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Pathogenesis of Influenza D Virus in Cattle

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ABSTRACT

Cattle have been proposed as the natural reservoir of a novel member of the virus family *Orthomyxoviridae*, which has been tentatively classified as influenza D virus (IDV). Although isolated from sick animals, it is unclear whether IDV causes any clinical disease in cattle. To address this aspect of Koch's postulates, three dairy calves (treatment animals) held in individual pens were inoculated intranasally with IDV strain D/bovine/Mississippi/C00046N/2014. At 1 day postinoculation, a seronegative calf (contact animal) was added to each of the treatment animal pens. The cattle in both treatment and contact groups seroconverted, and virus was detected in their respiratory tracts. Histologically, there was a significant increase in neutrophil tracking in tracheal epithelia of the treatment calves compared to control animals. While infected and contact animals demonstrated various symptoms of respiratory tract infection, they were mild, and the calves in the treatment group did not differ from the controls in terms of heart rate, respiratory rate, or rectal temperature. To mimic zoonotic transmission, two ferrets were exposed to a plastic toy fomite soaked with infected nasal discharge from the treatment calves. These ferrets did not shed the virus or seroconvert. In summary, this study demonstrates that IDV causes a mild respiratory disease upon experimental infection of cattle and can be transmitted effectively among cattle by in-pen contact, but not from cattle to ferrets through fomite exposure. These findings support the hypothesis that cattle are a natural reservoir for the virus.

IMPORTANCE

A novel influenza virus, tentatively classified as influenza D virus (IDV), was identified in swine, cattle, sheep, and goats. Among these hosts, cattle have been proposed as the natural reservoir. In this study, we show that cattle experimentally infected with IDV can shed virus and transmit it to other cattle through direct contact, but not to ferrets through fomite routes. IDV caused minor clinical signs in the infected cattle, fulfilling another of Koch's postulates for this novel agent, although other objective clinical endpoints were not different from those of control animals. Although the disease observed was mild, IDV induced neutrophil tracking and epithelial attenuation in cattle trachea, which could facilitate coinfection with other pathogens, and in doing so, predispose animals to bovine respiratory disease.

In addition to influenza A (IAV), B (IBV), and C (ICV) viruses, influenza D virus (IDV) has been proposed as a new member of the family *Orthomyxoviridae*. IDV is a single-strand, negative-sense RNA virus with 7 genomic segments that are predicted to encode 9 proteins: a glycoprotein hemagglutinin-esterase fusion (HE); polymerases PB2, PB1, and P3; nucleoprotein; matrix proteins (M1 and CM2); and nonstructural proteins (NS1 and NEP). IDV shares less than 50% protein sequence identity with ICV, which is the genetically closest member of the influenza virus family (1). There is no cross-reactivity between IDV and human ICV-generated serum (1–3).

IDV was first identified from swine with respiratory disease in 2011 and has subsequently been found in both healthy and sick cattle from multiple geographic areas across the United States, France, and China (1–7). The virus was recently detected in sheep and goats (8). Serological studies have shown that IDV has been present in beef cattle at least since 2004 (4). Cattle have been proposed as the reservoirs for IDV, and young, weaned, comingled, and immunologically naive calves seem to be most susceptible to infection due to dwindling maternal antibody levels after 6 months of age (4). The pathogenesis of IDV in cattle, however, is unresolved.

A metagenomic study in dairy calves with bovine respiratory diseases (BRD) found that IDV was present in 62% of 50 calves that were coinfected with bovine adenovirus 3, bovine rhinitis A virus, or another BRD-associated pathogen (5). Our virologic surveillance found that IDV was present in 23.6% of 55 calves affected with BRD but in only 2.4% of 82 healthy calves upon arrival at the same facility (4). Other studies have shown that the IDV positivity rates in cattle with BRD in the United States and France were 4.5% and 4.8%, respectively (3, 6). The prevalence of IDV in China among healthy adult cattle populations was reported to be 0.7% (7). Robust serologic evidence among small ruminants across multiple states in the United States suggests IDV exposure in 13.5% and 13.3% of sheep and goat farms, respectively, but not among chickens or turkeys (8). Lastly, inconclusive serologic evidence from human serum samples found that 1.3% of 316 samples

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were positive, leading to the speculation that IDV could have potential public health risks (1).

