each test also included titration of (i) a positive standard antiserum to constituent vaccinal viruses from a hyperimmunised calf and (ii) the dose of virus contained in the reaction. CPE-based VN tests were read 5 days afterwards and titres expressed as the reciprocal of the highest serum dilution in log_2 scale completely neutralising a virus. End points for neutralisation of NCRPVs were developed and scored after superinfection of monolayers with CP-BVDV on day 5 as described previously[13]. Values with half digits represent a dilution giving complete neutralisation in 1 of 2 monolayers but in both monolayers at lower dilutions. Values shown as <2.0 denote no neutralisation by 4-fold diluted sera.

Animal procedures

Nasopharyngeal swabs (NS) were taken using a 12 cm cotton-tipped plastic swab and extracting each swab in 2 mℓ HSMM.

Lung washings (LW) were collected from unanesthetised calves by irrigating lungs with 40 mℓ of HSMM using a coaxial soft plastic catheter and recovering between 20–25 mℓ for each lavage sample.

Buffy coat cells from blood samples taken in equal volume of isotonic 0.7 % (w/v) tri-sodium citrate solution were washed in phosphate-buffered saline (PBS, pH 7.4) and layered onto 4 mℓ of 63 % (v/v) percol™ (density 1.13 g/mℓ, Amersham Pharmacia Biotech AB, Uppsala, Sweden) cushion in PBS and centrifuged (2000 g, 10 minutes at room temperature) and collecting leukocyte band at sample-cushion interface was collected. This leukocyte fraction was suspended in HSMM diluent at a concentration of 10^6 cells/mℓ and frozen at –70 °C until tested.[13,14]

Challenge inocula

All 4 challenge inocula described below were sterile, mycoplasma free, 2nd to 4th passage stocks grown in BEL cells propagated in HSGM and maintained in HSMM. All viruses except BRSV were grown at 37 °C; BRSV stocks were grown at 35 °C.

BHV-1

Strain 532 was isolated in-house from a nasopharyngeal swab from a field calf (No. 532) with a severe febrile rhinotracheitis. This isolate at a titre of 7.7 log_{10} $TCID_{50}$ per millilitre was administered intranasally (IN, 2 mℓ) and intratracheally (IT, 5 mℓ) to a 3-month-old Friesian-Hereford calf (No. 31), 5 days afterwards, the calf had a high fever (41.5 °C) and severe rhinotracheitis accompanied by anorexia, severe depression, tachypnoea and dyspnoea when the calf was lavaged (lung washings) and virus re-isolated in BEL cells. The challenge inoculum consisted of an equal volume pool of the nasal swab isolate from calf 532 and the lung isolate from calf 31. For challenge each calf was administered 2 mℓ IN containing 6.0 ± 0.3 log_{10} $TCID_{50}$.

PI3

The challenge inoculum consisted of a pool of in-house lung isolates from 5-month-old calves (Nos 444 and 465) that had to be euthanased due to severe tachypnoea, dyspnoea, pyrexia (39.5–40.5 °C) and anorexia over several days. The 2nd passage BEL cell stocks of strains 444 and 465 had titres of 8.3 and 8.7 log_{10} $TCID_{50}$ per millilitre, respectively. For challenge an equal volume pool of the stocks was diluted to contain 6.0 log_{10} $TCID_{50}$ per millilitre and each calf was given 2 mℓ IN and 5 mℓ IT.

BVDV

Calves were challenged with a mixture of 11 isolates of NCRPVs, 3 of which were authentic border disease virus (BDV) strains. The isolation history or source and titre of the constituent strains in the pool are shown in Table 1. The inoculum contained 7.2 log_{10} $TCID_{50}$ per millilitre.

BRSV

The study involved an equal volume pool of 4 field isolates of BRSV (strain AC_2C1_3, Compton, ZW and PL-4). Prior to incorporation into the challenge pool, each isolate was individually passaged once in 2–3-week-old BRSV seronegative and NCRPV free Friesian bull calves. For this, each calf was inoculated IN and IT with 4 and 5 mℓ, respectively, of BEL cell grown virus suspension and lung washings collected 5 and 7 days afterwards and then passaged twice (strain AC_2C1_3 and Compton) or thrice (ZW and PL-4) in BEL cell monolayers at 35 °C in the presence of mycoplasma removal agent (ICN Biomedical, Thame, UK). The latter was a precautionary measure and the incubation period for the serial passages was 5–7 days. The pooled challenge inoculum contained BRSV at a titre of 5.4 log_{10} $TCID_{50}$ per millilitre and for challenge each calf was inoculated IN with 4 mℓ and IT with 5 mℓ.

