Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Veterinary Microbiology 199 (2017) 47–53

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

An inactivated influenza D virus vaccine partially protects cattle from respiratory disease caused by homologous challenge

Ben M. Hause[a,b,*], Lucas Huntimer[c], Shollie Falkenberg[c,1], Jamie Henningson[a,b], Kelly Lechtenberg[d], Tom Halbur[c]

[a] Kansas State Veterinary Diagnostic Laboratory, Kansas State University, Manhattan, KS, USA
[b] Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS, USA
[c] Elanco Animal Health, Greenfield, IN, USA
[d] Midwest Veterinary Services, Oakland, NE, USA

ARTICLE INFO

Article history:
Received 26 August 2016
Received in revised form 16 December 2016
Accepted 19 December 2016

Keywords:
Influenza
Bovine
Respiratory disease
Vaccine
Pathogenesis

ABSTRACT

Originally isolated from swine, the proposed influenza D virus has since been shown to be common in cattle. Inoculation of IDV to naïve calves resulted in mild respiratory disease histologically characterized by tracheitis. As several studies have associated the presence of IDV with acute bovine respiratory disease (BRD), we sought to investigate the efficacy of an inactivated IDV vaccine. Vaccinated calves seroconverted with hemagglutination inhibition titers 137–169 following two doses. Non-vaccinated calves challenged with a homologous virus exhibited signs of mild respiratory disease from days four to ten post challenge which was significantly different than negative controls at days five and nine post challenge. Peak viral shedding of approximately 5 $TCID_{50}$/mL was measured in nasal and tracheal swabs and bronchoalveolar lavage fluids four to six days post challenge. Viral titers were significantly ($P < 0.05$) decreased 1.4 $TCID_{50}$/mL, 3.6 $TCID_{50}$/mL and 5.0 $TCID_{50}$/mL, respectively, in the aforementioned samples collected from vaccinated animals compared to non-vaccinated controls at peak shedding. Viral antigen was detected in the respiratory epithelium of the nasal turbinates and trachea by immunohistochemistry from all unvaccinated calves but in significantly fewer vaccinates. Inflammation characterized by neutrophils was observed in the nasal turbinate and trachea but not appreciably in lungs. Together these results support an etiologic role for IDV in BRD and demonstrate that partial protection is afforded by an inactivated vaccine.

1. Introduction

First isolated from pigs with acute respiratory disease, the proposed influenza D virus has subsequently been identified in bovines in numerous countries, including the United States, Mexico, Italy, France, and China, as well as a sow in Italy (Hause et al., 2013, 2014; Ferguson et al., 2015; Collin et al., 2015; Mitra et al., 2016; Ducatez et al., 2015; Chiapponi et al., 2016; Jiang et al., 2014). While serological surveys of swine and human sera found low antibody prevalence (9.5 and 1.3%, respectively), several studies have found that IDV antibodies are nearly ubiquitous in bovines (Hause et al., 2013, 2014; Ferguson et al., 2015). These results, in addition to the common detection and isolation of IDV from bovines with acute respiratory disease, led to the proposal of cattle as the reservoir for IDV (Hause et al., 2014). Besides swine, humans and bovines, IDV antibodies have also been detected in goats and sheep but were absent from poultry (Quast et al., 2015).

Viral metagenomic sequencing of calves with acute respiratory disease and asymptomatic controls at a large ranch was used to identify viruses associated with bovine respiratory disease (BRD) (Ng et al., 2015). Bovine adenovirus 3, bovine rhinitis A virus and IDV were the only viruses significantly associated with BRD ($P < 0.01$). Similarly, viral metagenomic sequencing of feedlot cattle with acute BRD and healthy pen mates at ten sites in Mexico and the U.S. identified twenty one different viruses however IDV was the only virus moderately associated with BRD (odds ratio

* Corresponding author. Present address: Cambridge Technologies, 508 Oxford Street, Worthington, MN, USA.
E-mail address: bhause@cambridgetechnologies.net (B.M. Hause).
[1] Present address: National Animal Disease Center, United States Department of Agriculture, Ames, IA 50010, USA.

http://dx.doi.org/10.1016/j.vetmic.2016.12.024
0378-1135/

2.94, $P = 0.134$) (Mitra et al., 2016). The amount of IDV RNA in samples from animals with BRD was also significantly greater than IDV-positive asymptomatic animals. ($P = 0.04$). A separate study found a much higher incidence of IDV in respiratory swabs taken from calves with acute BRD (29.1%) as compared to healthy calves (2.4%) in a Mississippi cattle order-buyer facility (Ferguson et al., 2015).

Recently, the pathogenesis of IDV was investigated in a limited number of calves (Ferguson et al., 2016). While calves inoculated with IDV displayed mild respiratory disease similar to controls, virus replication was detected in the respiratory tract associated with a significant increase in neutrophils in the trachea. IDV transmitted to contact animals and all inoculated and exposed animals seroconverted.

While not conclusively established, mounting evidence suggests that IDV is part of the bovine respiratory disease complex (BRDC), the most economically significant disease affecting the U.S. cattle industry. The objectives of this study were to investigate IDV pathogenesis and to determine if an inactivated vaccine can afford protection to disease caused by IDV.

2. Materials and methods

2.1. Animals and animal procedures

Animal procedures were performed at Midwest Veterinary Services (MVS) using biosafety level 2 practices with protocols approved by the Institutional Animal Care and Use Committees (IACUC) of MVS.