This study aimed to assess the pathogenesis and transmission of IDV in seronegative cattle. We hypothesized that IDV would cause clinical disease in cattle and that the virus would have the ability to be transmitted to other cattle by direct contact. In addition, we aimed to assess the transmissibility of the virus from cattle to ferrets through a fomite vector to assess the potential risks to public health.

MATERIALS AND METHODS

Biosafety and animal handling. Laboratory and animal experiments were conducted under biosafety level 2 (BSL2) conditions in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Mississippi State University.

Viruses. The strain of IDV (D/bovine/Mississippi/C00046N/2014) used in the experimental challenge was isolated from a 6-month-old beef calf, which had been suffering from chronic BRD (4). The virus was propagated in human rectal tumor (HRT-18G) cells (ATCC, Manassas, VA) under the following growing conditions: 1× minimal essential medium (MEM) with 1% penicillin-streptomycin (Pen-Strep) and 5% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA) for 5 days at 37°C with 5% CO_2. The virus from the second passage in HRT-18G cells was aliquoted and stored at −80°C until use. The viral titers were determined by 50% tissue culture infective doses ($TCID_{50}$) in HRT-18G cells, and the $TCID_{50}$ was calculated using the Reed-Muench method (9).

Animals. Nine 4-month-old healthy male dairy calves were purchased from the dairy operation at Mississippi State University. Four 4-month-old healthy female ferrets were purchased from Triple F Farms (Sayre, PA). All the calves and ferrets were seronegative for two antigenically distinct IDV strains, IDV strain D/bovine/Mississippi/C00046N/2014 and IDV strain D/bovine/Mississippi/C00013N/2014, using hemagglutination inhibition (HAI) assays.

RNA extraction, molecular cloning, PCR, and quantitative PCR. Viral RNA was extracted using a GeneJet viral DNA/RNA purification kit according to the manufacturer's protocol (Thermo Scientific, Pittsburgh, PA). Gene-specific amplification primers were designed according to the D/swine/1334/Oklahoma/2011 sequence and a reverse transcription (RT) primer based upon the conserved noncoding regions, as described previously (4).

To quantify the copies of viral PB1 genes, a 135-bp-long region was amplified from D/bovine/C00046N/Mississippi/2014 using a previously described primer set (4) and then cloned into the pJET1.2/blunt plasmid vector using a CloneJET PCR cloning kit according to the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA). The plasmids were used to establish a standard curve, and the resulting formula, which was used to quantify the viral PB1 genes in the samples collected from the animals, was as follows: $\log_{10}$ (x [copy number of PB1 genes]) = -0.33 (ΔC_T) + 14.265 ($R^2 = 0.9929$; $P < 0.001$). A cutoff value of ΔC_T of ≤35 from quantitative RT-PCR was classified as IDV positive (4).

HA and HAI assay. Hemagglutination (HA) and HAI assays were performed as described previously (4). Briefly, sera were treated 1:3 with receptor-destroying enzyme (RDE) (Denka Seiken Co., Tokyo, Japan) at 37°C for at least 18 h, followed by heat inactivation at 55°C for 30 min. The inactivated serum was diluted to a final concentration of 1:10 with 1× phosphate-buffered saline (PBS). The HA and HAI assays were conducted using D/bovine/Mississippi/C00046N/2014 and 0.5% turkey red blood cells at room temperature.

$TCID_{50}$. Nasal swab samples or the homogenate supernatants from tissue samples were serially diluted from 10^{-1} to 10^{-6} and titrated in HRT-18G cells. The $TCID_{50}$ was calculated by using the Reed-Muench method (9). Each sample was tested in triplicate.

