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How efficacious are vaccines against bovine respiratory syncytial virus in cattle?

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ABSTRACT

Bovine respiratory syncytial virus (BRSV) is a paramyxovirus that is the major cause of pneumonia in calves. Vaccines for this important pathogen have been available since the late 1970's. This review is a critical assessment of the literature including, experimental challenge studies and field trials, that address the efficacy of commonly used vaccines to control respiratory disease caused by BRSV.

1. The respiratory syncytial viruses: a brief natural history

Sixty years ago a syncytiagenic agent was recovered from a chimpanzee with a cold that had infected the entire colony, as well as its handler; the virus was aptly named the "chimp coryza agent" (Blount et al., 1956). About a year later in 1957 similar (identical?) viruses were isolated from infants with respiratory disease. Initial epidemiologic studies were conducted documenting high prevalence infection, and the agents were named "respiratory syncytial" viruses based on their characteristic cytopathic effect *in vitro* (Chanock et al., 1957; Chanock and Finberg, 1957). The first indication that cattle were frequent hosts for a similar virus was provided by Dogget and colleagues in 1968 who demonstrated an "inhibitor" (neutralizing antibody) of (human) respiratory syncytial virus in bovine sera (Doggett et al., 1968).

The first reports of isolation of bovine respiratory syncytial virus (BRSV) from cattle with respiratory disease occurred almost simultaneously in Europe (Paccaud and Jacquier, 1970; Wellemans et al., 1970) and Japan (Inaba et al., 1970) in 1970, and four years later in the United States (Rosenquist, 1974) in 1974. In the years since then, BRSV and human respiratory syncytial virus (HRSV), representatives of the *Pneumovirinae (Paramyxoviridae)*, have been shown to be genetically and antigenically closely related pathogens, that cause seasonal respiratory disease of variable severity that effects primarily the young (Baker et al., 1997; Van der Poel et al., 1994; Valarcher and Taylor, 2007). The resulting clinical signs of respiratory distress with the causative bronchiolytic and alveolar lesions are very similar (Bryson, 1993; Hall, 2012; Johnson et al., 2007; Pirie et al., 1981; Viuff et al., 1996). The glaring disparity in the biology of the two viruses is that there still is no vaccine for HRSV; whereas, a range of vaccines for BRSV have been developed, tested, and are commonly used in cattle production. That process is the subject of this review.

2. The challenge of a challenge model for BRSV infection

It is difficult to determine a preventable or mitigated fraction, or otherwise assess clinical immunity, if a challenge model produces little or no disease to prevent; the latter reality plagued early studies concerning pathogenesis and immunoprophylaxis of BRSV infections. Although suggested (Stott and Taylor, 1985), but poorly formally documented in the published literature, the respiratory paramyxoviruses, notably the RSVs and parainfluenza viruses attenuate rapidly upon culture *in vitro*. Investigators who have attempted to experimentally reproduce RSV-associated disease with culture passaged virus, generally the inocula that, for practical purposes, are provided by regulatory agencies for vaccine licensing trials, can readily attest to the veracity of this statement. Perhaps the best published circumstantial evidence of this phenomenon can be found in early attempts to experimentally reproduce disease with repetitively passaged BRSVs, sometimes in the context of efficacy studies of BRSV vaccines; mild disease, or often no disease, was consistently reported in the principals or unvaccinated controls that were infected with *in vitro* passaged BRSVs (Belknap et al., 1995; Castleman et al., 1985; Mohanty et al.,

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1981). Pyrexia and viral shed were usually the only primary outcome variables (of infection) reported. Moreover, attempts at experimental reproduction of disease often involved the use of multiple and invasive (intratracheal [IT]) routes of administration, and when disease was produced (Bryson et al., 1983; Belknap et al., 1991) it was not, therefore, representative of natural transmission, begging the question of its utility in truly assessing vaccine efficacy which may involve stimulating responses in the upper respiratory tract.

The mechanism of attenuation is poorly understood. It could be as simple as the *de facto* selection of variants in the quasispecies comprising clinical BRSV isolates (Larsen et al., 2000) that grow preferentially in cultured cells which have rarely been primary bovine pulmonary epithelial cells the natural target cell (Viuff et al., 1996), but usually fibroblasts or monkey kidney (Vero) cells, (Stott and Taylor, 1985). Addressing an earlier suggestion (Stott and Taylor, 1985), the seminal published documentation that *in vivo* passage (in calf lungs) could maintain the virulence of field isolates of BRSV, and indication that this type of inoculum would be more relevant to study BRSV infections was made by Van der Poel and colleagues in the mid-1990's (Van der Poel et al., 1996). As a variation on this theme, the use of aerosolization as the sole delivery method, either by individual mask (West et al., 1999), or more naturally by group exposure to low doses of virus in an enclosed space (Ellis et al., 2005), resulted in the consistent reproduction of clinical disease and lesions typical of naturally occurring BRSV, using a mode of transmission that as closely as possible mimics natural transmission. The resultant consistent degree of disease severity allowed the outcome variables of affected lung lesional area and (decreased) arterial pO2, in addition to (respiratory) clinical signs, to be precisely quantitated, so that the totality of response in vaccinated and sham vaccinated animals could be more effectively comparatively measured to better assess vaccine efficacy (West et al., 1999; West et al., 2000; Ellis et al., 2013b). As well, sequence analyses of multiple *in vivo* passages indicated that the particular isolate used in Canadian challenge studies, although derived from a clinical case in Asquith, Saskatchewan, is in a clade containing European viruses, and thus, *de facto*, provides a heterologous challenge for many vaccines that are based on the North American 375 isolate or other related BRSVs (J. Ellis and J. Marx, unpublished data, 2006), thereby addressing some potential concerns regarding BRSV variation and subgrouping (Schrijver et al., 1996).

Obviously, the immune status of the host is another confounding variable in the success of challenge of immunity studies. Given the high prevalence and, in fact, endemicity, of BRSV in many cattle populations (Baker et al., 1997), natural exposure early in life can prime young passively immune animals to respond differently (anamnestically) than truly naïve animals to vaccination later in life; therefore, depending on calf age at time of enrollment, an individual could be seronegative, but not naïve. To avoid the logistical difficulties and inconsistencies in obtaining calves from BRSV-low prevalence herds (Blodorn et al., 2015), or the management and disease issues resulting from total failure of passive transfer in colostrum-deprived (CD) calves (Vangeel et al., 2007), the use of BRSV antibody-negative colostrum has been used to provide passive protection (West et al., 2000) to more closely approximate "normal" calf rearing. This increases the likelihood of using truly naïve subjects, and avoids the artificial nature and potentially confounding variables attendant to the rearing of CD calves, or gnotobiotic calves in isolation for use as experimental surrogates for "barnyard" calves, the usual candidates for vaccination.