Vaccine

The pre-betapropiolactone (BPL) inactivation antigen content per 2 mℓ dose in Bovilis® Qarat comprised 7.7 log_{10} $TCID_{50}$ of IBR virus, 7.7 log_{10} $TCID_{50}$ of PI3 virus, 7.4 log_{10} $TCID_{50}$ of CP-BVDV strain C86[14] and 0.5 × 10^6 cells from the persistently infected BRSV cell line MDBK-PL4[12] and 1 and 1.5 % (w/v), respectively, of aluminium hydroxide and aluminium phosphate.

Experimental studies

Efficacy determination

The study involved 3 matched groups, each with 5 or 6 seronegative (all 4 vaccine viruses), 2-week-old Frisean–Hereford cross bull calves. The basic intramuscular (IM) vaccination regime of 2 inoculations a month apart was staggered between the long-term (≥6 months) and the short-term (≤4 months) by about 5 months so that the 2 vaccinated and the unvaccinated control groups could be challenged at the same time. Challenges were performed sequentially at intervals of 3–8 weeks in the order: IBR virus, PI3 virus, BVDV and BRSV as described above

Table 1: **Constituent noncytopathic ruminant pestivirus** (NCRPV) **strains and titres used in a single pool to challenge calves.**

Strain	Passage level in BEL cells	Source[a]	Genotype[b]	Titre (log_{10} $TCID_{50}$/mℓ)
MDBK	4	Madin Darby bovine kidney cell line, UK	BVDV Type 1 (not in subgroups a, b, c or d	8.0
CK 47	4	Calf kidney cells, UK	BVDV Type 1a	7.0
WB	4	IBR virus stock, Ireland	BVDV Type 1a	7.2
ZW	3	BRSV stock, UK	BVDV Type 1d	7.0
BEL-101	3	Bovine embryo lung cell line (embryo 101), UK	BVDV Type 1d	7.4
BEL-110	3	Bovine embryo lung cell (embryo 110), UK	BVDV Type 1a	6.4
Stott	3	BRSV infected bovine cell strain, UK	BVDV Type 1c	7.7
PEN 2	4	Bovine serum, Belgium	BVDV Type 1a	6.0
Moredun 2	2	Moredun Research Institute Edinburgh, Scotland	BDV	6.0
L 83/84	2	Serum isolate from Welsh Mountain × Suffolk lamb, UK	BDV	7.2
R 2727	2	Central Vet. Labs Weybridge, UK	BDV	6.4
Pool of 8 BVDV and 3 BDV strains				7.2

[a]All strains were in-house isolates except Moredun 2 and R 2727. The former was kindly supplied by P F Nettleton, Moredun Institute, Edinburgh, and the latter by S Edwards, VLA, Weybridge.
[b]Typing based on N-Pro sequence homology by H-J Thiel and P Becher, Institute for Virology, Justus-Liebig University, Giessen, Germany.

under challenge inocula. Following each challenge, calves were monitored for clinical reactions (day –2 to day +21), virus shedding in nasal mucus (day 0 to day 14) and bled for serology at intervals until challenge and afterwards. Additionally, for the BVDV challenge, the incidence of viraemia (both serum and leukocytes) was assessed from day 1 through to day 14 and after BRSV challenge and shedding in lungs was assessed by virus isolation from lung washings on alternate days for 2 weeks.

Clinical monitoring

Pyrexia (rectal temperature of ≥39.5 °C) was scored as 1 (39.5–40.0 °C), 2 (40.1–40.5 °C), 3 (40.6–41.0°C) and 4 (>41.0 °C). Respiration rate per minute of 40 and above was scored as 1 (40–50), 2 (51–60), 3 (61–70) and 4 (>70). Calves that were depressed but eating were given a score of 1, those that were depressed with reduced appetite were scored as 2 and anorexia was given a score of 4. Hyperpnoea was given a score of 2 and dyspnoea a score of 4. Nasal discharge was scored as 1 for marked serous, mild mucopurulent as 2, marked mucopurulent as 4 and ocular discharge as 1 for lachrymation, 2 for mild mucopurulent and 4 for marked mucopurulent. Mild conjunctivitis was given a score of 2 and marked conjunctivitis a score of 4. Oral ulcers were scored individually until healing and the size-based scores given were 1 for 1–5 mm, 2 for 6–10 mm and 3 for over 10 mm in diameter. Vomiting of food plus clear fluid was given a score of 1, with bile as 2 and with blood as 3. Diarrhoea was scored as 1 if soft with mucus, 2 if watery, mucoid and profuse and 3 if haemorrhagic. Injection site lesions were scored as 1 for swelling of 5–20 mm, 3 for swelling of 21–50 mm, 5 for swelling larger than 50 mm and abscess as 10.

Calves were clinically examined for all the individual signs at the same time once daily.