Transmission and pathogenesis experiments in cattle. The calves in the treatment group were inoculated intranasally using a catheter with 10^7 $TCID_{50}$ of D/bovine/C00046N/Mississippi/2014 in a volume of 10 ml. The calves in the control group were inoculated with sterile PBS. One day postinoculation (dpi), seronegative calves of the in-contact group were randomly paired to be housed in pens occupied by inoculated calves in the treatment group. Nasal swabs, rectal swabs, and blood were obtained 1, 2, 4, 6, 9, and 21 dpi or days postexposure (dpe). Temperature, heart rate, and respiratory rate were recorded twice a day, in the morning and the evening; in addition, clinical observations were recorded in the morning. Lung auscultation scores on the left and right side of each animal were recorded. Lung auscultation scores ranged from 1 (normal) to 10 (severely abnormal). A complete blood count (CBC) was performed on blood obtained at each collection point by Diagnostic Laboratory Services, College of Veterinary Medicine, Mississippi State University.

To assess the IDV-induced pathology, one calf from the treatment group was euthanized at 4 dpi and one calf at 6 dpi, and one calf from the control group on each day was also euthanized. Necropsies were performed, and the tissue samples collected included lung tissues from different locations (left cranial lung [LCR], left caudal lung [LCD], right cranial lung [RCR], right caudal lung [RCD], right middle lung [RMD], and right accessory [RA]), trachea (upper trachea [U-TR], middle trachea [M-TR], and distal trachea [D-TR]), bronchus (BR), and turbinate (ethmoid turbinate [Nasal Eth Tur], dorsal turbinate [Nasal Dor], middle turbinate [Nasal Mid], and ventral turbinate [Nasal Ven]). To quantify IDV, 1 mg of each tissue was weighed, homogenized, and frozen and thawed three times prior to RNA extraction. Another set of tissues were fixed in 10% neutral formalin and embedded in paraffin, followed by sectioning at 5 μm and staining with hematoxylin and eosin (H&E). Nasal Eth Tur was used for viral titration only.

Transmission experiments between cattle and ferrets. To test the potential transmission of IDV from cattle to ferrets, four ferrets were separated into two groups, one contact group and one control group. Each ferret was housed in a separate cage. A ferret plastic ball toy was used as a fomite and soaked with nasal discharge from the external nostrils of one calf (at 3 dpi) in the treatment group and then placed into the ferret cages so that the ferrets could be exposed to the fomite. The fomite was kept in a sterile plastic bag and on ice after sampling and spent no more than 20 min on ice between sampling and exposure to the ferrets. Observations were made to confirm that the ferrets had an opportunity to play with the fomite. The fomite stayed in the cage for 24 h and was replaced daily with a new fomite with fresh cattle nasal discharge. The control ferrets were exposed to a fomite soaked with sterile PBS buffer. Nasal discharge and fecal swabs were collected daily from the ferrets, and the fomite was replaced daily for 8 days. Nasal washes were collected from the ferrets at 3, 5, 7, and 10 dpe. Blood was obtained from each ferret at 7, 10, and 21 dpe to determine seroconversion to IDV. Clinical signs, weight, and temperature were recorded daily.

IHC assay. To confirm the presence of virus in cattle tissues, an immunohistochemistry (IHC) assay was performed. Briefly, tissue sections (5 μm) were held at 65°C overnight. The slides were deparaffinized and retrieved in antigen retrieval solution, pH 6.0 (Dako, Carpinteria, CA). The slides were washed in Tris-buffered saline (TBS)–0.5% Tween prior to quenching endogenous peroxidase activity in 3% H_2O_2 and rinsed in sterile water, followed by washing in TBS-0.5% Tween. The slides were blocked with 10% normal goat serum (Invitrogen, Carlsbad, CA) for 1 h, followed by incubation with D/bovine/Mississippi/C00046N/2014 ferret antiserum at 1:50 dilution for 24 h at 4°C in antibody diluent (Dako, Carpinteria, CA). Following incubation, the slides were rinsed in TBS-0.5% Tween and incubated with a biotinylated goat anti-ferret IgG polyclonal secondary antibody diluted in TBS-0.5% Tween at 1:500 for 30 min. The slides were washed and incubated in ABC reagent (Vectastain, Burlingame, CA) according to the manufacturer's instructions. The slides were then dehydrated, counterstained with hematoxylin, and coverslipped.