Sheep have been proposed and used as a model for BRSV infection, for more than 30 years (Cutlip and Lehmkuhl, 1979), and more recently for HRSV (Gazquez et al., 2014; Sow et al., 2011). As well, there has been increased use of this model to study pneumoviral-induced inflammatory responses (Gazquez et al., 2014; Redondo et al., 2014; Sow et al., 2011). Certainly, sheep can be experimentally infected with BRSV, replicate the virus and develop some level of clinical disease and lesions associated with the infection. However, the lack of evidence that BRSV (or a closely related pneumovirus; Evermann et al., 1985) is a significant pathogen in sheep, suggest that differences in the overall response to this viral quasispecies preclude the use of RSV-infected sheep as the most relevant host/model to study pathogenesis or assess vaccine efficacy for cattle.

One of the most controversial and unresolved issues in RSV pathobiology that has affected approaches to vaccine development for RSVs in both human, and to a lesser extent veterinary medicine is the role of the host immune response in disease. The concern of immunopotentiation of disease by vaccination derived from the observation that children vaccinated with a formalin-inactivated (FI) alum-adjuvanted vaccine in the late 1960s developed apparently more severe (enhanced) disease, than unvaccinated children, following natural exposure to HRSV (Kapikian et al., 1969). As an (the primary?) investigative response to these unfortunate events, thousands of unfortunate mice have been "infected" with HRSV resulting in hundreds of papers, often in prestigious journals, which together with more recent studies in BRSV-infected mice (Spilki et al., 2006; Maptletoft et al., 2010) speculate regarding immunopathologic mechanisms of RSV-associated disease, usually in the context of vaccination. In this still expanding literature there is a near uniform heedlessness to the revealed inconvenient truths that xenoinfection models of pneumoviral infections result in: rare, if any, clinical disease, insignificant viral replication after inoculation of usually large (relative) amounts of cultured HRSV (*i.e.* indicative of abortive infection by a species specific (Schlender et al., 2000, 2003) pathogen), and are absent, except in selective high power photomicrography, gross and histologic lesions that are characteristic of those found in either BRSV-infected cattle (Bryson, 1993; Viuff et al., 1996, 2002), or HRSV-infected humans (Johnson et al., 2007). In other words, there is virtually no disease to prevent (or enhance) in this model; certainly no disease characteristic of naturally-occurring pneumoviral infections in their target species. Nevertheless, the findings in mice (and cotton rat)-HRSV models have been commonly extrapolated to BRSV-infected cattle and issues concerning vaccination of cattle against BRSV (Gershwin, 2008; Sacco et al., 2014; Valarcher and Taylor, 2007). Unarguably, they are assumptive, potentially misleading, and of limited value, given species differences in viral infectivity and host response; they will not be given further airtime here.

In spite of few published investigations in the target species, cattle, available data suggest a role for the innate/inflammatory response in disease expression subsequent to BRSV infection. Circulating leukocytes from colostrum-fed calves experimentally-infected with BRSV had enhanced secretion of proinflammatory cytokines including, IL-6, interferon gamma (IFNγ), TNFα (Grell et al., 2005a,b) This effect was age-dependent, with youngest (1–5 day old) calves having more pronounced innate (cytokine) responses, which could account for more severe BRSV-associated disease observed in those animals (Grell et al., 2005b). Perhaps more relevant to the lung, are data that document a direct association between the concentrations of IL-4, IL-6, IFN-γ and, in particular,TNFα in bronchoalveolar lavage fluid and the severity of lung lesions subsequent to BRSV infection (Rontved et al., 2000; Antonis et al., 2010). But, in contrast to the previous studies, experiments conducted in CD calves found more severe clinical disease (but less viral replication and pulmonary pathology) in young (6 week old) calves than in one day old neonates (Antonis et al., 2010); however there was relatively little pulmonary

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pathology in either group (≤5% affected lung area). This apparent disparity was attributed to a more mature (pronounced) innate (TNFα) response in the older calves. Age-dependent bovine innate/inflammatory responses to BRSV beg additional investigation in the context of vaccination, especially since there is now a tendency in the field to vaccinate 1 day old neonates with modified live virus (MLV) intranasal (IN) vaccines. Acknowledging the relative dearth of information concerning the bovine inflammatory response to BRSV, it is likely that control of viral replication and resultant dampening of inflammation is ultimately an important mechanism by which vaccination spares disease.

3. BRSV vaccines and efficacy testing

Modified-live BRSV vaccines first became available in Europe in 1978 (Delforge and Zygraich, 1978) and shortly thereafter in North America in early 1980's (Bohlender, 1984) whereas, commercial inactivated BRSV vaccines were first marketed in the early 1980's in Europe (Howard et al., 1987). In the intervening years between the initial development of BRSV vaccines and the availability of a challenge model that reproduced disease to better test vaccine efficacy, several studies were performed that examined the immune responses to BRSV vaccines. Generally, these documented the immunogenicity of BRSV vaccines and the generic features that both types of vaccines stimulated lymphokine responses (IL-2 and IFN-γ) and IgG responses as measured by ELISA; whereas, MLV vaccines stimulated, in addition, functional, i.e. neutralizing and fusion – inhibiting, antibody responses (Ellis et al., 1992a,b; West and Ellis, 1997). Subsequent studies better defined the vaccine-stimulated T cell responses in blood *in vitro* (Platt et al., 2006; Sandbulte and Roth, 2003). None of these studies included a challenge of immunity. For the sake of brevity and topicality, the remainder of this review will focus on BRSV vaccines that have been assessed by challenge, or in the field since late 1990's, especially vaccines that are/were commercially available and used by practitioners of bovine medicine, and those prototype vaccines of historical or biological interest.

