Onset of immunity of a quadrivalent vaccine against bovine respiratory diseases

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Abstract

Four experiments demonstrated the onset of immunity at 42 days of a vaccine including the following components: bovine viral diarrhoea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine parainfluenza (Pi3) and *Mannheimia haemolytica* (MH). Colostrum-deprived calves were vaccinated at 2 and 5 weeks of age and challenged with virulent strains 6 weeks after the 1st vaccine injection. The challenge strains caused respiratory symptoms in unvaccinated animals, while the vaccinates were clinically protected and excreted much reduced quantities of viral particles in the viral challenge tests. The MH experiment also demonstrated protection in vaccinated animals against clinical signs, lung lesions, and presence of the pathogen in the lungs.

Cattle, vaccination, BRD, BRDC, Bovalto

Bovine respiratory disease (BRD) is the most important health and economic issue affecting growing cattle. It is a multifactorial disease including viral and bacterial components as well as management and stress-related issues. In the context of increasing awareness and spread of antimicrobial resistance (O'Neill 2016) and the associated constraints placed on farmers regarding antimicrobial usage, appropriate use of effective multivalent vaccines has an important role to play. The purpose of the study was to demonstrate the efficacy of each vaccine component by challenge under laboratory conditions after administration of the quadrivalent vaccine Bovalto Respi 4 (Boehringer Ingelheim Vetmedica, Ingelheim, Germany) to calves of minimal age.

Materials and Methods

Four *in vivo* challenge tests were conducted with Bovalto Respi 4, one for each vaccine antigen component, namely, bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus (Pi3), bovine viral diarrhoea virus (BVDV) and *Mannheimia haemolytica* (MH). Each test comprised twelve to eighteen 2-week-old calves, which were colostrum-deprived to ensure absence of antibodies being investigated (BRSV, Pi3, MH) or conventional but seronegative to the component being tested (BVDV). All calves received a course of injectable amoxicillin during the 1st week of life and were confirmed seronegative for the virus/bacteria to be tested– using virus neutralising titres (VNT) for viruses and ELISA (in house) against MH and MH leukotoxin. The calves were of dairy breeds and mostly males, housed on straw, fed milk replacer and supplementary mixture, and had access to water *ad libitum*. For each test, the calves were allocated to a vaccine and a control group on the basis of age and/or bodyweight and maintained in pens sharing the same airspace. The quadrivalent vaccines used were formulated with the minimal antigen quantities described in the formulation file, and the relative potency of each component determined (Table 1). Vaccinated animals received two subcutaneous injections of the vaccine, given at Day 0 and Day 21 (on the left and right shoulder respectively). The control animals were either left untreated (BRSV and BVDV) or received a placebo (based on the adjuvant used in the vaccine formulation) at Day 0 and 21 (Pi3 and MH).

An overview of the study design is shown in Fig 1. Calves were vaccinated at around two weeks of age at Day 0 and 21, and challenge was administered to all calves at Day 41 (BVDV) or Day 42 (all other components) using an appropriate route (intranasal for the viral components, specifically the BRSV challenge was administered using

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an ultrasonic nebulizer (UltraNeb 99, DeVilbiss, Scottsdale, USA), or intratracheal (for MH). Each challenge strain was heterologous to the corresponding vaccinal strain (Table 2). Post-challenge observation period lasted 7 (MH) to 14 days (all viral components).

All animals were blood sampled at intervals, from before the 1st vaccination to the end of the challenge period, in order to confirm seronegativity at the time of the 1st vaccination, and to monitor the immune response to vaccination and challenge, using Virus Neutralising Titres (VNT) for the viral challenge tests and ELISA for the MH trial. Post-challenge observations included rectal temperature, respiratory symptoms, viral excretion in the nostrils and postmortem lung lesions (BRSV and MH). Respiratory clinical signs were scored daily using a scoring system that was identical across all studies, based on evaluating the presence and severity of apathy, cough, nasal and ocular discharge and dyspnoea (Table 3); total clinical scores for each animal were calculated by summing all daily scores for that animal. Nasal swabs were collected daily; a diluted fraction of the liquid collected was inoculated into Madin-Darby bovine kidney cell line suspension on 96-well plates and incubated for 5 days. Virus titres (expressed as the tissue culture infectious dose, $TCID_{50}$) were determined using specific cytopathogenic effect (Pi3) or immunofluorescence staining (BVDV and BRSV). Animals were euthanized at the end of the observation period (except for Pi3). Individual lung lesions were evaluated in the MH trial using lung scores (on a scale from 0 to 20 depending on extent of lesions and signs of gravity, such as pleurisy); in the BRSV trial, the percentage affected in each lobe weighted by the lobe size was calculated for each animal.