Sixty five approximately six-month old calves which received colostrum were purchased from a single farm and transported to MVS. All animals had hemagglutination inhibition (HI) titers ≤ 40 to D/bovine/Kansas/162655/2012 and were negative for bovine viruses detected by the Kansas State Veterinary Diagnostic Laboratory (KSVDL) BRD PCR panel (IDV, bovine viral diarrhea virus, bovine herpesvirus 1, bovine respiratory syncytial virus, and bovine coronavirus) prior to transport and at study initiation.

2.2. Cells and viruses

Human rector tumor 18G (HRT18G) and swine testicle (ST) cells were maintained in minimal essential media (MEM) supplemented with 7.5% fetal bovine serum and L-glutamine at 37 °C with 5% CO_2. Strain D/bovine/Kansas/162655/2012 was isolated on HRT18G cells from a nasal swab submitted to the KSVDL for BRD diagnostic testing. Virus was passaged on HRT18G cells and titrated on ST cells to determine the 50% tissue culture infective dose per ml ($TCID_{50}$). Propagation of IDV in HRT18G utilized MEM supplemented with 0.5 μg/mL trypsin while in ST's trypsin was omitted.

2.3. PCR

Samples (nasal swabs, tracheal swabs and bronchoavleolar lavage fluids) were analyzed by quantitative reverse transcription PCR (qRT-PCR) for IDV as previously described (Hause et al., 2013). RNA extracted from a culture of D/bovine/Kansas/162655/2012 with a titer of 4.2 $TCID_{50}$/mL was serially diluted 10-fold and analyzed by qRT-PCR to generate a standard curve to correlate cycle threshold (Ct) values to $TCID_{50}$/mL.

2.4. Serology

Hemagglutination and hemagglutination inhibition assays were performed using turkey red blood cells as previously described (WHO, 2002). Titers were log_2 transformed and mean group titers were calculated for each time point.

2.5. Vaccine

Several T225 cm2 flasks of HRT18G were inoculated with D/bovine/Kansas/162655/2012 at a multiplicity of infection of 0.001. Cells were harvested by a freeze/thaw cycle and viral titer determined by the hemagglutination assay (HA). Virus was inactivated with 0.1% β-propiolactone for 24 h at 37 °C. Antigen was diluted in MEM and commercial adjuvant (Emulsigen® D, MVP Biologics) was added to the inactivated viral fluids to 30% final volume so that the HA concentration of the vaccine was 320 HA units per mL. Vaccine was administered as a 2.0 ml dose given subcutaneously to the neck.

2.6. Experimental design

Calves were randomly assigned to one of three treatment groups. Treatment group 1 (T1, n = 15 calves) received vaccine on days 0 and 14 and were moved to separate housing prior to challenge of treatment groups 2 and 3. Treatment group 2 (T2, n = 25 calves) was mock vaccinated on days 0 and 14 with MEM and challenged on day 35 with D/bovine/Kansas/162655/2012. Treatment group 3 (T3, n = 25 calves) was vaccinated on days 0 and 14 and challenged on day 35 with D/bovine/Kansas/162655/2012. The challenge virus, diluted to 6.0 $TCID_{50}$/mL in MEM, was administered intranasally with 2.5 ml instilled into each nostril.

2.7. Monitoring and sample collection

All animal experimental procedures were conducted by blinded study members. Body weight and temperature (determined rectally) were recorded on day 0. Temperature was recorded beginning day 35 daily until day 45. Blood was collected from on days 0, 14, 21, 35 and 42. Nasal swabs were collected on days 0, 35, 37, 39, 41, 43 and 45. The calves were observed for adverse events following each vaccine administration. Calves were also observed daily beginning day 35 for clinical signs of disease and assigned a 0–4 score (0 = normal, 1 = mild, 2 = moderate, 4 = severe) separately for depression, respiratory distress and body condition. Mild clinical signs included lethargy, slightly increased respiratory rate, abnormal nasal or ocular discharge and/or cough, and a fat flank with some dehydration or rough hair coat. Moderate clinical signs included inactivity, increased respiratory rate, muco-purulent nasal or ocular discharge, and a concave flank with dehydration and/or rough hair coat. Severe clinical signs include recumbence, respiratory distress and prominent skeleture with severe dehydration. A composite clinical score was calculated by the summation of the depression, body condition and respiratory distress scores for each treatment group on each day divided by the number of calves in the treatment group.

Three calves from T1 and five calves from both T2 and T3 were humanely euthanized each on days 37, 39, 41, 43 and 45. On necropsy, the lungs were removed *in toto* and bronchoalveolar lavage fluid (BALF) and tracheobronchial lymph nodes specimens were collected. The percentage of lung consolidation was estimated for each lung lobe. The total lung consolidation score was calculated by estimating lung lobe volume as a percentage of the whole (apical lobes, 10% each; diaphragmatic lobes, 25% each; cardiac lobes, 10% each; accessory lobe, 10%). Tracheal swabs were also collected at necropsy on days 41, 43 and 45.

2.8. Histopathology and immunohistochemistry

Tissue samples including lung, trachea, and nasal turbinate were collected from euthanized cattle and were preserved in 10% neutral formalin. Representative tissues were collected from the middle of the trachea and from different lung lobes from different

Table 1
Log 2-transformed hemagglutination inhibition titers and standard deviation in calf sera. Treatment groups T1 and T3 received at inactivated vaccine on days 0 and 14. Treatment groups T2 and T3 were challenged with a homologous virus on day 35. Different letters (A, B, C) indicate significant differences ($p \leq 0.05$) between groups within a column. Groups with the same letter indicate no difference between them.