3.1. Modified-live BRSV parenteral vaccines

The first study to assess the efficacy of any commercially available vaccines in a disease-producing challenge model was a comparison of two MLV combination (BRSV, bovine parainfluenza virus-3 [BPIV-3], bovine herpesvirus-1 [BHV-1], bovine viral diarrhea virus [BVDV]) vaccines (West et al., 2000) Twenty-seven neonatal calves were fed a pool of BRSV antibody-negative colostrum at birth and randomized into 4 groups: unvaccinated controls (n = 9); vaccine (Bovishield-4, SmithKline Beecham Animal Health, 375 isolate of BRSV, n = 6) 2 ml intramuscularly (IM) twice at 3 wk intervals; vaccine (Bovishield-4, SmithKline Beecham Animal Health, n = 6) 2 ml IM once; adjuvanted vaccine (Pyramid 4, Fort Dodge/Ayerst, n = 6) 2 ml IM once. Calves received their first (or only) vaccination at 2–4 week of age and were challenged via individual mask aerosolization of approximately 10^5 plaque-forming units (pfu) of *in vivo*-passaged Asquith isolate BRSV 3 weeks after the last (or only) vaccination. Compared to unvaccinated controls, all vaccine groups had significantly ($p < 0.05$) less lung lesions, less nasal shed of BRSV, less hypoxemia, and less clinical disease overall. Interestingly, the response to 1 dose of adjuvanted vaccine was equivalent to 2 doses of unadjuvanted MLV vaccine. The disease-sparing was most closely associated with cellular immunity, IFN-γ and pulmonary MHC I-restricted cytotoxic T cells, but anamnestic systemic and mucosal antibody responses after challenge were observed as well in vaccinated groups. A limitation of this study was the relatively small numbers of cattle in the vaccine groups.

Three studies were conducted in the mid 2000's with a combination (BRSV, BHV-1, BPIV-3, BVD-1) vaccine (Rispoval 4, Pfizer Animal Health; 375 isolate of BRSV, approximately $10^{5.3}$ tissue culture infective dose [TCID] $_{50}$/5 ml dose) that is commercially available in Europe. All used a challenge model(s) that resulted in minimal clinical disease, and pulmonary pathology was not examined, limiting the conclusions that could be drawn relative to clinical protection. In the first study that examined duration of immunity (Peters et al., 2004), six month old, colostrum fed, seronegative (<2 VN), pasture-reared randomized calves (of unspecified breed) were vaccinated twice IM at a three week interval (n = 10), or received saline IM (n = 10). Twelve months after the second injection, calves were challenged intranasally with $4 \times 10^{6.1}$ $TCID_{50}$ of the 165 isolate of BRSV. Housing arrangements after challenge were not indicated. The frequency and degree of nasal shedding of virus was significantly ($P < 0.05$) reduced in the vaccinated vs sham vaccinated calves. There were no differences in the mild clinical signs of nasal discharge and pyrexia (>39.5 °C) between the 2 groups. Although this study supports the efficacy of parenteral immunization against BRSV, there are several limitations: it is unclear based on the source and rearing whether the calves were BRSV-naïve or only seronegative (decayed maternal antibodies) at the time of vaccination; there was minimal clinical disease; no evaluation of pulmonary pathology; and apparent differences in antibody responses between groups were not analyzed.

In the second study (Harmeyer et al., 2006) that examined vaccination in the face of maternal antibodies (IFOMA; passive immunity), colostrum-fed approximately 6 week old, weaned, commingled calves were grouped according to the variable BRSV antibody titers, and were vaccinated twice IM at a 4 week interval with 5 ml of the same vaccine (Rispoval 4, n = 10), or left as untreated controls (n = 10). Thirty-five days after the second vaccination all calves were challenged intranasally with $10 \times 10^{7.3} TCID_{50}$ of the Snook isolate of BRSV. As in the previous study, the frequency and degree of nasal shedding of virus was significantly ($P < 0.05$) reduced in the vaccinated versus untreated calves. Neutralizing serum antibody responses to BRSV were significantly ($P < 0.05$) higher after vaccination and challenge; but, there were no apparent (statistical) difference in the mild clinical signs observed in both groups. In addition to the very mild disease in this challenge model, the claim that these data document the generic ability of this vaccine to successfully immunize parenterally IFOMA is limited by the relatively low concentration of BRSV-neutralizing maternal antibodies (least square mean approximately 1:16) at the time of vaccination; considerably lower than that found in many, if not most, candidates for calfhood vaccination.

The third study (Salt et al., 2007) is very similar to the first except that the time between vaccination and challenge was shorter (3 weeks vs 12 months) and the animals were challenged both intranasally and intratracheally with the Snook strain of BRSV (dose not indicated). As in the first study with this vaccine (Rispoval 4), the frequency and degree of nasal shedding of virus was significantly ($P < 0.05$) reduced in the vaccinated vs sham vaccinated calves, but there were no differences in the mild clinical signs of nasal discharge and pyrexia (>39.5 °C) between the 2 groups. The decreased shedding was associated with a significant rise in BRSV-reactive IgG as determined by ELISA. The limitations of this study are those of the first with this vaccine, and are related primarily to the low stringency of the challenge model vis-à-vis the severity of disease that is frequently associated with BRSV infection in the field.

A recent study re- addressed the controversy concerning parenteral immunization in the context of passive immunity (Ellis et al., 2014) using a combination (BRSV, BHV-1, BPIV-3, BVDV1,2) commercial vaccine (Vista 5 SQ; Merck Animal Health, Summit,

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New Jersey, USA, 375 isolate of BRSV). Thirty-four neonatal calves were fed a pool of BRSV high antibody-positive colostrum at birth, to assure uniform high passive immunity, randomized into 2 groups, and vaccinated once at 3–9 days of age. One group (n = 17) received the combination BRSV vaccine, and the other group (controls, n = 17) received a similar vaccine without BRSV (Vista 3 SQ, Merck Animal Health). Eleven weeks later calves were group challenged via aerosol with a total dose of approximately 10^7 pfu of *in vivo*-passaged Asquith isolate of BRSV. Calves in both groups developed moderate to severe respiratory disease characteristic of BRSV, and there was no difference in the decrease in arterial oxygen between groups. Although the vaccinated calves had significantly ($P = 0.05$) less affected lung than controls, median 33% vs 39%, respectively, this difference was not considered biologically relevant. The lack of anamnestic antibody and IFN-γ and IL-4 responses after challenge was consistent with conclusion that the passively immune calves were not immunologically primed, and that maternal antibody typical of good passive transfer in neonates inhibited the responses to parenteral delivery of the BRSV vaccine.