RESULTS

Vaccine efficacy

Against IBR virus

The 1st of 4 sequential challenges was with 6.0 log_{10} $TCID_{50}$ of IBR virus administered intranasally, 24 (group 1, long-term immunity) or 4 (group 2, short-term immunity) weeks after the recommended vaccination regime. Group 2 calves were vaccinated 5 months after group 1 calves using the same batch of +4 to +8 °C stored vaccine. It resulted in marginally but not significantly higher circulating group mean VN antibody titre in the younger group 1 calves and activity did not significantly decline until 6 months later (Table 2). Concurrently monitored group 2 and 3 (unvaccinated control) calves, respectively, remained seronegative until after vaccination or challenge.

At the level of average amount of virus shed daily by each group shown as group mean titres in Table 2, differences between vaccinated groups 1 and 2 and unvaccinated calves in group 3 were between 80–40 000-fold. Values shown in italics in Table 2 represent an average amount of virus shed by a calf in a group calculated by averaging the amount shed by an individual and then using these values to obtain the group mean shown as mean titre per calf. These values were 4.3 ± 0.5, 5.1 ± 0.5 and 6.4 ± 0.2 log_{10} $TCID_{50}$ per millilitre, respectively, for groups 1, 2 and 3. Hence both sets of means showed a significant (P = <0.05) protection against virus shedding for a period up to 6 months. Furthermore, results in Table 2 show a significantly shortened shedding period due to the vaccine (P = 0.01–0.02, respectively, for groups 2 and 1).

Although the daily clinical monitoring was from day –2 through to day +21, cumulative daily group mean scores for only day –2 through to day +14 are shown since the main reaction occurred between days 2 and 8 after challenge (Table 2). Between days 2 and 8 the daily group mean scores in group 1 calves were 13.6, 14.2, 16.6, 4.5, 2.1, 2, 1.2 and 2.0-fold lower compared to those in group 3 control calves. During this period the group mean scores in group 2 calves were 17.0, 10.6, 14.7, 5.2, 3.0, 1.4, 1.9 and 1.7-fold lower compared to the control group 3 calves. The group mean score per calf, calculated for days 1 to 14 were 3.0 ± 0.6, 3.1 ± 0.8 and 9.9 ± 2.3, respectively, for groups 1, 2 and 3. Hence calves in both vaccinated groups were significantly (P = <0.05) protected. In keeping with the challenge virus shedding data, control calves seroconverted with VN antibody titres of between 5 and 7 whereas the VN titre in all vaccinated calves rose above 9.0 (Table 2). Individually, the serum VN titre rise in vaccinated calves ranged in excess of between 8 and 128-fold (data not shown). Results in Table 2 support the claim that a normal course of 2 intramuscular vaccinations would significantly protect seronegative cattle up to a period of 6 months.

Against PI3 virus

Findings for the PI3 virus component are shown in Table 3. After the course of 2 IM vaccinations, all calves became positive for circulating VN antibody but overall the response was about 4-fold higher in the older (group 2) calves while unvaccinated control calves remained seronegative until after challenge. The latter was administered intranasally as well as intratracheally 3 weeks after the

Table 2: **Responses to IBR virus component.**

(i) Serum VN antibody activity.

Group and treatment[a]	Geometric group mean VN antibody titre[b] at time (weeks)									
	0	4	8	12	16	20	24	28	31	35
ONE	**Vac**	**Vac**						**CH**		
Challenge 6 months after vaccination	<2	2.04	5.44	5.48	4.93	3.71	4.08	2.14	9.0	9.0
TWO						**Vac**	**Vac**	**CH**		
Challenge 1 month after vaccination	<2	<2	<2	<2	<2	<2	4.04	4.04	9.0	9.0
THREE								**CH**		
Unvaccinated control	<2	<2	<2	<2	<2	<2	<2	<2	6.39	6.13

[a]2 × 2.0 mℓ vaccine intramuscularly a month apart.
[b]Titre in $\log_2$ scale; values shown a <2 denote no activity detected in 4-fold diluted serum.

(ii) Challenge IBR virus shedding in nasal mucus.

Group and treatment	Group mean titre ($\log_{10}$ $TCID_{50}$) per mℓ nasal swab on day after challenge												Mean titre per calf ($\log_{10}$ $TCID_{50}$)	Mean duration (days)
	1	2	3	4	5	6	7	8	9	10	11	12		
ONE														
Challenge 6 months after vaccination	1.1	2.7	4.4	4.7	5.5	6.2	5.9	3.2	1.0	0.3	–	–	*4.3 ± 0.5*	8.0 ± 1.4
TWO														
Challenge 1 month after vaccination	0.8	3.9	5.3	6.3	6.2	6.2	5.8	3.6	1.3	–	–	–	*5.1 ± 0.5*	7.8 ± 0.8
THREE														
Unvaccinated control	5.4	7.9	8.2	8.4	8.2	8.1	8.2	6.5	4.3	4.3	3.6	–	*6.4 ± 0.2*	11.0 ± 0
Difference														
Group 3 – Group 1	4.3	5.2	3.8	3.7	2.7	1.9	2.3	3.3	3.3	4.0	3.6	–	*2.1*	2.0
Difference														
Group 3 – Group 2	4.6	4.0	2.9	2.1	2.7	1.9	2.4	2.9	3.0	4.3	3.6	–	*1.3*	2.2

(iii) Pyrexia and respiratory disease score after challenge.