Treatment Group	Day 0	Day 14	Day 21	Day 28	Day 35	Day 42
T1 (n = 15)[a]	4.7 ± 0.5^{A}	5.7 ± 0.6^{A}	6.9 ± 1.1^{A}	7.1 ± 1.3^{A}	7.1 ± 1.2^{A}	7.1 ± 0.8^{A}
T2 (n = 25)	4.7 ± 0.5^{A}	5.2 ± 0.3^{B}	4.5 ± 0.4^{B}	4.9 ± 0.5^{B}	5.0 ± 0.5^{B}	5.3 ± 0.0^{B}
T3 (n = 25)	4.7 ± 0.5^{A}	5.5 ± 0.5^{AB}	6.7 ± 0.9^{A}	7.3 ± 1.0^{A}	7.4 ± 0.9^{A}	8.0 ± 1.1^{A}

[a] n, number of calves.

animals selected grossly based on lung consolidation. All tissue samples were routinely processed by the histopathology section of the Kansas State Veterinary Diagnostic Laboratory (KSVDL). Histopathological examination was performed on hematoxylin and eosin stained slides. Immunohistochemical staining was performed on formalin fixed paraffin embedded tissues that were sectioned at 4 μm thickness onto positively charged slides. Slides were stained using the Leica Bond-Max autostainer with the Polymer Refine Red Detection kit. Polyclonal antisera from rabbits hyperimmunized with D/swine/Oklahoma/1334/2011 was diluted to 1:5000 with Bond Primary Antibody Diluent (Leica Biosystems, Tris-buffered saline). Heat mediated epitope retrieval was performed using EDTA pH 9.0 for 20 min at 100 °C. Tissue sections were incubated with the primary antibody for 15 min at ambient temperature. Polymerization was performed with Polymer-AP α-Rabbit (Leica Biosystems) for 25 min at ambient temperature. Visualization was done with fast red and slides were counterstained with hematoxylin. Immunohistochemistry was recorded as positive or negative and presented as the number of animals in each treatment group that were positive.

2.9. Statistical analysis

Lung consolidation, HI titers, and IDV titers in nasal swabs, tracheal swabs and BALF were analyzed by analysis of variance (ANOVA) using the JMP software package (SAS, Cary, NC). A $P < 0.05$ was considered significant. Where significant differences were identified between study groups, mean pairwise comparisons were performed with the Tukey-Kramer test. Composite clinical signs (depression, body score and respiratory distress) at each individual day post-challenge and the presence of IDV in nasal turbinates and trachea by IHC were analyzed by analysis of variants (ANOVA) using GraphPad Prism, GraphPad Softare, La Jolla, CA. A value of 1 was assigned for positive IHC samples and a value of 0 was assigned for negative IHC samples. Kruskal–Wallis test was used to analyze composite clinical scores and IHC scores. Response variables shown to have a significant effect by treatment group were subjected to pair-wise comparisons using the Tukey–Kramer test or the Dunn's test with Bonferroni correction

3. Results

3.1. Seroconversion in vaccinated calves

At study commencement, mean group IDV HI titers were 4.7 $\log_2$ and were statistically indistinguishable (Table 1). A 2-fold increase or less in titers were observed on day 14 when the booster vaccine was administered. On days 21, 28 and 35, the vaccinated groups (T1 and T3) had significantly higher mean HI titers (6.7–7.4 $\log_2$) than the non-vaccinated group (4.5–5.0 $\log_2$). A less than 2-fold increase in HI titers was observed for the non-vaccinated animals from day 0 to 35, suggesting a lack of IDV exposure.

3.2. Infection with IDV causes mild respiratory disease

Prior to challenge, all calves were clinically normal. All 15 calves in T1 (vaccine only) remained clinically normal throughout the study (composite clinical signs = 0). There were no significant differences in rectal temperatures between groups on any day during the challenge phase of the experiment. Groups challenged with IDV all had mean clinical composite scores >0.2 beginning on day 40 throughout the remainder of the study (Table 2). The mean composite clinical scores of the non-vaccinated/challenge group (T2) on days 40 and 44 were significantly greater than negative challenge controls (T1), suggesting that IDV causes mild respiratory disease. A subset of nasal swabs collected on day 0 (three per group) were additionally analyzed by the full KSVDL BRD PCR panel to detect viruses and bacteria associated with BRD (bovine viral diarrhea virus, bovine herpesvirus 1, bovine respiratory syncytial virus, bovine coronavirus, IDV, *Mycoplasma bovis*, *Pasteurella multocida*, *Mannheimia haemolytica* and *Histophilus somni*). The only organism detected was *P. multocida* with Ct values 30.7–34.8. *P. multocida* is known to commensally inhabit the bovine respiratory system and is an opportunistic pathogen (Biberstein, 1990).

3.3. IDV shedding is decreased in vaccinated calves

All calves were negative for IDV by PCR on the day of challenge (day 35) and T1 remained negative through the challenge phase of the study. T2 had significantly higher IDV titers in nasal swabs than

Table 2
Mean composite clinical score for calves challenged with IDV on day 35. Calves were assigned a 0–4 score (0 = normal, 4 = severe) separately for depression, body condition and respiratory distress. A mean composite clinical score was calculated by the summation of the depression, body condition and respiratory distress scores for each treatment group on each day divided by the number of calves in the treatment group. Different letters (A, B, C) indicate significant differences ($p \leq 0.05$) between groups within a column. Groups with the same letter indicate no difference between them.