Available data based on challenge of immunity indicate that parenteral delivery of MLV BRSV (in combination) can stimulate disease-sparing, antibody and cell-mediated immune responses. Interestingly, the findings of the first study (West et al., 2000), especially, document that *parenteral* administration can prime for anamnestic responses on the *mucosal surface*, in bronchoalveolar lavage fluid; however, this priming is probably variably subject to passive immune status (Ellis et al., 2014).

3.2. Modified-live BRSV intranasal vaccines

Given that clinical BRSV infections are most severe in calves with waning and variable concentrations of maternal antibodies that may inhibit immunization by parenteral vaccination, mucosal (intranasal) administration of MLV vaccines early in life provides a potentially more effective method of administration to engender protective immunity. This possibility was supported by the disease-sparing results reported in 2 studies with prototypical experimental IN attenuated BRSV vaccines (Kimman et al., 1989b; Woolums et al., 2004a,b). Those studies were limited by the small numbers of calves, and apparently variable and mild pulmonary lesions which were not statistically evaluated.

The first study that evaluated IN administration of a commercial BRSV vaccine in a disease-producing challenge model was conducted with a single component BRSV vaccine, and a commonly used combination vaccine that were licensed for parenteral administration (Ellis et al., 2007). Thirty nine neonatal calves were fed a pool of BRSV antibody negative colostrum at birth and allocated into randomized groups. In two preliminary experiments, nine week old calves received IN 1 (n = 3) or 2 (n = 3) doses (three weeks apart) of the single component BRSV vaccine (Bovishield-BRSV. Pfizer Animal Health, Kalamazoo, MI) or no vaccine (8 controls per experiment), and were group challenged by aerosol with approximately 10^6 $TCID_{50}$ *in vivo*-passaged BRSV three weeks after the first or second vaccination. In a third experiment, 2 week old calves (n = 9) received an IN combination (BRSV, PIV-3, BHV-1, BVDV) vaccine (Bovishield-4. Pfizer Animal Health, Kalamazoo, MI), or the same combination vaccine without BRSV (n = 8, Bovishield-3, Pfizer Animal Health, Kalamazoo, MI) and were group challenged by aerosol with approximately 10^6 $TCID_{50}$ *in vivo*-passaged BRSV 8 days later. Vaccinated calves in the preliminary experiments did not develop clinical signs and shed minimal BRSV compared to controls, and were not euthanized. In the third experiment, compared to sham-vaccinated controls, the IN vaccines had significantly ($P < 0.05$) less lung lesions, less frequent nasal shed of BRSV, less clinical disease overall, and higher concentrations of arterial oxygen. None of the calves in the third experiment had systemic BRSV-specific IgG responses after vaccination, or after challenge; however, the observed disease-sparing was associated with a significantly ($P = 0.02$) higher concentration of BRSV-specific IgA in nasal secretions after challenge.

BRSV vaccines specifically licensed for IN use became commercially available, virtually simultaneously in Europe and North America in 2007. Although traditionally, and legally, vaccine licensing trials have been conducted in individuals that are seronegative for the antigens in questions, many if not most calves that are BRSV vaccine candidates will have potentially inhibitory concentrations of maternal antibodies at the time of vaccination. To address this biological and practical reality several studies with IN vaccines have been reported in calves with passive BRSV immunity.

The first published report (Vangeel et al., 2007) of efficacy testing of commercial IN BRSV vaccines concerned a European combination (375 isolate of BRSV, PIV-3) vaccine (Rispoval RS+Pi3 IntraNasal, Pfizer, Ltd). In one study 39 colostrum-deprived calves were randomized into 2 groups, and at three weeks of age received one IN dose of the vaccine or one IN dose of saline (controls). Calves were challenged individually by nebulization of $10^{3.2}$ $TCID_{50}$ *in vivo* passaged Odijk strain of BRSV at 5 days (6 vaccinees/6 controls), 10 days (7 vaccinees/7 controls), or 21 days (6 vaccinees/6 controls after vaccination. Resulting clinical signs were mild with few differences between groups, but there was significantly ($P < 0.05$) reduced shedding of BRSV in the calves that were challenged 10 and 21 days after vaccination. In a second study, 20 calves that had received maternal colostrum were grouped according to their moderate to high BRSV titers. Ten received a single dose of the vaccine, and 10 received saline; all were similarly challenged as in the first experiment except with $10^{4.3}$ $TCID_{50}$ of the same BRSV inoculum at 66 days after vaccination. Resulting clinical disease was more severe; 2 controls and 1 vaccinee died or were euthanized and had interstitial pneumonic lesions typical of BRSV. However, aside from significantly ($P < 0.05$) decreased nasal shed of BRSV, there were no significant differences between groups. In both studies there was no significant change in BRSV neutralizing serum antibodies after vaccination; but, after challenge there was a significant ($P < 0.05$) increase, compared to the respective controls, in the seronegative vaccinated calves challenged at day 21 after vaccination, and in the vaccinated seropositive calves. Both studies supported the clinical efficacy of IN vaccination, including in passively immune calves; however, the variable and often mild clinical disease, overall, and a lack of quantitative evaluation of pulmonary pathology are limitations of these studies.

The efficacy of the first combination IN vaccine commercialized in North America was tested in studies using neonatal (3–8 day old) BRSV seropositive and seronegative calves (Ellis et al., 2010; Xue et al., 2010). In one experiment 11 BRSV-seropositive calves that had been fed a pooled colostrum replacer, and 10 BRSV-seronegative calves that had been fed a pool of BRSV antibody negative colostrum were administered 1 ml of a combination (BRSV, BPIV-3, BHV-1, BVDV 1, 2) vaccine (Vista Once 5 SQ, [marketed as Onset for intranasal use], Merck Animal Health; 11 seropositive calves received 1 ml of diluent (controls). Calves were housed in individual hutches on a group-wise basis (to prevent exposure to shedding vaccine virus) for 60 days, then commingled and then group challenged with approximately 10^6 $TCID_{50}$ of nebulized *in vivo* passaged BRSV (Asquith strain) 4.5 months after vaccination. Calves in all groups developed moderate to severe disease, and there were few significant differences, overall, in clinical responses, nasal shed of BRSV, mortality, extent of pulmonary pathology, or hypoxemia; in other words under the conditions of experiment, the vaccine failed to protect. This apparent failure raised questions regarding the efficiency of