Bias reducing methods were used in all tests and consisted of personnel conducting clinical and laboratory examinations being blind to treatment group, whereas the treatment dispenser was unblinded.

A phylogenetic analysis of the heterologous challenge viral strains was also conducted, in order to establish the relevance of the challenges in comparison to published sequences from Genbank® (NCBI, Bethesda, USA; these sequences were assumed to be representative of field-circulating strains). For each strain the analysis focused on a specific target gene known to be associated with protection and variability – HN gene for Pi3, G gene for BRSV, E2 gene for BVDV. Sequence comparison used clustal alignment within Seaview Software version 4 (PRABI, Lyon, France) and phylogenetic tree diagrams, with the neighbour-joining distance method, the Kimura correction and 500 bootstraps replicates; only the bootstrap values > 70% were considered significant. The percentages of homology were deducted from alignment.

Table 1. Vaccines used in the challenge studies and number of animals in each study.

	BVDV	BRSV	Pi3	MH	Placebo (Pi3 and MH)
Composition					
BVDV	RP = 1.09	RP = 1.09	RP = 1.07	RP = 1.07	Phosphate buffered
BRSV	RP = 1.06	RP = 1.06	RP = 0.95	RP = 1.03	saline,
Pi3	RP = 1.21	RP = 1.21	RP = 1.18	RP = 1.18	Aluminium hydrogel,
MH	RP = 1.19	RP = 1.19	RP = 1.16	RP = 1.16	Thiomersal
Dose volume and administration regimen	2 ml, subcutaneous injection, left shoulder at D0 and right shoulder at D21 booster				
Number of calves in the study	12	12	12	18	

BVDV = bovine viral diarrhoea virus; BRSV = bovine respiratory syncytial virus; Pi3 = bovine parainfluenza virus; MH = *Mannheimia haemolytica*; RP = relative potency

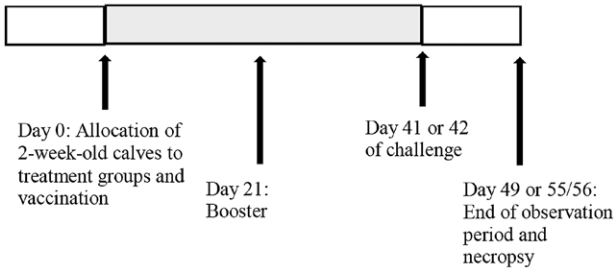

Fig. 1. Study design

Table 2. Challenge strains used in the studies.

Study	Strain	Titre	Dose and route of administration
BVDV	HAN81-MDBK4 strain, 22146 genotype 1f, non-cytopathogenic biotype, supplied by Merial	Target titre 10^5 $TCID_{50}$/ml	2 ml applied in each of the 2 nostrils using an intranasal applicator
BRSV	Snook strain (Compton 4-Merial)	10^5 $TCID_{50}$/ml	10 ml applied intranasally using a nebuliser face mask
Pi3	Strain S265, VRI, Brno, CZ	$10^{7.8}$ $TCID_{50}$/ml	2 ml applied in each of the 2 nostrils using an intranasal applicator
MH	*Mannheimia haemolytica,* serotype A1, strain Dolní Újezd, isolated from calf lungs by SVI Hradec Králové	5.43×10^{10} CFU/dose	15 ml administered intratracheally

BVDV = bovine viral diarrhoea virus; BRSV = bovine respiratory syncytial virus; Pi3 = bovine parainfluenza virus; MH = *Mannheimia haemolytica*; VRI = Veterinary Research Institute; SVI = State Veterinary Institute; TCID = tissue culture infectious dose; CFU = colony forming unit

Table 3. Recorded signs and clinical post-challenge scoring in the studies.