Treatment Group	Day 35	Day 36	Day 37	Day 38	Day 39	Day 40	Day 41	Day 42	Day 43	Day 44	Day 45
T1	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}
T2	0.0 ± 0.0^{A}	0.0 ± 0.2^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.1 ± 0.2^{A}	0.5 ± 0.6^{B}	0.5 ± 0.5^{AB}	0.3 ± 0.5^{A}	0.3 ± 0.5^{A}	1.0 ± 0.0^{B}	0.6 ± 0.5^{A}
T3	0.0 ± 0.0^{A}	0.0 ± 0.2^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.3 ± 0.5^{AB}	0.5 ± 0.5^{B}	0.2 ± 0.4^{A}	0.4 ± 0.5^{A}	0.4 ± 0.5^{AB}	0.6 ± 0.5^{A}

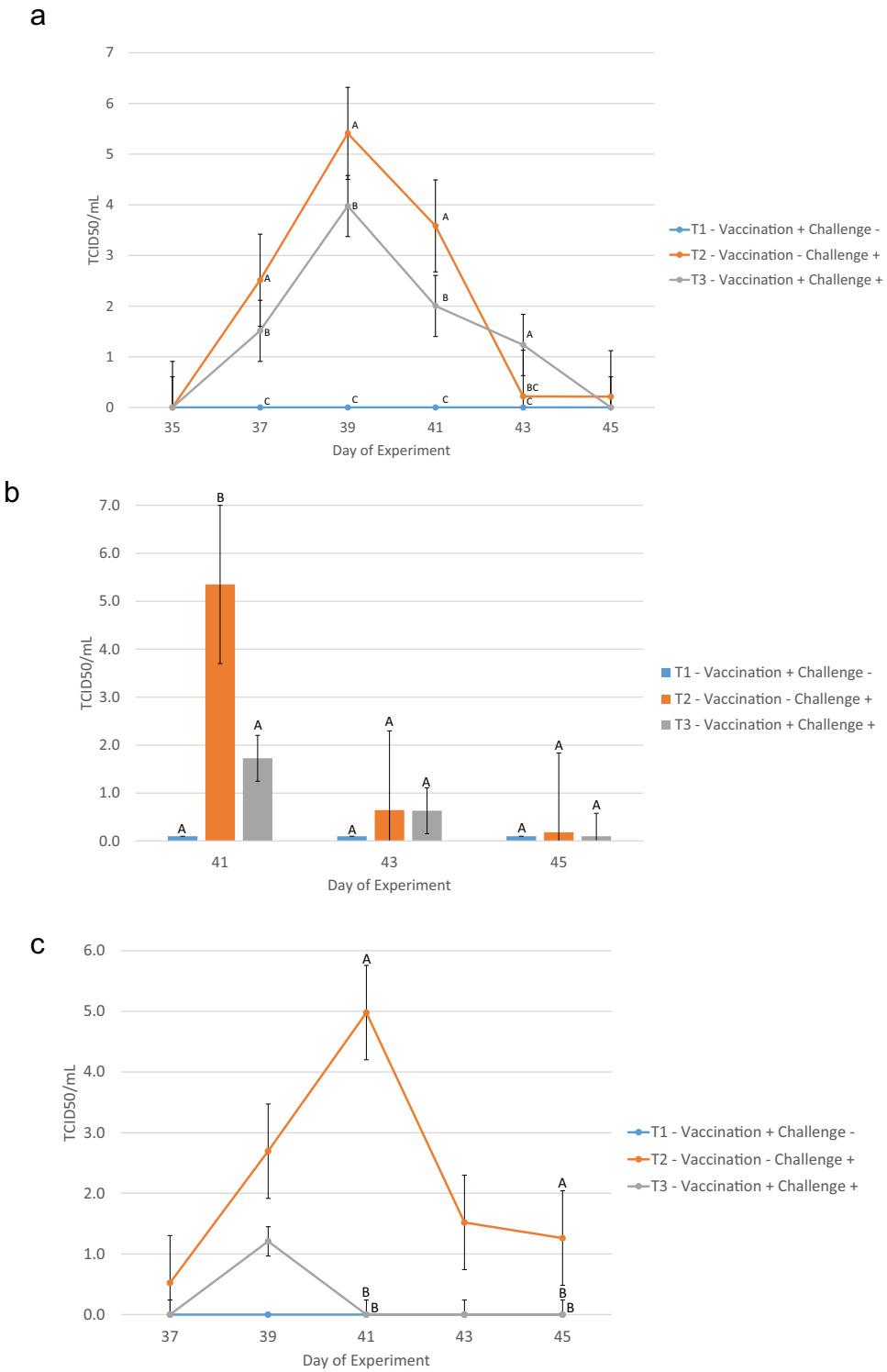

Fig. 1. Influenza D virus titers in (a) nasal swabs, (b) tracheal swabs, and (c) bronchoalveolar lavage fluids. Calves in treatment group T1 received vaccine only while those in T2 were only challenged with IDV on day 35 post vaccination. Calves in T3 were both vaccinated and challenged. Different letters (A–C) indicate significant differences ($p \leq 0.05$) between groups at that time point. Groups with the same letter indicate no difference between them. Error bars indicate standard error of the mean (SEM).

all other groups on days 37, 39 and 41 (Fig. 1a). Peak shedding of approximately 5.4 $TCID_{50}$/mL was observed on day 39. The homologous IDV vaccine significantly reduced IDV shedding in nasal swabs ~1–2 $TCID_{50}$/mL. Maximal IDV titers in tracheal swabs were similar to those determined in nasal swabs, with 5.3 $TCID_{50}$/mL detected in T2 at day 41 (Fig. 1b). The significant difference in IDV titers in tracheal swabs for vaccinated and non-vaccinated animals (5.3 versus 1.7 $TCID_{50}$/mL, respectively) on day 41 was greater than observed for nasal swabs. IDV was detected in BALF for T2 calves from day 37–45, with the highest titers at day 41 (Fig. 1c). Interestingly, vaccinated calves in group 3 were only positive on day 39 with a relatively low titer (1.2 $TCID_{50}$/mL).