Group and treatment	Group mean score on day																
	–2	–1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ONE																	
Challenge 6 months after vaccination	0	0	1.3	0.2	0.5	1.2	1.8	4.2	7.0	5.0	6.2	2.8	2.8	2.8	2.3	2.0	2.0
TWO																	
Challenge 1 month after vaccination	0.2	0.8	1.2	1.0	0.4	1.6	1.6	4.8	5.6	6.6	3.8	3.2	4.0	3.2	2.8	2.8	2.6
THREE																	
Unvaccinated control	0.5	1.7	2.7	0.8	6.8	17.0	26.5	21.7	14.8	9.8	7.2	5.5	4.2	8.3	9.7	3.7	3.3

IBR virus challenge.

Although the challenge inoculum contained low passage lung isolates from 2 pneumonic calves, it did not result in marked clinical signs despite the fact that the inoculum had replicated in all calves (results for up to 14 days shown in Table 3) However, during the main virus growth phase between days 1 and 8 there was 50–10 000-fold less virus recovered from vaccinated groups than that from unvaccinated group 3 calves (Table 3). Unsurprisingly, virus titres in nasal mucus between vaccinated groups 1 and 2 were not significantly different (P = 0.6036). The significant protective effect due to the vaccine was also evident when comparing the average amount of virus shed by a calf for which the values were 2.5, 2.3 and 4.3 $\log_{10}$ $TCID_{50}$ per millilitre swab, respectively, for groups 1, 2 and 3 (values in italics). Furthermore, both vaccinated groups shed virus for a much (P = 0.005, group 1 and 0.003, group 2) shorter period than unvaccinated calves and in this respect vaccinated group 2 was at the borderline of significance (P = 0.058) compared to vaccinated group 1. As expected from the virus shedding data all calves in the study seroconverted but overall, the level of seroconversion was at least 4-fold lower in the control group than that in the vaccinated groups. Based on virus shedding data, a conclusion from results in Table 3 is that the vaccine would significantly protect seronegative cattle for a period of up to at least 6 months.

Against ruminant pestiviruses

Findings for the BVDV component (Strain C86) are shown in Table 4. Both the younger (group 1) and the older (group 2) calves responded with a similar high (≥8.0) level of circulating virus neutralis-

Table 3: **Responses to PI3 virus component.**

(i) Serum VN antibody activity.

Group and treatment[a]	Geometric group mean VN antibody titre[b] at time (weeks)									
	0	4	8	12	16	20	24	28	31	35
ONE	**VAC**	**VAC**							**CH**	
Challenge 27 weeks after vaccination	<2	1.3	5.6	5.7	6.5	6.5	4.1	4.1	4.2	9.0
TWO						**VAC**	**VAC**		**CH**	
Challenge 7 weeks after vaccination	<2	<2	<2	<2	<2	<2	6.8	7.5	7.6	9.0
THREE									**CH**	
Unvaccinated control	<2	<2	<2	<2	<2	<2	<2	<2	<2	6.9

[a]2 × 2.0 mℓ vaccine intramuscularly a month apart.
[b]Titre in $\log_2$ scale: values shown a <2 denote no activity detected in 4-fold diluted serum.

(ii) Challenge PI3 virus shedding in nasal mucus.

Group and treatment	Group mean titre ($\log_{10}$ $TCID_{50}$) per mℓ nasal swab on day after challenge										Mean titre per calf ($\log_{10}$ $TCID_{50}$)	Mean duration (days)
	1	2	3	4	5	6	7	8	9	10		
ONE												
Challenge 27 weeks after vaccination	–	0.6	1.6	1.4	1.9	3.0	3.1	0.8	0.3	–	–	*2.5 ± 0.2*	5.0 ± 1.1
TWO												
Challenge 7 weeks after vaccination	–	0.3	0.3	–	1.9	2.1	2.6	0.4	–	–	–	*2.3 ± 0.5*	3.2 ± 1.5
THREE												
Unvaccinated control	–	2.3	4.3	4.5	5.5	5.5	5.8	3.5	2.4	0.4	–	*4.3 ± 0.4*	8.0 ± 0
Difference												
Group 3 – Group 1	–	1.7	2.7	3.1	3.6	2.5	2.7	2.7	2.1	0.4	–	*1.8*	3.0
Difference												
Group 3 – Group 2	–	2.0	4.0	4.5	3.6	3.4	2.6	3.1	2.4	0.4	–	*2.0*	4.8

(iii) Pyrexia and respiratory disease score after challenge.