Histologic tracheal inflammation score. A histologic scoring system was developed to assess neutrophil infiltration within the tracheal epithelium. Scoring was performed on 3 sections of trachea at different locations

TABLE 1 Serological responses in experimental cattle using HAI assay against D/bovine/C00046N/Mississippi/2014

Group	Calf no. (pen no.)	Titer at day postinoculation or postexposure[a]:							
		0	1	2	4	6	9	13	21
Treatment	24 (I)	<10	<10	<10	<10	80	320	NA	640
	26 (III)	<10	<10	<10	<10	20	NA	NA	NA
	30 (II)	<10	<10	<10	<10	NA	NA	NA	NA
Contact	18 (I)	<10	<10	<10	<10	<10	20	320	320
	27 (II)	<10	<10	<10	<10	<10	<10	160	320
	29 (III)	<10	<10	<10	<10	<10	10	40	160

[a] NA, not done because sera were not available for HAI assay.

(upper, middle, and distal) and 1 section of bronchus from each calf. For each tissue section, 10 ×400-magnification fields were examined and scored as follows: 0, no neutrophils; 1, 1 to 10 neutrophils; or 2, >10 neutrophils per ×400 field. Each tracheal area (upper, middle, and distal) and bronchus was scored, and the 10 scores for each of the four tissue groups were averaged. Statistical analysis was performed by Mann-Whitney test.

Statistical analyses of clinical data. The effect of IDV exposure on the heart rate, respiratory rate, rectal temperature, $\log_{10}$ virus titer, and white blood cell parameters was tested by general linear mixed models with random intercept and repeated measures of subject (SAS 9.4, Cary, NC) for 1 to 9 dpe. A first-order autoregressive correlation structure was defined.

RESULTS

Infected calves can shed and seroconvert in response to IDV inoculation. To determine the susceptibility of calves to IDV infection, we infected IDV-seronegative animals and monitored them for seroconversion and viral shedding over the course of 21 days. Serological characterization showed that the two infected treatment calves tested at 6 dpi had already seroconverted, with HAI titers of 1:20 and 1:80. We continued to monitor one of the animals, and the titers rose to 1:320 at 9 dpi and 1:640 at 21 dpi, at which time the calf was euthanized (Table 1). To determine the time course of viral shedding we used quantitative RT-PCR to quantify the viral PB1 gene and the $TCID_{50}$ to quantify viral titers. Nasal swabs collected from the infected treatment animals from 1 to 6 dpi were IDV positive (Tables 2 and 3). However, all rectal swabs and environmental samples (feces and hay) were IDV negative (data not shown). The results from quantitative RT-PCR showed that a subset of tissues collected at 4 dpi were IDV positive, including the Nasal Eth Tur, Nasal Mid, Nasal Dor, Nasal Ven, D-TR, M-TR, U-TR, BR, and RMD, all of which except Nasal Mid were confirmed to be IDV positive in $TCID_{50}$ titration (Table 4). The highest $\log_{10}$ copy number of viral RNA was found in the Nasal Eth Tur (7.303 ± 0.058), whereas the lowest $\log_{10}$ copy number of viral RNA was found in the RMD. Viral titers decreased in tissues collected from the upper (nasal cavity to trachea) to the lower (bronchus and right medial lung lobe) respiratory tract. Likewise, the highest $\log_{10}$ $TCID_{50}$ titer was found in the Nasal Eth Tur (4.584 ± 0.118); however, the RMD had the second highest $\log_{10}$ $TCID_{50}$ titer (3.916 ± 0.589), followed by Br, Nasal Ven, L-Tr, M-Tr, U-Tr, and Nasal Dor (Table 4). A small subset of tissues, including the Nasal Eth Tur, Nasal Mid, Nasal Dor, and Nasal Ven, collected at 6 dpi were IDV positive, but none had a detectable $TCID_{50}$ titer (Table 4).