Clinical sign	Observation	Score
Apathy	Absent (bright, alert, responsive)	0
	Mild: animal is slow to rise, but otherwise still active	1
	Moderate: animal is able to rise and move but inactive other than eating or drinking	2
	Severe: animal is recumbent, unable to rise and refuses food and/or drink	3
	Death	10
Cough	Absent	0
	Mild: induced by gentle tracheal palpation. Animal coughed once during clinical observation	1
	Moderate: spontaneous/frequent coughing. Animal coughed at least twice during clinical observation	2
	Severe: spontaneous/very frequent coughing. Animal has persistent and prolonged cough	3
Nasal discharge	Absent (includes normal moist nose)	0
	Mild: serous (clear, watery) discharge - could be intermittent	1
	Moderate: persistent mucopurulent discharge	2
	Severe: severe mucopurulent discharge	3
Ocular discharge*	Absent - eyes normal	0
	Mild: evidence of excessive tear production (some secretion at the corner of the eye or eyes brimming with tears)	1
	Moderate: thin and clear, serous (clear and watery) flowing out of the eye	2
	Severe: mucopurulent discharge flowing out of the eye/swollen eye/conjunctivitis	3
Dyspnoea	Absent: normal respiratory effort	0
	Mild: slightly increased respiratory effort	1
	Moderate: obvious abdominal breathing	2
	Severe: severe abdominal breathing	3

*Not monitored in the *Mannheimia haemolytica* challenge study

Data analysis for the animal tests

Areas under the virus excretion curve (AUC) were calculated for each animal from the challenge to the end of the study using the formula shown below:

$$AUC = \sum_{i=0}^{n} \frac{(T_i + T_{i+1})}{2} \cdot (t_{i+1} - t_i)$$

where i = 0, n; n = 13

t_i and t_i+1 are 2 adjacent days of sampling

T_i (or T_i+1) is virus titre determined in samples from 2 adjacent days (t_i and t_i+1) and expressed in log10 $TCID_{50}$/ml.

Total clinical scores and virus excretion AUCs were compared between treatment groups within each study, using one-sided, non-parametric, Mann-Whitney tests. In addition, rectal temperature, respiratory rates, percentage lung lesions (BRSV) or lung lesion scores (MH) were compared between groups using one-sided parametric *t*-test or non-parametric Mann-Whitney test. Isolations of MH from lung fragments were compared between groups using Fisher's exact test. The level of significance used was set at $P < 0.05$.

Protocol approval and welfare compliance

The study was carried out in accordance with the Act on Animal Health and Animal Welfare of the Czech Republic. The facility has accreditation which allows biological testing on laboratory animals (No. 163622/2012-MZE-17214). All study procedures were carried out under this licence. All personnel involved in the *in vivo* phase of the study were fully trained and provided with necessary instructions and support to ensure that the care for and welfare of the animals was not compromised during the study period.

Results

Exclusions

In the Pi3 test, on Day 11 one vaccinated animal showed diarrhoea, anorexia, and severe apathy, and was euthanized on Day 12. Necropsy showed pathological findings of peritonitis, enteritis, and omphalophlebitis. This was thought to be unrelated to vaccination.

In the BVDV test, a vaccinated calf died on Day 30 following two days of diarrhoea. Postmortem findings were not reported.

In the other two tests, all animals completed the procedures described.

Clinical signs post challenge

Respiratory clinical signs were seen in animals from all control groups. In each challenge test, vaccinated animals showed significantly lower clinical scores, associated with milder symptoms expressed for a shorter duration. In the MH trial one control animal died two days after challenge with signs of severe pneumonia.

The BVDV daily clinical scores post challenge were generally lower for vaccinates than for control animals: the vaccinates showed fewer clinical signs, particularly during the 5 to 10 days post-challenge period. The BVDV total clinical scores (sum of daily individual scores) were significantly lower in vaccinates compared with control animals ($P = 0.04$).

The BRSV daily clinical scores post challenge were generally lower for vaccinates than for control animals: the vaccinates showed fewer clinical signs, particularly during the 5 to 10 days post-challenge period. The BRSV total clinical scores were significantly lower in vaccinates than in control animals ($P = 0.007$).

The Pi3 daily clinical signs post challenge were discrete and transient, generally lower for vaccinates than for control animals. The Pi3 total clinical scores were lower in vaccinates, but the difference was not significant ($P > 0.05$). A significant difference was observed when comparing only the upper respiratory signs (cough + nasal discharge) ($P = 0.034$).