Group and treatment	Group mean score on day																
	–2	–1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ONE																	
Challenge 27 weeks after vaccination	2.8	0.7	0.8	1.0	1.0	2.0	3.3	3.2	2.5	3.2	2.7	8.0	0.8	1.7	2.2	2.0	1.2
TWO																	
Challenge 7 weeks after vaccination	3.8	2.2	1.8	2.0	1.0	2.4	2.8	3.4	3.0	3.8	5.8	8.0	2.8	2.0	2.8	2.0	0.8
THREE																	
Unvaccinated control	3.5	2.0	1.3	0.8	2.2	2.2	2.8	3.7	5.3	4.5	4.5	2.7	3.8	5.5	3.0	2.3	2.0

ing antibody activity after the standard vaccination regime. The high activity declined about 4-fold in group 1 calves over the 30 weeks to challenge but not in group 2 calves during 10 weeks to challenge. The latter was administered IN 3 weeks after the IN plus IT P13 virus challenge. The challenge resulted in a mild pyrexia and nasal discharge in unvaccinated calves on days 8 and 9. These reactions did not occur in vaccinated calves in groups 1 and 2. A much more significant protection due to the vaccine was evident at the level of challenge virus isolations from nasal mucus and blood. Between days 1 and 9 all unvaccinated calves in group 3 shed virus in nasal mucus for 7–9 days (mean 7.7 ± 0.8). Although calves in both vaccinated groups also became infected, the amount shed during the peak period (days 2–8) was significantly (10–300-fold P = 0.02–0.03) lower than that shed by the unvaccinated calves. While the shedding by both vaccinated groups was significantly (P = 0.005 group 1 and 0.007 group 2) shorter (3.5 ± 1.6 and 1.6 ± 0.5 *vs* 7.7 ± 0.8 days), more recently (group 2) vaccinated calves were significantly (P = 0.049) better protected.

Both leukocytes and serum were tested for the presence of virus. Isolation results are shown in Table 5. Although all vaccinated calves had infected leukocytes for 1–4 days, none of the animals yielded virus from their sera. By contrast, all unvaccinated calves were viraemic in both fractions, normaly for 6–9 days in leukocytes (mean 7.5 ± 1.2 day) and for 5–8 days in serum (mean 6.2 ± 1.2 days) between days 1 and 10 (Table 5) but not afterwards (individual results not shown). In serum the titres ranged between 1.7 and 2.4 $\log_{10}$ $TCID_{50}$ per mℓ and daily individual mean titres were between 1.9 and 2.2 (data not

Table 4: **Responses to BVD virus component.**

(i) Serum VN antibody activity.

Group and treatment[a]	Geometric group mean VN antibody titre[b] at time (weeks)											
	0	4	8	12	16	20	24	28	31	33	34	37
ONE	**VAC**	**VAC**									**CH**	
Challenge 30 weeks after vaccination	<2	1.9	8.0	8.1	7.5	6.8	6.8	6.3	6.3	6.0	5.5	9.0
TWO						**VAC**	**VAC**				**CH**	
Challenge 7 weeks after vaccination	<2	<2	<2	<2	<2	<2	2.7	8.6	8.5	8.3	8.3	9.0
THREE											**CH**	
Unvaccinated control	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	5.5

[a]2 × 2.0 mℓ vaccine intramuscularly a month apart.
[b]Titre in $\log_2$ scale; values shown a <2 denote no activity detected in 4-fold diluted serum.

(ii) Challenge BVDV shedding in nasal mucus.

Group and treatment	Group mean titre ($\log_{10}$ $TCID_{50}$) per mℓ nasal swab on day after challenge										Mean titre per calf ($\log_{10}$ $TCID_{50}$)	Mean duration (days)
	1	2	3	4	5	6	7	8	9	10		
ONE												
Challenge 30 weeks after vaccination	0.6	1.2	1.2	1.0	1.0	1.0	0.4	–	0.3	–	*1.9 ± 0.1*	3.5 ± 1.6
TWO												
Challenge 10 weeks after vaccination	–	0.3	1.4	0.8	0.3	–	–	–	–	–	*1.8 ± 0.2*	1.6 ± 0.5
THREE												
Unvaccinated control	0.3	2.2	2.7	2.8	2.8	2.3	2.3	2.0	0.9	–	*2.4 ± 0.2*	7.7 ± 0.8
Difference												
Group 3 – Group 1	0.3	1.0	1.5	1.8	1.8	1.3	1.9	2.0	0.6	–	*0.5*	4.2
Difference												
Group 3 – Group 2	0.3	0.9	1.3	2.0	2.5	2.3	2.3	2.0	0.9	–	*0.6*	6.1

(iii) Pyrexia and respiratory disease score after challenge.