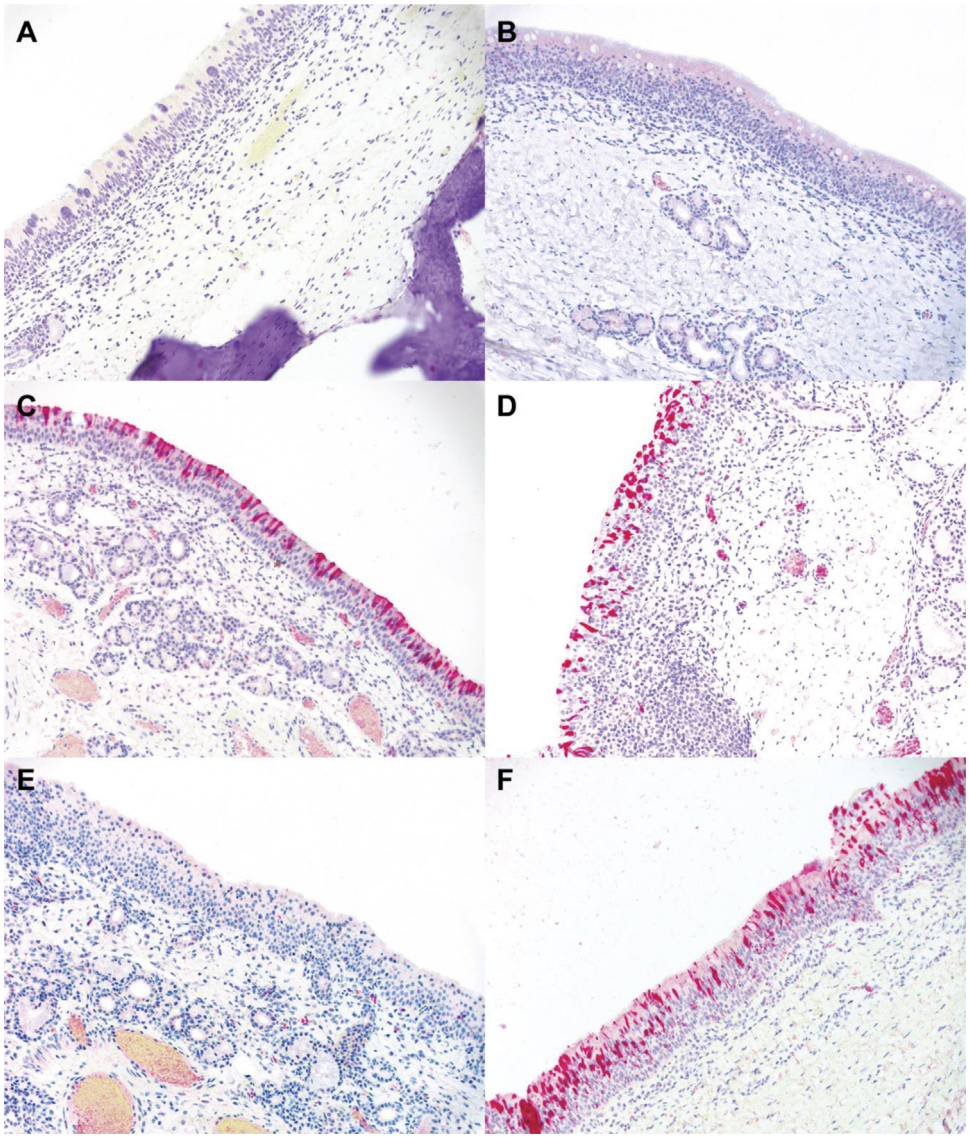

Fig. 2. IDV antigen staining from nasal turbinate and trachea of each treatment group. (A). Negative staining in nasal turbinate respiratory epithelium from a calf in T1. (B) Negative staining in tracheal epithelium from a calf in T1. (C) Positive intense cytoplasmic staining in nasal turbinate respiratory epithelium from a calf in T2. (D) Positive intense cytoplasmic staining in trachea respiratory epithelium from a calf in T2. (E) Negative staining in nasal turbinate respiratory epithelium from a calf in T3. Nasal turbinates from all calves in group 3 were negative. (F) Positive intense cytoplasmic staining in trachea respiratory epithelium from a calf in T3. In all figures, A–E, the antibody polyclonal produced some non-specific background staining in the blood vessels of the submucosa and in (B) which has some non-specific staining in the cilia and mucous layer of the respiratory epithelium. All figures taken at 200× magnification.

3.4. IDV replication is mainly limited to the upper respiratory tract

On routine histology of the nasal turbinates, challenged calves had small numbers of neutrophils in the submucosa, neutrophil exocytosis, and mild multifocal loss of cilia (Fig. 2). Three calves had suppurative rhinitis characterized by neutrophils admixed with mucous overlying the respiratory epithelium. Lesions were similar in the trachea and consisted of small numbers of neutrophils in the submucosa, neutrophil exocytosis, occasional loss of cilia and flattening of the epithelium, and mild lymphoplasmacytic tracheitis. Two calves had a layer of mucous admixed with neutrophils overlying the tracheal mucosa. One calf had a mild suppurative bronchitis and bronchiolitis in the lung. There were no significant lesions observed in the tracheobronchial lymph node.