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mucosal priming by vaccine IFOMA and/or the duration of immunity (DOI) of neonatal vaccination. In a second experiment, typical of a conventional licensing trial using seronegative calves, 11 calves received 1 ml IN of the vaccine with a minimal immunizing dose (MID approximately 1/100 of release dose), 12 calves received 2 ml of the MID-containing vaccine subcutaneously (according to label dose) and 11 calves received 1 ml of diluent IN (controls). Calves were maintained in individual hutches as before for 21 days, then commingled and challenged on day 21 as in the first experiment. Calves in all groups developed respiratory disease typical of BRSV infection and there were no significant differences in clinical responses, overall; however, both groups of vaccinated calves shed significantly ($P < 0.05$) less BRSV and had significantly ($P < 0.05$) less hypoxia and lung lesions compared to controls. Notwithstanding the disease-sparing efficacy of this vaccine in experimental BRSV infection under licensing conditions, due to field reports of adverse reactions/failure to protect, this intranasal vaccine was withdrawn from the market in 2009. Unfortunately the reason(s)/co-factors for the apparent failure of this vaccine in the field have never been resolved, or at least documented in the literature.

A subsequent study conducted in 3–11 day old Holstein calves readdressed the issues of blockade of mucosal priming by maternal antibodies, and the DOI of a single neonatal IN vaccination (Ellis et al., 2013a) using a commercial combination (BRSV [375 isolate], temperature sensitive (ts)BPIV-3 and ts BHV-1) IN vaccine (INFORCE 3, Pfizer Animal Health, Kalamazoo MI) that had been licensed using the disease-producing challenge model with the *in vivo*-passaged Asquith strain of BRSV (J Ellis unpublished data, 2009; on file at the United States Deparment of Agriculture). In the first experiment 16 BRSV-seronegative calves were given one, 1 ml dose of the vaccine with an MID concentration of BRSV IN and another 10 were given the same vaccine without BRSV (controls). All were commingled and group challenged approximately 7 weeks later with approximately 10^6 $TCID_{50}$ of nebulized BRSV. In a second experiment, 15 BRSV seropositive calves were given the 3-way commercial INFORCE 3 vaccine and 15 were given the control 2-way vaccine. All were commingled and group challenged 9 weeks later with the same lot and amount of nebulized BRSV. The third experiment was a repeat of the second with 14 vaccinees and 14 controls, except that challenge occurred approximately 16 weeks after vaccination. Vaccinees in the first 2 experiments had significantly ($P < 0.05$) less clinical disease overall, less affected lung, less hyoxemia, and less mortality (death or euthanasia due to severe disease); although the differences were generally more pronounced in the seronegative calves. In contrast, there were no significant differences between vaccines and controls in these outcome variables in the third experiment with the lengthened interval between neonatal IN vaccination and challenge. The disease-sparing was generally associated with both systemic (IgG) and mucosal (IgA) responses, but these 2 immunological variables do not elucidate all of the protective mechanisms.

In totality, the currently available data from the last 10 years confirm earlier observations and indicate that IN administration of MLV BRSV (in combination) can prime for protective immunity IFOMA, but that the duration of that immunity is rather short-lived. Whether this brevity is related to the relative immaturity of the immune system when the vaccines are commonly given, in neonatal or young calves, or the difficulty in establishing memory with mucosal administration, remain to be examined, optimally in a direct comparison of IN and parenteral delivery.

3.3. Inactivated BRSV parenteral vaccines

As discussed, the history of inactivated RSV vaccines began in the late 1960's with the aforementioned debacle involving a formalin-inactivated alum-adjuvanted single component HRSV vaccine. Those unfortunate events have colored inactivated RSV vaccines ever since, including those in cattle.

Two studies evaluating alum-adjuvanted FI-BRSV vaccines in different disease producing challenge models were independently conducted nearly simultaneously in the late 1990's, with differing outcomes. In both studies the "recipe" for the "lot 100" FI-HRSV vaccine (Kim et al., 1969) was followed to produce equivalent FI-BRSV vaccines and relevant tissue culture control (sham) vaccines absent the BRSV, both using bovine cells. In one study, (Gershwin et al., 1998) 19 conventionally-reared 7–8 week old calves with "low" BRSV serum antibodies were "divided" into 3 groups: vaccinated/sham infected (V/SI, n = 6); sham vaccinated/infected (SV/I, n = 6); and vaccinated/infected (V/I, n = 7). The calves were vaccinated twice IM on days 0 and 14 with 0.2 mg of protein in the 1.3 ml and then with 0.1 mg protein in 1 ml, respectively. Thirty days after the second vaccination calves were individually challenged in 2 groups on two subsequent days by nebulization with approximately 10^4 $TCID_{50}$ of plaque-purified cultured (passage level not indicated) BRSV isolate CA-1 in 5 ml; sham infected calves were similarly treated with uninfected cell culture supernatant. Compared to the unvaccinated infected controls (SV/I), the FI-BRSV vaccinated calves had significant increases in total clinical scores on only 2 days (late) after infection, questionable differences in decreased arterial oxygen (group mean 59 vs 53 mmHg, respectively), and "increased (neutrophilic) alveolitis" in the caudal lung lobes; however, there were no significant differences in effected lung lesional area between the 2 infected groups.

In the second study (West et al., 1999), "lot 100" FI-BRSV vaccine was compared to a prototypical MLV vaccine. Twelve pooled (BRSV antibody positive) colostrum fed, VN seronegative, naïve 4–5 month old Holstein calves were randomly divided into 3 groups of 4 calves: FI-BRSV, MLV-BRSV, and tissue culture controls (2 FI and 2 MLV). The FI calves were vaccinated twice three weeks apart with 1.3 mg of protein (approximately 6× the dose in the previous study) in 1 ml intradermally divided among 4 cervical sites; the MLV group received $10^{3.5}$ pfu of cultured BRSV (RB94 strain) in one intradermal site. The respective controls were similarly treated. Thirty four days after the second vaccination, calves were challenged individually by nebulization of approximately 10^5 pfu of *in vivo* passaged Asquith strain of BRSV. In contrast to the previous study, the FI-vaccinated calves had a significantly earlier onset of clinical disease; however, 3 of the 4 (compared to 2 of the 4 MLV vaccinates) had reduced pneumonic lung area relative to sham- vaccinated controls. These findings were associated with peribronchial eosinophilic infiltrates, and higher BRSV-specific lymphoproliferative and total IgG responses in the FI-BRSV vaccinees. Neutralizing antibodies increased after challenge in both vaccinated groups.