Group and treatment	Group mean score on day																
	–2	–1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ONE																	
Challenge 30 weeks after vaccination	1.8	1.3	1.3	1.8	1.2	1.5	1.7	0.8	1.2	0.8	1.3	1.2	1.0	1.0	0.8	0.8	1.2
TWO																	
Challenge 10 weeks after vaccination	1.6	1.6	1.2	1.6	0.8	0.8	2.4	0.8	0.8	1.8	1.6	0.6	1.0	0.8	1.6	0.6	1.6
THREE																	
Unvaccinated control	2.5	2.3	1.2	1.2	1.2	2.5	2.3	2.5	3.0	3.7	11.3	10.2	4.3	2.7	2.5	1.7	1.3

shown). While the vaccine did not prevent infection of leukocytes, the duration of leukocyte viraemia in vaccinated groups was significantly (P = 0.004, group 1; and 0.007, group 2) short-lived compared to the unvaccinated group. Despite considerable local and systemic virus replication, the level of seroconversion in control calves after challenge was surprisingly moderate with circulating VN antibody titres in the range of 4–6 whereas the response in all vaccinated calves was beyond the highest (9.0 $\log_2$) dilution in the titration. Data in Tables 4 and 5 allow the conclusion that the vaccine would significantly protect seronegative calves for a period of at least 6 months. The fact that there was no serum viraemia in vaccinated calves allows the conclusion that the vaccine would also significantly prevent transplacental infection.

Against BRSV

Findings for the BRSV component are shown in Table 6. Unlike other components the VN response to BRSV in both vaccinated groups 1 and 2 was just detectable. Nonetheless in group 1 calves activity persisted until 31 weeks in all 6 calves and thereafter in 2–3 of 6 calves up to 36 weeks but not at challenge 42 weeks after vaccination. All 5 group 2 calves were VN antibody positive at challenge 18 weeks after vaccination. However, no activity was detected in any of 6 unvaccinated calves until after challenge.

In daily sampling of nasopharyngeal swabs for 10 days after BRSV challenge, none of the calves in vaccinated groups 1 and 2 shed virus. During this period between 16.7 and 83.3 % of unvaccinated calves excreted virus, the daily average of excretors was 48.3 ± 25.4 % (Table 6). Between days 1 and 10 after challenge, sampling for virus shedding in lung

Table 5: **Serum and leukocyte-associated viraemia after intranasal challenge with a pool of ruminant pestiviruses.**

(i) Serum.

Group and treatment	Group mean titre (log_{10} $TCID_{50}$) per mℓ serum on day after challenge										Mean titre[b] per calf (log_{10} $TCID_{50}$)	Mean duration (days)
	1	2	3	4	5	6	7	8	9	10		
ONE												
Challenge 30 weeks after vaccination	0[a]	0	0	0	0	0	0	0	0	0	*0 ± 0*	0 ± 0
TWO												
Challenge 10 weeks after vaccination	0	0	0	0	0	0	0	0	0	0	*0 ± 0*	0 ± 0
THREE												
Unvaccinated control	0	0.6	2.0	2.4	2.1	2.1	1.8	0.9	0.9	0	*2.1 ± 0.1*	6.2 ± 1.2

[a]No virus isolated from 4 × 200 µℓ of undiluted serum samples from any of the calves in the group.
[b]Mean titre per calf: sum of individual mean titres (sum of daily virus recovered divided by numbers of days virus shed) divided by number of animals in group.

(ii) Leukocytes.

Group and treatment	Calf No.	Isolation (+) on day										Duration (days)	
		1	2	3	4	5	6	7	8	9	10	Individual	Group mean
ONE													
Challenge 30 weeks	1				+	+	+	+				4	
after vaccination	2			+			+					2	
	3			+	+	+						3	2.7 ± 1.0
	4			+	+	+						3	
	5		+	+	+							3	
	6				+							1	
TWO													
Challenge 10 weeks	7			+								1	
after vaccination	8				+	+						2	
	9			+		+						2	1.6 ± 0.5
	10			+								1	
	11				+	+						2	
THREE													
Unvaccinated controls	12			+	+	+	+	+	+	+		7	7.5 ± 1.2
	13		+	+	+	+	+	+	+	+	+	9	
	14			+	+	+	+	+	+	+		7	
	15			+		+	+	+	+	+	+	7	
	16				+	+	+	+	+	+		6	
	17		+	+	+	+	+	+	+	+	+	9	

washings was on alternative days for ethical reasons. During this period no BRSV was recovered from lung washings of any calves in vaccinated groups 1 and 2. However, at this time between 33.3 and 100 % of unvaccinated calves shed virus in lungs and daily average of excretors was 88.9 ± 27.2 % (Table 6). Clinically, however, the challenge infection did not result in significant respiratory disease. Results in Table 6 allow the conclusion that the recommended course of 2 IM vaccinations would provide significant protection for the claimed period of 6 months.