Positive antigen deposition was present as cytoplasmic staining of the respiratory epithelium of nasal turbinates and trachea (Fig. 2). Nasal turbinates from one of five T2 calves only were positive on days 37 and 39 for IDV by IHC (Fig. 3a). On day 41, all samples from T2 were positive (5/5) while samples from all other groups remained negative. A single calf from T2 was positive on day 43 while on day 45, all T2 samples were negative. In contrast to PCR where IDV was detected in nasal and tracheal swabs and BALF for T3, all T3 nasal turbinate samples were negative by IHC. Two of the five animals sampled from T3 were only positive by IHC of tracheal samples on day 39 (Fig. 3b). This was lower than the 4/5 positive tracheal samples on day 39 for T2. Similar to nasal turbinates, IHC on tracheal samples were positive for all T2 samples on day 41 (Fig. 3b.). IHC was also performed on lung tissue however only two T2 calves were weakly positive in the respiratory

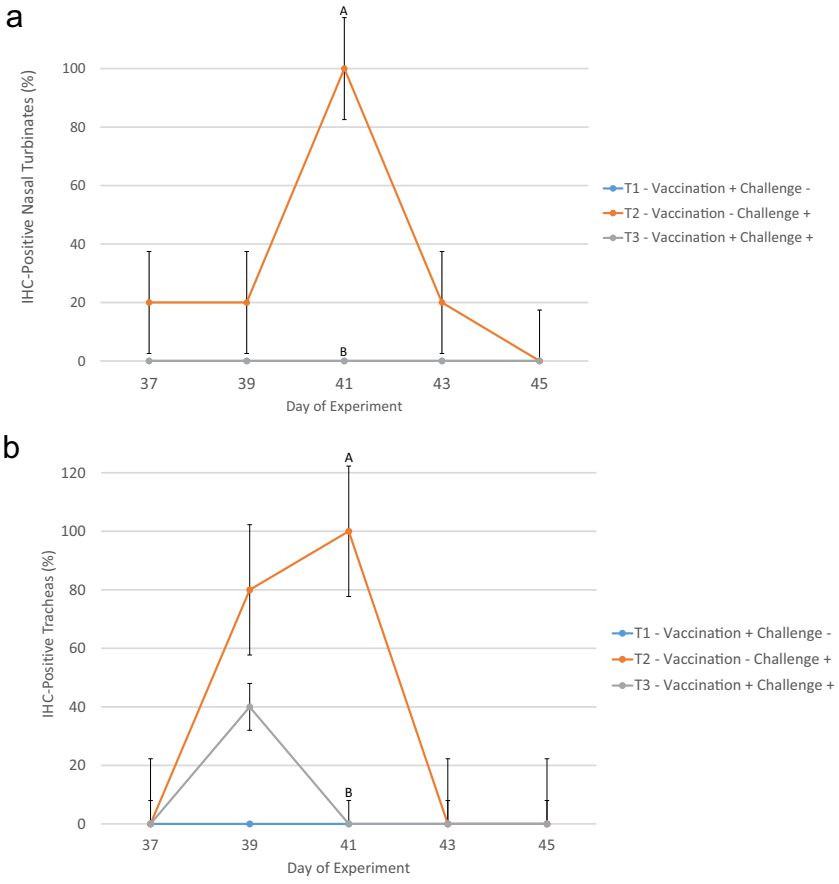

Fig. 3. Percentage of calves positive for influenza D virus by immunohistochemistry of (a) nasal turbinate, and (b) trachea. Calves in treatment group T1 received vaccine only while those in T2 were only challenged with IDV on day 35 post vaccination. Calves in T3 were both vaccinated and challenged. Different letters (A, B) indicate significant differences ($p \leq 0.05$) between groups at each time point. Groups with the same letter indicate no difference between them. Error bars indicate standard error of the mean (SEM).

epithelium of the airways. All tracheobronchial lymph nodes were negative by IHC for IDV.

4. Discussion

Bovine respiratory disease is the most significant disease affecting the United States cattle industry, causing annual estimated losses in excess of one billion dollars (McVey, 2009; Griffin, 1997; Snowder et al., 2006). Numerous commercial vaccines variably consisting of combinations of inactivated and modified live bacteria and viruses are widely used; however, the incidence of BRD has been increasing for the past several decades (USDA, 2011). The etiology of BRD involves interactions between the host, environment and pathogens (Mosier, 2014). Dogma posits that animal stress, often due to movement and animal management, predisposes cattle to a primary viral insult followed by secondary bacterial pneumonia due to commensal or exogenous pathogens. A number of viruses have established roles in BRD pathogenesis, including bovine viral diarrhea virus (Ridpath, 2010), bovine herpesvirus 1 (Muylkens et al., 2007), bovine respiratory syncytial virus (Gershwin et al., 2000), bovine parainfluenza virus 3 (Ellis, 2010), bovine rhinitis virus (Mohanty et al., 1969; Betts et al., 1971), and most recently, IDV (Ferguson et al., 2016). Commercial vaccines for the latter virus are not available however, likely in part due to its recent discovery and our developing understanding of its role in BRD pathogenesis. Here we show that an inactivated IDV vaccine provides partial protection to disease caused by a homologous challenge. Significant reductions in viral titers in nasal swabs and tracheal swabs and BALF were all observed, as well as the number of animals positive for antigen in nasal turbinate and tracheal tissues.