The MH daily clinical scores post challenge were generally much lower for vaccinates (2 out of 9 showing signs and not during the the first four days after challenge) than for the control animals (8/9 showing signs, including one dead). The MH total clinical scores were lower in vaccinates than in controls, the difference was significant ($P = 0.011$) (Fig. 2).

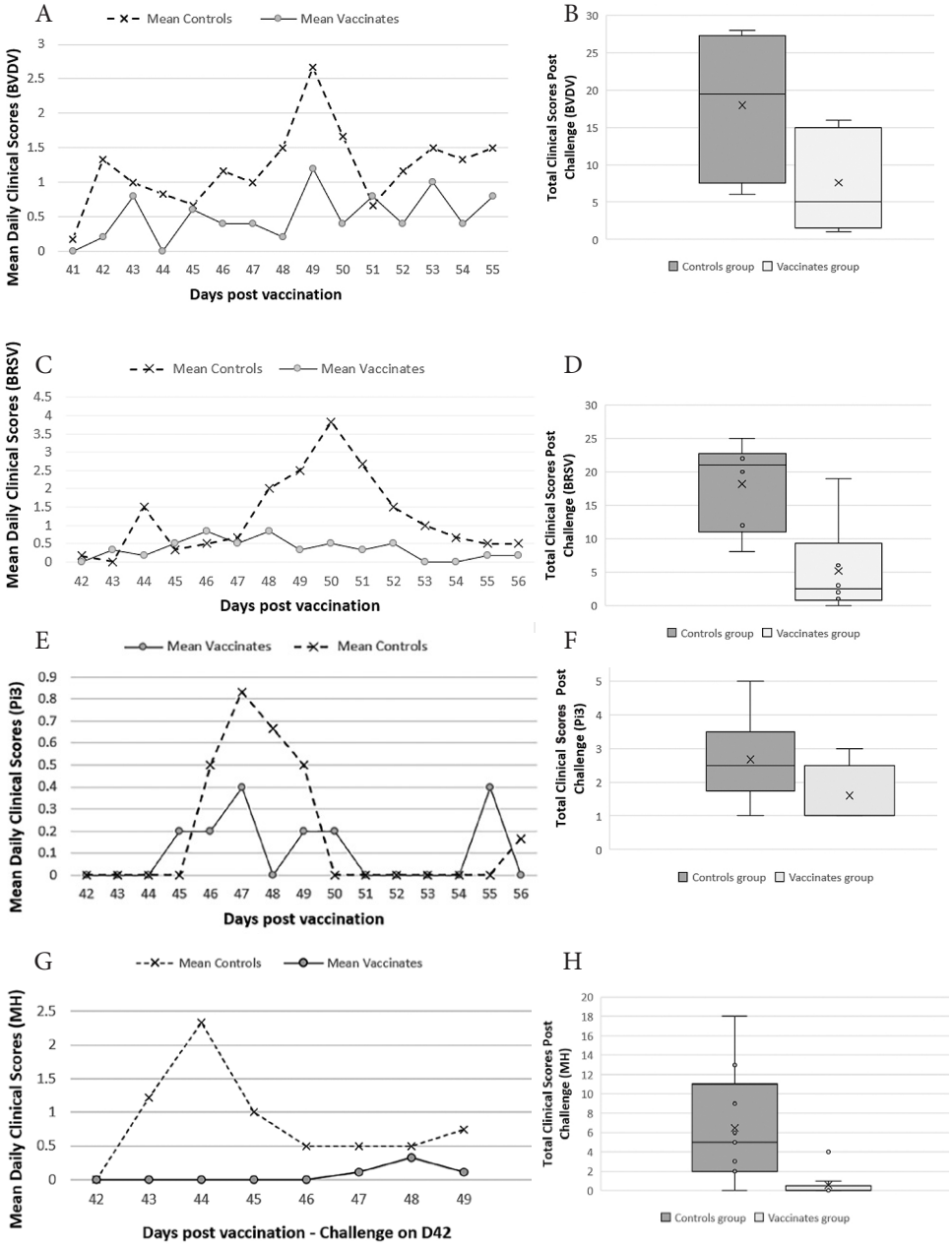

Fig. 2. Clinical signs post challenge in treated and control groups (BVDV, BRSV, Pi3, MH). Graphs on the left show the daily mean score in each group, graphs on the right show dispersion of total individual clinical scores over the post-challenge period in each group (boxplots): (A) BVDV daily clinical scores; (B) BVDV total clinical scores (sum of daily individual scores); (C) BRSV daily clinical scores; (D) BRSV total clinical scores; (E) Pi3 daily clinical signs; (F) Pi3 total clinical scores; (G) MH daily clinical scores; (H) MH total clinical scores.