DISCUSSION

The principle of multivalent inactivated cattle vaccines is not new but there is scanty published data indicating the quality of protection that should be expected from such vaccines. No doubt the available multivalent vaccines do meet the licensing requirements of countries where such products are marketed, but it is important that the data for experimental validation of efficacy and safety are more widely and readily available to practising clinicians and other interested parties not directly connected with the product licensing authorities. The present inactivated vaccine was formulated to reduce the impact of 4 most commonly implicated viruses in the aetiology of bovine respiratory disease and also the reproductive disease due to IBR virus and BVDV. It is important to point out that controlled efficacy determinations made are of naïve target species and challenge infections were sequential in order to mimic the field situation. The latter, however, was at long enough intervals in order to avoid interference due to the preceding challenge. The fact that all 3 experimental groups were challenged at the same time allowed a direct comparison of short-term (≥1 month) and long-term (≥6 months) immunity.

Immune stimulation due to the vaccine was measured by the virus neutralisation (VN) test since it is the most virus specific serological response and VN is a recognised defence mechanism in many viral infections. After the recommended course of 2 IM vaccinations high VN activity was recorded against all vaccine components except BRSV. In natural hosts, BRSV is known to produce a low-grade VN antibody response[1]. Furthermore, the VN activity to BRSV was comparable to that after live virus challenge[18] (Table 6). Also, VN activity due to IBR, PI3 and BVD vaccinations was comparable to that after

Table 6: **Response to BRSV component.**

(i) Serum VN antibody activity.

Group and treatment[a]	Geometric group mean VN antibody titre[b] at time (weeks)													
	0	4	8	12	16	20	24	28	31	33	34	37	42	45
ONE	**VAC**	**VAC**											**CH**	
Challenge 38 weeks after vaccination	1.3	1.3	2.8	3.2	2.6	2.3	2.6	2.1	2.1	1.6	1.4	1.3	1.0	7.2
TWO						**VAC**	**VAC**						**CH**	
Challenge 18 weeks after vaccination	<2	<2	<2	<2	<2	<2	1.2	3.1	3.5	3.3	2.9	3.8	3.6	7.2
THREE													**CH**	
Unvaccinated control	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	4.04

[a]2 × 2.0mℓ vaccine intramuscularly a month apart.
[b]Titre in $\log_2$ scale; values shown on a <2 denote no activity detected in 4-fold diluted serum.

(ii) Isolation from nasal mucus.

Group and treatment	No. of calves	No. of positive calves on day after challenge										Daily mean excretors (%)	Mean duration (days)
		1	2	3	4	5	6	7	8	9	10		
ONE													
Challenge 38 weeks after vaccination	6	0	0	0	0	0	0	0	0	0	0	0 ± 0	0
TWO													
Challenge 18 weeks after vaccination	5	0	0	0	0	0	0	0	0	0	0	0 ± 0	0
THREE													
Unvaccinated control	6	2	5	5	5	3	1	2	2	2	2	48.3 ± 25.4	4.8 ± 2.1

[a]Daily mean excretors = sum of per cent of shedding calves divided by number of days of shedding.

(iii) Isolation from lung washings.

Group and treatment	No. of calves	No of positive calves on day after challenge										Daily mean[c] excretors (%)
		1	2	3	4	5	6	7	8	9	10	
ONE												
Challenge 38 weeks	3	–[a]	–	0[b]	–	–	0	–	0	–	–	0 ± 0
after vaccination	3	–	–	–	0	–	–	0	–	0	–	
TWO												
Challenge 18 weeks	3	–	–	0	–	–	0	–	0	–	–	0 ± 0
after vaccination	2	–	–	–	0	–	–	0	–	0	–	
THREE												
Unvaccinated control	3	–	–	1	–	–	3	–	3	–	–	88.9 ± 27.2
	3	–	–	–	3	–	–	3	–	3	–	

[a]Denotes no sampling to avoid stress.
[b]No virus isolated upon 2 serial passages, each for 4 days at 37 °C.
[c]Daily mean excretors = sum of per cent of shedding calves divided by numbers of days of shedding.

(iv) Pyrexia and respiratory disease score after challenge.

Group and treatment	Group mean score on day															
	–2	–1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ONE																	
Challenge 9 months 2 weeks after vaccination	1.2	0.5	0.5	0.0	0.3	0.8	0.7	1.0	0.5	0.2	1.4	0.4	0.6	0.6	1.0	0.4	1.2
TWO																	
Challenge 4 months 2 weeks after vaccination	1.6	0.8	0.4	0.4	0.6	0.8	0.4	0.6	0.8	1.2	0.4	1.4	1.6	0.4	0.8	0.2	0.8
THREE																	
Unvaccinated controls	0.7	0.5	0.3	0.2	0.5	1.5	1.8	1.2	1.3	1.7	1.5	1.0	0.8	1.3	0.5	1.0	0.7