This study expands upon an earlier IDV pathogenesis and transmission study performed in a small number of calves. Similar to that work, IDV replication was limited to the upper respiratory tract and the main pathology observed was rhinitis and tracheitis in this study. Ferguson et al. showed that IDV causes mild respiratory disease with the highest viral titers present in the nasal turbinates, decreasing in tissues lower in the respiratory tract. While we also measured high (>5 $TCID_{50}$/mL) titers in the turbinates and trachea, similar titers were determined in BALF. Surprisingly, only two calves were weakly positive for IDV by IHC on lung tissue. We suspect that virus identified in BALF is largely a result of virus deposition due to the large volume of mucous and exudate observed in the upper respiratory tract as opposed to significant replication in the lung. Pathology was only seen in the lung in one calf and IDV antigen deposition in the lung of two calves, which demonstrates that this virus, although primarily an upper respiratory tract pathogen, does have the ability to replicate into the lung. This extension into the lung is important to consider in cases of highly stressed cattle that develop BRD. IDV is thought to utilize 9-*O*-acetyl-N-acetylneuraminic acid as a cellular receptor (Hause et al., 2013). This receptor is also utilized by bovine coronavirus for cellular attachment and challenge studies have shown that BCV can cause lung pathology and be readily detected in lung tissue by IHC (Vlasak et al., 1988; Park et al., 2007). Factors other than cell receptors may restrict IDV replication mainly to the

upper respiratory tract. IDV replication was similarly restricted to the upper respiratory tract in swine and ferrets but in contrast, high lung titers and abundant antigen was detected in guinea pig lungs (Hause et al., 2013; Sreenivasan et al., 2015).

In swine, inactivated influenza A virus vaccines often yield sterilizing immunity to homologous challenge (Vincent et al., 2008). While significant reductions in viral titers in nasal and tracheal swabs and BALF were observed, in addition to significant reductions in the number of animals positive for IDV by IHC on turbinate and tracheal tissue, viral titers significantly greater than non-challenged controls were observed. The calves used in this study received colostrum at birth and had measurable IDV antibody titers at study commencement (4.7–4.9 $\log_2$ HI titer). While we cannot be certain that these antibodies were maternally derived, previous work found that 94% of neonatal beef cattle were seropositive for IDV (Ferguson et al., 2015) and our prescreening of these calves >1 month before study enrollment showed they were negative for IDV by PCR. As maternal antibodies are known to interfere with vaccination and the development of active immunity, the presence of antibodies at the time of vaccination may have lessened the immune response to the vaccine (Sandbulte et al., 2014). For humans, a HI titer of 40 (5.3 $\log_2$) is generally considered protective however does not fully alleviate disease symptoms (Memoli et al., 2016). Two doses of the inactivated vaccine here resulted in HI titers 137–169 (7.1–7.4 $\log_2$) which yielded partial protection. Interestingly, the reduction in viral titers was most pronounced in samples collected lower in the respiratory tract (BALF < trachea < turbinates). As inactivated influenza vaccines largely elicit protection through humoral immunity, this result may suggest a greater role for humoral immunity in the lower respiratory tract.

These results add to the growing body of data that IDV can play an etiologic role in BRD pathogenesis. Although the significant clinical disease caused by IDV was mild, it may be sufficient to allow infiltration and colonization of bacteria to both the upper and lower respiratory tract (Ferguson et al., 2016). Future studies are needed to test this hypothesis. Additionally, an inactivated IDV vaccine was moderately protective to a homologous challenge. Inclusion of IDV into commercial BRD vaccines may improve their efficacy in preventing BRD. While several studies have failed to find evidence of widespread IDV infections in humans (Hause et al., 2013; Smith et al., 2016), a recent study found that 97% of humans occupationally exposed to cattle were serologically positive to IDV by microneutralization in contrast to 18% of non-exposed humans (White et al., 2016). While it is unknown whether infection of humans with IDV causes disease, vaccination of cattle could help limit zoonosis.

References

Betts, A.O., Edington, N., Jennings, A.R., Reed, S.E., 1971. Studies on a rhinovirus (EC11) derived from a calf. J. Comp. Pathol. 81, 41–49.

Biberstein, E.L., 1990. Our understanding of the Pasteurellaceae. Can. J. Vet. Res. 54, S78–82.

Chiapponi, C., Faccini, S., De Mattia, A., Baioni, L., Barbieri, I., Rosignoli, C., Nigrelli, A., Foni, E., 2016. Detection of influenza D virus among swine and cattle, Italy. Emerg. Infect. Dis. 22, 352–354.

Collin, E.A., Sheng, Z., Lang, Y., Ma, W., Hause, B.M., Li, F., 2015. Cocirculation of two distinct genetic and antigenic lineages of proposed influenza D virus in cattle. J. Virol. 89, 1036–1042.

Ducatez, M.F., Pelletier, C., Meyer, G., 2015. Influenza D virus in cattle, France, 2011–2014. Emerg. Infect. Dis. 21, 368–371.

Ellis, J.A., 2010. Bovine parainfluenza-3 virus. Vet. Clin. North Am. Food Anim. Pract. 26, 575–593.

Ferguson, L., Eckard, L., Epperson, W.B., Long, L.P., Smith, D., Huston, C., Genova, S., Webby, R., Wan, X.F., 2015. Influenza D virus infection in Mississippi beef cattle. Virology 486, 28–34.

Ferguson, L., Olivier, A.K., Genova, S., Epperson, W.B., Smith, D.R., Schneider, L., Barton, K., McCuan, K., Webby, R.J., Wan, X.F., 2016. Pathogenesis of influenza D virus in cattle. J. Virol. doi:http://dx.doi.org/10.1128/jvi.03122-15.

Gershwin, L.J., Gunther, R.A., Anderson, M.L., Woolums, A.R., McArthur-Vaughan, K., Randel, K.E., Boyle, G.A., Friebertshauser, K.E., McInturff, P.S., 2000. Bovine respiratory syncytial virus-specific IgE is associated with interleukin-2 and -4, and interferon-gamma expression in pulmonary lymph of experimentally infected calves. Am. J. Vet. Res. 61, 291–298.