Calves in the control group had no viral shedding or seroconversion, and their tissues were negative for IDV.

Transmission between calves through contact is possible after inoculation with IDV. If cattle are indeed a natural reservoir for IDV, the animals not only must be able to be infected, they must also be able to transmit virus. To assess this, we introduced IDV-seronegative contact animals to the infected calves 24 h postinoculation. All three contact animals seroconverted, showing that IDV was readily transmitted from animal to animal. Two of the contact calves seroconverted at 9 dpe with HAI titers of 1:20 and 1:10. The third animal seroconverted at 13 dpe with an HAI titer of 1:160 (Table 1). Viral titration of nasal swabs showed that the calves shed from 6 dpe until at least 9 dpe (Tables 2 and 3). Virus was not detected in rectal swabs collected from the contact calves.

IDV caused mild disease in experimentally infected cattle. We have previously shown that IDV detection rates are higher in clinically sick cattle than in apparently healthy cattle, suggesting

TABLE 2 Titration of viral shedding in treatment and in-contact cattle using quantitative RT-PCR

Day postinoculation or postexposure	$\log_{10}$ (copies of virus) (±SD)[a]					
	Pen I		Pen II		Pen III	
	Treatment (calf 24)	Contact (calf 18)	Treatment (calf 30)	Contact (calf 27)	Treatment (calf 26)	Contact (calf 29)
1	4.781 ± 0.084	ND	5.998 ± 0.032	ND	5.010 ± 0.058	ND
2	5.867 ± 0.046	ND	7.139 ± 0.055	ND	6.763 ± 0.039	ND
4	6.705 ± 0.053	ND	7.210 ± 0.047	ND	7.684 ± 0.017	ND
6	5.089 ± 0.056	5.884 ± 0.147	NA	7.389 ± 0.047	ND	7.289 ± 0.062
9	ND	5.594 ± 0.053	NA	6.545 ± 0.027	NA	6.898 ± 0.021
21	ND	ND	NA	ND	NA	ND

[a] ND, not detected; NA, not available (the calf was euthanized).

TABLE 3 Titration of viral shedding in treatment and in-contact cattle using $TCID_{50}$

Day postinoculation or postexposure	Log_{10} ($TCID_{50}$) (±SD)[a]					
	Pen I		Pen II		Pen III	
	Treatment (calf 24)	Contact (calf 18)	Treatment (calf 30)	Contact (calf 27)	Treatment (calf 26)	Contact (calf 29)
1	2.556 ± 0.193	ND	3.055 ± 0.481	ND	2.278 ± 0.255	ND
2	4.000 ± 0.000	ND	3.00 ± 0.333	ND	2.423± 1.386	ND
4	3.616 ± 0.544	ND	3.167 ± 0.289	ND	3.389 ± 0.096	ND
6	3.222 ± 0.192	3.855 ± 0.326	NA	3.334 ± 0.472	ND	4.417 ± 0.118
9	3.556 ± 0.385	1.542 ± 0.938	NA	4.556 ± 0.419	NA	3.778 ± 0.385
21	ND	ND	NA	ND	NA	ND

[a] ND, not detected; NA, not available (the calf was euthanized).