the respective live virus challenge (see control group results in Tables 2, 3 and 4). The IN administered IBR virus challenge caused typical reactions of IBR in the unvaccinated control group but the reactions were significantly ($P < 0.05$) reduced in groups vaccinated a month or 6 months previously (Table 2). In keeping with the general experience[8,9], vaccination was not expected to completely prevent challenge virus growth and shedding from the upper respiratory tract; nonetheless, the amount and the duration of virus shedding in nasal mucous was significantly lower in vaccinated calves than that in the control group (Table 2). As expected, therefore, all calves showed a rise in VN antibody titre that was markedly higher in vaccinated calves than unvaccinated calves due to prior sensitization. In common with other experimental efficacy studies[15,2] the PI3 virus challenge by IN plus IT routes caused only mild clinical signs despite the fact that the low passage lung isolates used originated from calves with pneumonia. Nonetheless, the challenge virus recovery from nasal mucus (Table 3) showed a highly significant protection due to the vaccine administered 7 or 27 weeks previously. The fact that the challenge virus grew in all calves was also reflected in the VN antibody response that as expected , was significantly higher in vaccinated groups than the control group (Table 3).

Cattle infected with BVDV can present a variety of clinical signs both enteric and respiratory and usually as a trivial illness of a few days' duration.[5] However, the major economic impact of BVDV is fertility failure, abortions and other consequences of transplacental infection. In the present study only mild respiratory signs were observed in the unvaccinated calves despite an intranasal challenge consisting of 8 BVDV (types 1a, 1c, 1d and a strain not belonging to 1a,b,c, or d) and 3 BDV strains. Results shown in Table 4 do, however, demonstrate short-term (2 months and 2 weeks) and long-term (7 months and 2 weeks) efficacy against mild clinical signs. A far clearer measure of both the short and the long-term efficacy was evident at the level of virus isolation from nasal mucous, leukocytes and serum shown in Tables 4 and 5. In this regard it should be pointed out that it is not possible to prevent re-infection of cattle with BVDV even after a short period and even with a homologus strain[11,14]. A noteworthy finding shown in Table 5 is a complete lack of viraemic serum in vaccinated calves although the calves had infected leukocytes albeit for a significantly shorter duration than uninfected calves. In our experience[14] and that of others[11] it is not possible to completely prevent leukocyte-associated viraemia despite immune stimulation. An important conclusion, however, is that the lack of serum viraemia suggest that the quadrivalent vaccine like the monovalent vaccine[14] containing the same BVDV strain (C86) will also significantly prevent transplacental BVDV infection, abortion and fertility losses due to BVDV infection.

A major drawback in assessing vaccines against BRSV is the inability to experimentally reproduce respiratory disease[12,18]. The present study also did not record clinical reactions typically seen in natural BRSV infections. Nonetheless, virus isolation for up to 10 days from nasal swabs and lung washings from all 6 control calves with accompanying seroconversion indicates that the challenge was infectious. This is further supported by the fact that all calves in both vaccinated groups showed an anamnestic response for VN antibody despite no obvious virus shedding in nasal mucus or lung washings (Table 5). While the latter observation indicates a complete protection against challenge virus shedding from the upper and the lower respiratory tracts of calves in both vaccinated groups, data do not explain the booster sero-response in the absence of detectable virus growth. This has been recorded before[10] and the likely explanation is that the vaccinated calves did undergo a low-grade infection which remained undetected and/or the low amounts of shed virus was inactivated since BRSV is very labile. Nonetheless, results shown in Table 6 do support the conclusion that a course of 2 IM inoculations with the this vaccine protected calves against BRSV up to 9 months. This conclusion, however, would have been far more robust had the BRSV challenge produced a significant respiratory disease. Hence for the BRSV component in the present quadrivalent vaccine and for that matter any other BRSV vaccines, field trials are essential to further augment experimental data.

A matter of practical concern for any inactivated vaccine is the incidence and the severity of clinical reactions, particularly those at the injection site induced by the vaccine. In this regard we have, in experimental safety studies and in field trials, not observed clinical reactions that have given us cause for concern (data not shown). Usually the type and the nature of the adjuvant determines the persistence and the severity of injection site reactions. Findings for the aluminium salt adjuvant in the present quadrivalent vaccine indicate that it is innocuous and does not normally cause a severe injection site reaction other than a mild inflammatory reaction for a few days.

In summary, the experimental data in naive target species for the efficacy of the quadrivalent inactivated vaccine containing IBR virus, PI3 virus, BVDV, BRSV and aluminium salts demonstrated a significant protection for each component for at least 6 months. It is also noteworthy that for 3 (IBR virus, PI3 virus and BVDV) of the vaccine components, VN antibody persists for over 12 months (data not shown). Also under normal circumstances the vaccine will not induce significant adverse clinical reactions systemically or at the injection site.

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