Griffin, D., 1997. Economic impact associated with respiratory disease in beef cattle. Vet. Clin. North Am. Food Anim. Pract. 13, 367–377.

Hause, B.M., Ducatez, M.F., Collin, E.A., Ran, Z., Liu, R., Sheng, Z., Armien, A., Kaplan, B., Chakravarty, S., Hoppe, A.D., Webby, R.J., Simonson, R.R., Li, F., 2013. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. PLoS Pathog. 9, e1003176.

Hause, B.M., Collin, E.A., Liu, R., Huang, B., Sheng, Z., Lu, W., Wang, D., Nelson, E.A., Li, F., 2014. Characterization of a novel influenza virus in cattle and swine: proposal for a new genus in the Orthomyxoviridae family. mBio 5 (e00031-14).

Jiang, W.M., Wang, S.C., Peng, C., Yu, J.M., Zhuang, Q.Y., Hou, G.Y., Liu, S., Li, J.P., Chen, J.M., 2014. Identification of a potential novel type of influenza virus in bovine in China. Virus Genes 49, 493–496.

McVey, D.S., 2009. BRD research needs in the next 10–20 years. Anim. Health Res. Rev. 10, 165–167.

Memoli, M.J., Shaw, P.A., Han, A., Czajkowski, L., Reed, S., Athota, R., Bristol, T., Fargis, S., Risos, K., Powers, J.H., Davey Jr., R.T., Taubenberger, J.K., 2016. Evaluation of antihemagglutinin and antineuraminidase antibodies as correlates of protection in an influenza A/H1N1 virus healthy human challenge model. mBio 7, e00417–16.

Mitra, N., Cernicchiaro, N., Torres, S., Li, F., Hause, B.M., 2016. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. J. Gen. Virol..

Mohanty, S.F., Lillie, M.G., Albert, T.F., Sass, B., 1969. Experimental exposure of calves to a bovine rhinovirus. Am. J. Vet. Res. 20, 1105–1111.

Mosier, D., 2014. Review of BRD pathogenesis: the old and the new. Anim. Health Res. Rev. 15, 166–168.

Muylkens, B., Thiry, J., Kirten, P., Schynts, F., Thiry, E., 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. Vet. Res. 38, 181–209.

Ng, T.F., Kondov, N.O., Deng, X., Van Eenennaam, A., Neibergs, H.L., Delwart, E., 2015. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. J. Virol. 89, 5340–5349.

Park, S.J., Kim, G.Y., Choy, H.E., Hong, Y.J., Saif, L.J., Jeong, J.H., Park, S.I., Kim, H.H., Kim, S.K., Shin, S.S., Kang, M.I., Cho, K.O., 2007. Dual enteric and respiratory tropisms of winter dysentery bovine coronavirus in calves. Arch. Virol. 152, 1885–1900.

Quast, M., Sreenivasan, C., Sexton, G., Nedland, H., Singrey, A., Fawcett, L., Miller, G., Lauer, D., Voss, S., Pollock, S., Cunha, C.W., Christopher-Hennings, J., Nelson, E., Li, F., 2015. Serological evidence for the presence of influenza D virus in small ruminants. Vet. Microbiol. 180, 281–285.

Ridpath, J., 2010. The contribution of infections with bovine viral diarrhea viruses to bovine respiratory disease. Vet. Clin. Food Anim. 26, 335–348.

Sandbulte, M.R., Platt, R., Roth, J.A., Henningson, J.N., Gibson, K.A., Rajão, D.S., Loving, C.L., Vincent, A.L., 2014. Divergent immune responses and disease outcomes in piglets immunized with inactivated and attenuated H3N2 swine influenza vaccines in the presence of maternally-derived antibodies. Virology 464–465, 45–54.

Smith, D.B., Gaunt, E.R., Digard, P., Templeton, K., Simmonds, P., 2016. Detection of influenza C virus but not influenza D virus in Scottish respiratory samples. J. Clin. Virol. 74, 50–53.

Snowder, G.D., Van Vleck, L.D., Cundiff, L.V., Bennett, G.L., 2006. Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. J. Anim. Sci. 84, 1999–2008.

Sreenivasan, C., Thomas, M., Sheng, Z., Hause, B.M., Collin, E.A., Knudsen, D.E.B., Pillatzki, A., Nelson, E., Wang, D., Kaushik, R.S., Li, F., 2015. Replication and transmission of the novel bovine influenza D virus in a guinea pig model. J. Virol. 89, 11990–12001.

USDA, 2011. Feedlot 2011: Part IV: Health and Health Management on U.S. Feedlots with a Capacity of 1,000 or More Head. USDA-APHIS-VS-CEAHNAHMS, Fort Collins, CO.

Vincent, A.L., Lager, K.M., Janke, B.H., Gramer, M.R., Richt, J.A., 2008. Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine. Vet. Microbiol. 126, 310–323.

Vlasak, R., Luytjes, W., Spaan, W., Palese, P., 1988. Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. Proc. Natl. Acad. Sci. U. S. A. 85, 4526–4529.

WHO, 2002. Manual on Animal Influenza Diagnosis and Surveillance. World Health Organization, Geneva, Switzerland.

White, S.K., Ma, W., McDaniel, C.J., Gray, G.C., Lednicky, J.A., 2016. Serological evidence of exposure to influenza D virus among persons with occupational contact with cattle. J. Clin. Virol. doi:http://dx.doi.org/10.1016/j.jcv.2016.05.017.