Several subsequent studies further dissected the bovine immune response to prototypical FI-BRSV vaccines in the context of disease-producing BRSV challenges. One study reported decreased production of IFNγ by BRSV-stimulated PBMC after challenge in calves that had received the "lot 100" FI-BRSV vaccine compared to those that did not (Woolums et al., 1999). In contrast, another investigation found slightly higher production of IFNγ by pulmonary CD8+ T cells in FI-BRSV vaccinees after challenge compared to those that were vaccinated with MLV BRSV, and the authors concluded that several response patterns (Th1/Th2) coexist during BRSV infection (Antonis et al., 2006). Those results also seemingly contrast with another study (Woolums et al., 2004a,b) reporting no priming for CD8+ CTLs or IFNγ by FI-BRSV vaccine. Supportive of the observation that disease-sparing with FI-BRSV vaccines may be a dose effect (West et al., 1999), are 2 studies (Kalina et al., 2004, 2005) indicating that relatively high

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doses of vaccinal BRSV antigen can prime for disease-sparing mucosal IgG1 (thought to be a Th2/IL-4 regulated subtype in cattle (Estes and Brown, 2002)) responses in the lung.

Altogether, the data concerning FI-BRSV vaccines in cattle comprise a can of worms. The data render an extrapolation of the central dogma of the murine xenoinfection (with HRSV) model, i.e., the Th1 "good"/Th2 "bad" dichotomy (Openshaw and Tregoning, 2005), to BRSV vaccination/infection, overly simplistic at best. But, perhaps more importantly, especially in moving forward, although certainly inducing interesting (dose dependent?) immunological phenomena in a variety of species, the still studied FI- RSV vaccines, are arguably best viewed as relics of a bygone era in vaccine formulation, rather than a paradigm to further investigate. They are not particularly relevant to modern inactivation processes, that better preserve native protein (epitope) structure, or more targeted adjuvants; the elephant in the room being the documented efficacy of at least some commercial inactivated BRSV vaccines.

The first study to address the issues of efficacy and potential enhancement of disease by commercially available inactivated BRSV vaccines in a disease-producing challenge model was conducted with a single component vaccine (Bovine Respiratory Syncytial Virus Vaccine, Killed Virus, Merial Ltd.) that was adjuvanted with carbopol, a polymer of acrylic acid (Ellis et al., 2001). Twenty seven nine week old seronegative (fed BRSV antibody negative colostrum) Holstein calves were randomized into 3 groups of 9: unvaccinated controls; vaccine at reduced potency (the equivalent of a MID for MLV vaccines; inactivated BRSV vaccines have generally had a preinactivation titer of approximately 10^7 $TCID_{50}$, and the antigen content of the release dose is standardized using a capture ELISA or serological testing in animals [G. Gallo, personal communication, 2016]) IM twice at 3 wk intervals; and the same vaccine at commercial release dose administered twice IM. Forty-two days after the first vaccination calves were individually challenged with approximately 10^5 $TCID_{50}$ of *in vivo*-passaged BRSV (Asquith strain) that was nebulized into a mask. Compared to unvaccinated controls, both vaccine groups had significantly ($P < 0.05$) less lung lesions, less nasal shed of BRSV, less hypoxemia, and less clinical disease overall. A greater reduction in mortality in the "release" dose group (0 versus 2) suggested a dose effect that was not further examined. As in previous studies with inactivated BRSV vaccines (West and Ellis, 1997), vaccinated calves responded with predominately non-neutralizing (IgG) antibodies, however, this response, as well as enhanced production of IFNγ by peripheral blood leukocytes, was statistically associated with the observed disease sparing. Moreover, the IFNγ response suggested that this inactivated vaccine primed for Th1 responses. Subsequent experiments with reduced doses (250–500 μl) of this adjuvanted BRSV antigen delivered transdermally yielded similar results (J. Ellis and T. Leard, unpublished data, 2006). Unfortunately, this vaccine and similar combination vaccines with this BRSV antigen and adjuvant are no longer commercially available.

A saponin-adjuvanted inactivated combination (BRSV, BPIV-3, BHV-1, BVDV) vaccine (Triangle 4, Wyeth (Ft. Dodge) was assessed in another similar study, again using 8–9 week old BRSV seronegative calves (Ellis et al., 2005). Calves were randomized into 2 groups: 6 received 2 doses of the commercial vaccine at days 0 and 20; 8 were untreated controls. Twenty-six days after the second vaccination, the calves were group challenged with nebulized *invivo* passaged BRSV as previously. Again, compared to unvaccinated controls, the vaccinees had significantly ($p < 0.05$) less lung lesions, less nasal shed of BRSV (vaccinees did not shed virus), less hypoxemia, and less clinical disease overall. As with the other inactivated vaccine, calves responded with predominately non-neutralizing (IgG) antibodies. Although limited somewhat by small numbers of vaccinated calves the observed highly significant disease-sparing as measured in several outcome variables was similar to that with the previous commericial inactivated vaccine, as well as to that engendered by MLV parenteral and IN vaccines tested in the same challenge model.

Two studies in passively immune calves assessed the efficacy of a combination (BRSV, BPIV-3, *Manheimia hemolytica*) anydrogel and Quil A adjuvanted inactivated vaccine (Bovipast RSP, Intervet) that is marketed in Europe. In the first study (Mawhinney and Burrows, 2005) 21, 4–5 week old Holstein-cross maternal colostrum-fed calves were randomly blocked according to their "moderate" concentrations of ELISA-determined BRSV antibody into 3 groups of 7: unvaccinated controls, a group that received 5 ml of the inactivated vaccine IM, and a group that received 2 ml of a single component MLV BRSV vaccine (Rispoval RS, Pfizer Animal Health) IM. There was no masking of investigators. Three weeks after vaccination calves were challenged with $10^{5.8}$ plaque-forming units of "low passage" Snook strain of BRSV in 20 ml; 10 ml administered IN, 10 ml administered intratracheally. There was a significant ($P < 0.05$) increase in BRSV-reactive IgG in both vaccinated groups after challenge, indicative of priming by the vaccines. However, there were no statistical differences among groups in the mild clinical signs that resulted from the challenge, nor in nasal shed of BRSV, and no evaluation of pulmonary pathology, making it difficult to justify the authors' claim that these data were indicative of "protection".