BVDV = bovine viral diarrhoea virus, BRSV = bovine respiratory syncytial virus, Pi3 = bovine parainfluenza virus, MH = *Mannheimia haemolytica*

Viral excretion

In all 3 viral challenge tests, vaccinated animals excreted much reduced quantities of viral particles (2 to 5 $\log_{10}$ reduction $TCID_{50}$, on average, at the time of peak) and for a shorter duration, (1/3 to 1/2 of the duration) compared to control animals. Virus concentrations over time in AUCs, indicative of total viral shedding, were all significantly lower in the vaccinated calves.

All 6 control animals excreted BVDV, with a peak between D46 to D49. Only 1 out of 5 vaccinates excreted virus between D45 to D47. Virus excretion (as measured by the AUCs over the post-challenge period) was significantly lower in vaccinated animals compared to controls ($P = 0.004$).

All animals in the control group excreted BRSV for at least 1 day between D47 to D51, with a peak of excretion between D48 to D50. Only 1 animal out of 6 vaccinates had a transient nasal excretion of the virus on D48 (viral load of 2.3 $\log_{10}$ $TCID_{50}$/ml). Virus excretion (as measured by the AUCs over the post-challenge period) was significantly lower in vaccinated animals compared to controls ($P = 0.002$).

All 6 control animals shed Pi3 virus, for up to 7 days post-challenge, up to a titre of 6.8 $\log_{10}$ $TCID_{50}$/ml. In vaccinates, virus was isolated from nasal swabs of 3 out of 5 animals. Each of the 3 calves excreted virus for one day only and the titre was at the threshold of detection (2.1 $\log_{10}$ $TCID_{50}$ /ml). Virus excretion (as measured by the AUCs over the post-challenge period) was significantly lower in vaccinated animals compared with controls ($P = 0.007$) (Fig. 3).

Lung lesions – BRSV and MH

All control animals (6 for BRSV, 9 for MH) demonstrated lung lesions at postmortem, comparable with only one vaccinated calf in the BRSV test and three in the MH test. The lesions in the vaccinated animals were significantly less severe and extensive than those observed in the controls.

All 6 animals from the BRSV control group presented at least one lung lesion (from 2.8 to 35.5% of total lungs), associated in most cases with pathology signs of pneumonia. In contrast, only one vaccinate out of 6 showed a congestion lesion of 50% of the dorsal side of the right cranial lobe (2.8%) of total lungs. The difference was significant *($P = 0.002$).*

All 9 MH control animals showed pneumonia lesions, 7 animals showed solitary pneumonic lesions of moderate (one animal; 2–4 lesions) to severe extent (6 calves; 5 and more lesions). Two remaining calves suffered fibrinous pleuro-pneumonia and were scored with the highest possible score (20 points for extensive oedematous findings with pleuritis).

In the vaccine group, 3 out of 9 animals showed solitary lesions of severe extent (5 and more lesions) while 6 animals had a score of 0. The difference between groups was significant ($P = 0.007$).

In addition, MH was recovered from lung tissue samples from all 9 control animals, but only from the 3 vaccinates with lesions. This difference was significant ($P = 0.009$) (Fig. 4).

Phylogenetic analysis of viral challenge strains

The analysis confirmed the serotype of each challenge strain. The percentage homology of each challenge strain to published strains within their serotype were 71%, 92%, and 94% for BVDV, BRSV, and Pi3, respectively, and the percentage homology of each challenge strain with all strains were not determined, 93%, and 87%, for BVDV, BRSV, and Pi3, respectively (Table 4).