A followup study with the same inactivated vaccine (Bovilis RSP) focused on the cellular immune responses associated with vaccination and challenge of passively immune calves (van der Sluijs et al., 2010). Ten, approximately 2 week old Holstein calves that had been fed pooled BRSV-antibody positive colostrum neonatally, were blocked according to age and antibody concentration into 2 groups: 5 unvaccinated controls and 5 that received 5 ml of the vaccine SQ. These calves had markedly higher concentrations of Mab (approximately 9 log_2 VN) at the time of vaccination than in the previous study. Four weeks later they were challenged by individual nebulization of $10^{5.9}$ $TCID_{50}$ "wild-type" CA-1 strain of BRSV in 10 ml. There were no significant differences between groups in the resultant mild clinical disease and no apparent differences in the variable pulmonary pathology resulting from the challenge; however, vaccinees shed significantly ($P < 0.05$) less virus for fewer days. This was associated with a significant increase in BRSV- stimulated IFN-γ secretion by PBMC in the vaccinated calves after challenge, consistent with the priming of a Th1 response by inactivated BRSV IFOMA.

The efficacy of 2 doses of this vaccine, 1 month apart, was evaluated in a complicated study (Patel, 2004) involving groups of 5–6, seronegative (<2 VN) 2 week old Friesen-Hereford cross bulls that were sequentially challenged at 2 different times after vaccination (<4 months or >4 months; with 3–8 week intervals between challenges): BHV-1 > BPIV3 > BVDV, then *in vivo*-passaged BRSV (4 pooled field isolates, approximate total dose, 10^6 $TCID_{50}$ given IN and IT). Again, in this study only mild clinical disease resulted with no apparent differences between vaccinees and controls. Reportedly, no vaccinated animals shed virus after challenge compared to controls that shed variable amounts of virus on multiple days; however, no statistical evaluation of shed data was presented. This was associated with high (geometric mean 5.5–8.3 log_2) BRSV-neutralizing antibody at the time of challenge.

Despite the demonstrable clinical efficacy of some inactivated BRSV vaccines, certainly, incidents, both recorded (Kimman et al., 1989a; Schreiber et al., 2000) and anecdotal, of post-vaccinal disease enhancement in cattle that received commercial BRSV vaccines provide a caveat for any cavalier assumptions (of efficacy) concerning them. Especially in the case of inactivated vaccines,

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each vaccine formulation begs efficacy testing. The second reported case involved a beta propriolactone-inactivated alum and saponin adjuvanted vaccine. However, the irony that the first documented case of disease enhancement was subsequent to vaccination with MLV BRSV (Kimman et al., 1989a), underscores those authors' suggestion that host factors such as the stage of the immune response (after vaccination) at the time of infection may be as, or more, important than vaccine formulation, *per ce*, in the exacerbation of disease after vaccination. This is subject that begs further investigation, but is difficult to address from an experimental design perspective.

3.4. Field trials of BRSV vaccines

It is cliché to say that the ultimate test of any vaccine is in its application in the field; reduction of disease and, in the case of livestock, economic benefits, comprise passing grades. As in the laboratory, a discerning examination (of a vaccine) in the field is predicated upon some disease to prevent; in this case disease attributable to BRSV. Interestingly, the first publication concerning *any* BRSV vaccine was a field trial (Bohlender, 1984). Following up on the initial isolation of BRSV in Nebraska in 1978, a multi-year field trial involving >10,000 head of cattle on several commercial operations compared 1 (at 4–6 months of age) or 2 (second dose at 7–8 months of age) doses of a single component MLV BRSV vaccine (name not indicated, Norden Laboratories) to unvaccinated controls. Vaccinated cattle had dramatic reductions in undifferentiated respiratory disease and treatment costs, with the 2 dose regime being the better one. Although relatively short on details, largely absent specific diagnoses (of BRSV), and limited somewhat by the use of historical controls in some cases, and lack of statistical analyses, this large seminal study certainly suggested the clinical efficacy of BRSV vaccines (alone) in reducing respiratory disease in commercially-managed beef cattle. Several field trials with BRSV-containing vaccines have been conducted in the last 15 years; however, few have addressed the critical question as to whether an induced immune response (to BRSV) had any role in mitigating the occurrence of disease by actually implicating BRSV in a disease process. Admittedly, the latter proposition is not a trivial pursuit. BRSV is a labile virus and has short life cycle in cattle, often making it difficult to detect in clinical material (nasal swabs), and at post mortem coincident with secondary bacterial infections. As well, assessment of seroconversion after exposure is often tenuous in vaccinated populations (Baker et al., 1997). Absent those measurements, assessing disease and mortality in cohorts that receive similar vaccines with and without BRSV, as has been done in the laboratory, can provide good circumstantial evidence of the role of BRSV in any disease observed and, importantly, any disease-sparing role of vaccine stimulated responses to BRSV. Even in well- designed studies, no matter how large, a failure to assess these parameters, and simply look at the difference (in disease or economic outcomes) between vaccinated (with combination vaccine) and non-vaccinated cohorts (Windeyer et al., 2012), or the difference between 2 vaccines different in other ways besides containing BRSV or not (Wildman et al., 2008), it is nearly impossible to determine the specific contribution of vaccine-induced immunity to BRSV.

One "serendipitous" field trial (Larsen et al., 2001) was conducted in more than 750 calves on 2 Danish farms with a betapropriolactone-inactivated, alum and saponin adjuvanted vaccine that was not further identified. All calves were given 2 ml of vaccine SQ at approximately 6 and 9 weeks of age. Approximately 2 months later, there was subsequent high prevalence of respiratory disease in 4 to 7 month old calves; 8/500 and 20/250 calves died. BRSV was detected in 2/20 nasal swabs and 2 of 5 lungs from sampled calves; seroconversion to BRSV was common. Although limited by a lack of unvaccinated controls for a comparison of severity of disease in vaccinated vs not, these data certainly suggest a failure to protect. Given the age of the calves at the time of vaccination, the author's attribution of failure to inhibition of priming by maternal antibodies is reasonable. The identity of this vaccine although not specifically indicated appeared at least very similarly formulated to that held responsible in an incident of vaccine associated disease enhancement (Schreiber et al., 2000) This vaccine was withdrawn from the market in the late 1990's (Larsen et al., 2001).