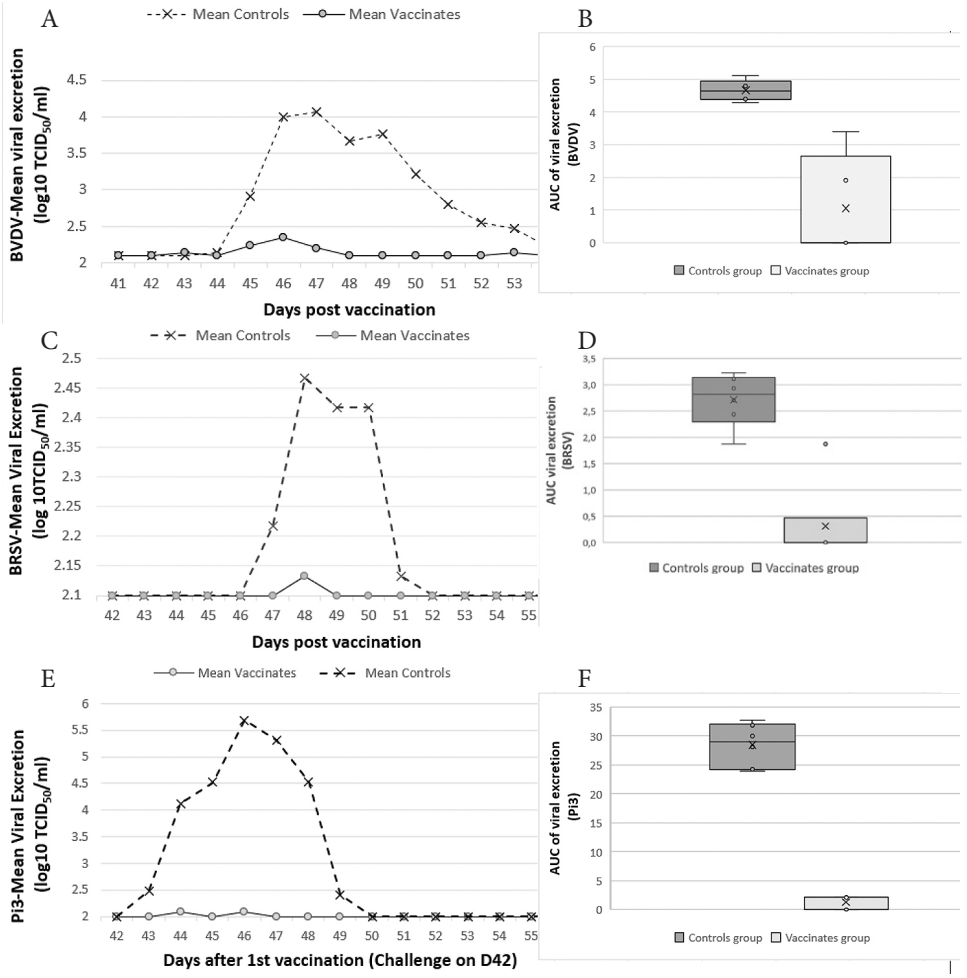

Fig. 3. Post-challenge viral excretion in nasal swabs (BVDV, BRSV, Pi3). Graphs on the left show daily mean virus concentration in nasal swabs in each group, graphs on the right show dispersion of total individual area under the virus excretion curve (AUC) over the observed post-challenge period in each group: (A) BVDV excretion; (B) BVDV excretion as measured by the AUCs; (C) BRSV excretion; (D) BRSV excretion as measured by the AUCs; (E) Pi3 excretion; (F) Pi3 excretion as measured by the AUCs.
BVDV = bovine viral diarrhoea virus, BRSV = bovine respiratory syncytial virus, Pi3 = bovine parainfluenza virus, $TCID_{50}$ = tissue culture infectious dose 50

Table 4. Phylogenetic analysis of the viral challenge strains.

Virus - strain	Target gene	No. of published sequences	Challenge strain serotype	Observed homology of challenge strain	
				vs genotype	vs all strains
BVDV - 22146	E2	70	1*	71%	Not determined
BRSV - Snook-(Compton 4-Merial)	G	31	A*	92.3%	93%
Pi3 - S265	HN	17	A*	94%	87%

*Confirmed by analysis

BVDV = bovine viral diarrhoea virus; BRSV = bovine respiratory syncytial virus; Pi3 = bovine parainfluenza virus