One field trial (Ferguson et al., 1997) compared (milk) production and reproductive parameters in 385 Holstein randomized cows and heifers given either 2 doses of "4-way" (Cattle Master 4, Pfizer, Inc., Exton, PA, USA; MLV-BRSV, tsBHV-1, tsBPI3V, inactivated BVDV) or "3-way" (Cattle Master 3, minus the BRSV) vaccines prior to parturition (second dose 2–3 before calving). The "4-way"-vaccinated heifers had significantly (ANOVA, $P < 0.05$) higher milk production during the first 21 days of lactation and higher first insemination conception rates. Although no apparent respiratory disease was reported during the trial, these data uniquely addressed the impact of subclinical BRSV infections on production.

Another study addressing the efficacy of BRSV antigen in the context of its absence from a combination vaccine was examined in a 2 year trial (MacGregor and Wray, 2004) involving yearling feedlot cattle that were blocked by sex and randomly allocated into 2 groups: One group received a "4-way" MLV vaccine (BRSV, BHV-1, BPI3V, BVDV), the other a "3-way" (minus the BRSV). The cattle that received BRSV in the "4-way" vaccine had significantly ($P < 0.05$) less morbidity and mortality attributable to undifferentiated respiratory disease, less overall mortality, overall case fatality, and "respiratory mortality"; however, feeding performance outcomes were not different between groups. Those results, that suggested the contribution of BRSV to the observed disease, as well as the field efficacy of inclusion of parenteral MLV BRSV in vaccine protocol, were very similar, with regard to reduced treatment rates, in 7 of 8 groups comprising more than 8000 cattle in 5 trials conducted in the late 1980's, shortly after the introduction of BRSV vaccines (van Donkersgoed et al., 1990).

4. Discussion

It is customary in reviews such as this to conclude that there is need for more research, especially when the author(s) is actively working in the subject area reviewed, and/or actively seeking funding. Certainly, science is written in pencil, and continuing research is the way of progress. Supportive of this reasoning is a recent meta analysis of published studies of BRSV vaccines in cattle (Theurer et al., 2015), including those referenced herein, that concluded, " in experimental challenge trials evaluating MLV BRSV vaccines no significant difference; in morbidity or mortality risk was found between vaccinated and control calves."; in other words, essentially, current BRSV vaccines are not efficacious and begging further development. Admittedly, at least some models, especially those using *in vivo* passaged BRSV err on the side of stringency, and they are, like all experimental infections, by definition, "artificial". However, the latter analysis that was based primarily on the assessment of semi-subjective clinical findings in a usually subfatal infection, overlooks the highly significant disease-sparing in vaccinates, as measured entirely objectively by lung lesional area and blood oxygen, that was generally reported in those same manuscripts. The (reduction in) latter outcomes was generally directly associated with vaccine-induced immune responses.

One potential limiting factor in comparatively evaluating vaccine efficacy is the proprietary nature of vaccine formulation

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in veterinary medicine. Usually information concerning the particular isolate of BRSV and the dose of BRSV in (commercial) vaccines is not indicated, and, in the case of inactivated vaccines, often there are no details regarding adjuvants. As well, certainly much is still unknown concerning the genetics of protective immunological responsiveness (Glass et al., 2012) or potential for immunological enhancement of disease following vaccination (Schreiber et al., 2000) Acknowledging these caveats, relatively simple, whole virus, modified-live, and to a less frequently tested extent, inactivated BRSV vaccines have been shown to be efficacious in cattle, depending on the outcome variables that are assessed, and depending on ones' expectations for vaccination; sterile immunity vs disease reduction. From a safety perspective, and in contrast to other important viral pathogens, such as bovine herpesvirus-1 and bovine viral diarrhea virus, the long-observed restricted tropism of BRSV to respiratory epithelial cells makes it highly unlikely that a reversion to virulence can occur in intramuscularly or subcutaneously administered attenuated (cultured) vaccine virus populations. More likely, as has been long speculated BRSV effectively undergoes an abortive infection in dermal fibroblasts. In theory, there is a greater safety concern for the now commonly used MLV IN BRSV vaccines. However, given the difficulty in producing disease with cultured (attenuated) BRSV, especially when using intranasal inoculation alone, it is highly unlikely that these vaccines would "revert to virulence" or be "pathogenic", nothwithstanding the possibility of a recombinational event with wild-type BRSV's,

Certainly, future research should not exclude continuing vaccine development involving novel vaccine candidates. Some recent examples that show promise include: microparticle encapsulated BRSV glycoprotein peptides (Kavanagh et al., 2013); a BRSV SH deletion recombinant combined with HRSV peptides (Blodorn et al., 2014; and a human adenovirus-vectored HRSV F, N, and M2-1 construct (Taylor et al., 2015). It is, however, an open question whether such novel "high-tech" approaches will equal or improve on the efficacy and duration of immunity that has been, or may be demonstrated in the case of simple "conventional' whole virus vaccines; are they truly better, or just novel (and 'grant-worthy'). As well, beyond this burden of proof, new vaccine candidates must be "scale-upable", economically feasible to produce, and practical to use in the field. Arguably, more immediate progress in improving the overall efficacy of BRSV vaccines may be possible by simply rethinking the approach to vaccination for BRSV (with the current vaccines) and other respiratory pathogens, with an emphasis on early mucosal priming followed by a boosting and (immune) memory maintenance; a so-called "heterologous prime-boost" approach (Woodland, 2004). Conceptually this involves exposing the immune system to different forms of an antigen by different routes to achieve an immunologically balanced, durable response. Serological data from 2 recent field trials using currently available conventional vaccines in several hundred passively immune beef calves (Stotenow et al., 2011; Stokka et al., 2016) support this approach. In both studies neonatal IN vaccination and parenteral boosting at weaning compared to a more traditional 2 dose parenteral regimen resulted in significantly higher anamnestic responses to BRSV after the second vaccination. Further experimentation with various vaccine combinations and intervals between vaccinations in the laboratory and in the field is likely to provide a more enlightened answer to the question, "How efficacious are BRSV vaccines in cattle?"

Conflict of interest statement

The author has conducted efficacy studies for several veterinary biologics companies.

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