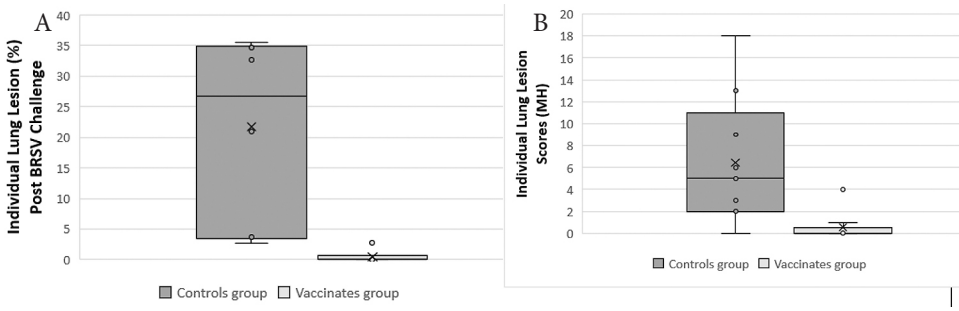

Fig. 4. Lung lesions: (A) BRSV study; (B) MH study

BRSV = bovine respiratory syncytial virus, MH = *Mannheimia haemolytica*

Discussion

The viral challenge strains used in these onset of immunity trials and submitted to phylogenetic analysis differed in part from those used in the duration of immunity trials, described by Philippe-Reversat et al. (2017). The Pi3 strain was the same, whereas the BRSV and BVDV strains were different. This change was driven by the European Pharmacopeia regulatory requirements: it is necessary that viral challenge strains cause clinical signs in most control animals, and the vaccinates must demonstrate significantly fewer clinical signs to validate the study, with a relatively low prescribed number of animals (at least 5 per group for viral challenge, and at least 8 animals per group for the MH challenge). The BRSV-Snook strain has now been used for a number of published BRSV challenge models with clinical signs (for example, Blodörn et al. 2015), and its phylogenetic analysis demonstrated a high degree of homology for the *G* gene against published sequences. Conversely the BVDV challenge strain only reached 71% homology for the *E2* gene against genotype 1 published sequences, compared with 87% homology for the strain used in the duration of immunity trial; the latter provides welcome reassurance regarding the relevance of the BVDV onset of immunity trial to field strains.

The excellent MH efficacy demonstrated by the vaccine, formulated at minimum potency, after a challenge which was severe enough to kill one control animal, was thought to be due in part to its leukotoxin content, and to the preparation method of the bacterial component. The MH vaccine strain, belonging to serotype A1, was grown under iron-deprived conditions. Iron acquisition *in vivo* is an important function in pathogenic bacteria invading a mammalian host and has been dubbed "the battle for iron" (Skaar 2010). Mammalian hosts maintain their cells virtually iron-free, as the iron is sequestered in proteins such as lactoferrin, transferrin, siderophores. When pathogenic bacteria infect mammalian host lung tissue for instance, the bacteria upregulate virulence genes allowing for host cell invasion, host cell death, and bacterial cell receptors for iron-containing proteins. Thus, the use of iron-deprived conditions in bacterial culture, and notably for *M. haemolytica*, mimics reactions in a mammalian host at the time of bacterial infection and triggers the expression of outer membrane proteins acting as iron receptors – so-called iron-repressible outer membrane proteins or IROMPs- and ABC-type transporters of transferrin, lactoferrin, haemoglobin, and siderophores (Deneer and Potter 1989; Roehrig et al. 2007). In addition, studies using convalescent calf sera have shown that specific IROMPs induced by iron restriction in the culture medium cross-react between all serotypes A1 to A12 (Deneer and Potter 1989). This is supported by *in vitro* work highlighting conservation of a highly immunogenic outer membrane protein between

A1 and A6 serotypes (Ayalew et al. 2006). An *in vitro* study examining transcriptional response to iron restriction in the swine pathogen *Actinobacillus pleuropneumoniae* also found a common core set of genes across serotypes (Kliitgard et al. 2010). Various MH serotyping studies in cattle have been conducted worldwide (US, EU, Japan) over the last 20 years (Al-Ghamdi et al. 2000; Katsuda et al. 2008; Andrés-Lasheras et al. 2019) and demonstrated generally Serotype A1 to be the most prevalent (approximately 60% of isolates), followed by serotype A6 (22%) and serotype A2 (18%). Cozens et al. (2019) have shown that serotype A1 bacteria, but not A2, invade differentiated bronchial epithelial cells. Therefore, while the vaccine contains the most prevalent serotype, evidence supports that the iron-deprived conditions of the vaccinal MH strain culture provide protection against other serotypes including A6 and A2 serotype bacteria.

In conclusion, our onset of immunity trials have demonstrated good vaccine efficacy under challenge as early as 42 days after vaccination at minimum age.

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