that the virus can cause respiratory disease in this host. To assess this further, we monitored the clinical symptoms in infected calves. Overall, clinical signs were minimal in IDV-infected cattle. Among the calves in the treatment group, one calf was noticed dry coughing at 2 dpi and had nasal discharge by 5 dpi, which was consistent with viral shedding patterns described previously. A second calf was noted to be depressed and had a lung auscultation score of 1 at 2 dpi while the calf was shedding virus, and the third treatment calf had no clinical signs despite shedding virus. Among the calves in the contact group, one had an auscultation score of 1 at 4 dpe with nasal discharge at 9 dpe, although we did not detect virus until 6 dpe. A second calf in the contact group had mucoid nasal discharge at 9 dpe and serous ocular discharge at 13 dpe, and the third calf had mucoid nasal discharge from both nostrils at 9 and 10 dpe. The three calves in the control group displayed no clinical signs, showing that IDV was likely responsible for the respiratory symptoms seen. The total white blood cell, lymphocyte, monocyte, and neutrophil counts were averaged based on the treatment group and sampling date and plotted over dpi/dpe to determine statistical significance (Fig. 1). Segmented neutrophils significantly differed across treatments and time postexposure ($P = 0.0139$). Other measures of clinical severity, such as heart rate, respiratory rate, rectal temperature, total white cell count, and lymphocyte counts, did not differ statistically between IDV-infected and control calves.

IDV caused tracheal inflammation in calves. To further examine the pathological consequences of IDV infection, H&E-stained tissue sections from the nasal turbinates, trachea, bronchus, and lung were examined. Tracheal inflammation was significantly elevated in IDV-infected calves and was characterized by multifocal areas of epithelial neutrophil infiltration and mild epithelial attenuation (Fig. 2A). Tracheal tissues were scored for neutrophil infiltration, and infected calves had a significant increase in neutrophils within the tracheal epithelium at 4 dpi and 6 dpi compared to control animals (P values of 0.026 and 0.028 for 4 dpi and 6 dpi, respectively) (Fig. 2B). In the lung, multifocal large airways contained small numbers of degenerate neutrophils and mucus within the lumen, regardless of treatment group. There was no evidence of pulmonary pathology in treated or control calves.

IHC was performed on all the tissue sections from two treatment calves (calves 30 and 26) and two control calves (calves 25 and 31). A subset of tissues from the 4-dpi treatment calf (calf 30) had IDV-immunoreactive cells via IHC (Table 4). All locations of trachea and nasal turbinates had scattered to clustered immunopositive epithelial cells (Fig. 2C). Antigen was also detected in the

TABLE 4 Viral detection in tissues from treatment calves[a]

Tissue	Log_{10} (copies of virus) (±SD)		Log_{10} ($TCID_{50}$) (±SD)		IDV detection via IHC[b]	
	Calf 30 (4 dpi)	Calf 26 (6 dpi)	Calf 30 (4 dpi)	Calf 26 (6 dpi)	Calf 30 (4 dpi)	Calf 26 (6 dpi)
Nasal Eth Tur	7.303 ± 0.058	5.031 ± 0.008	4.584 ± 0.118	ND	NA	NA
Nasal Mid	6.677 ± 0.029	4.631 ± 0.061	ND	ND	+	ND
Nasal Dor	6.305 ± 0.005	4.117 ± 0.026	1.667 ± 0.577	ND	+	ND
Nasal Ven	6.114 ± 0.020	3.126 ± 0.054	3.500 ± 0.707	ND	+	ND
U-TR	5.014 ± 0.037	ND	1.855 ± 0.742	ND	+	ND
M-TR	5.144 ± 0.032	ND	2.167 ± 0.289	ND	+	ND
L-TR	5.313 ± 0.044	ND	2.444 ± 0.096	ND	+	ND
BR	5.753 ± 0.035	ND	3.556 ± 0.096	ND	+	ND
RMD	4.655 ± 0.036	ND	3.916 ± 0.589	ND	ND	ND
RCR	ND	ND	ND	ND	ND	ND
LCR	ND	ND	ND	ND	ND	ND
RCD	ND	ND	ND	ND	ND	ND
LCD	ND	ND	ND	ND	ND	ND
RA	ND	ND	ND	MD	ND	ND

[a] The viral PB1 genes were quantified using quantitative RT-PCR, and the copy numbers were determined based on standard curves (data not shown). ND, not detected; NA, not available (the calf was euthanized).
[b] +, present.

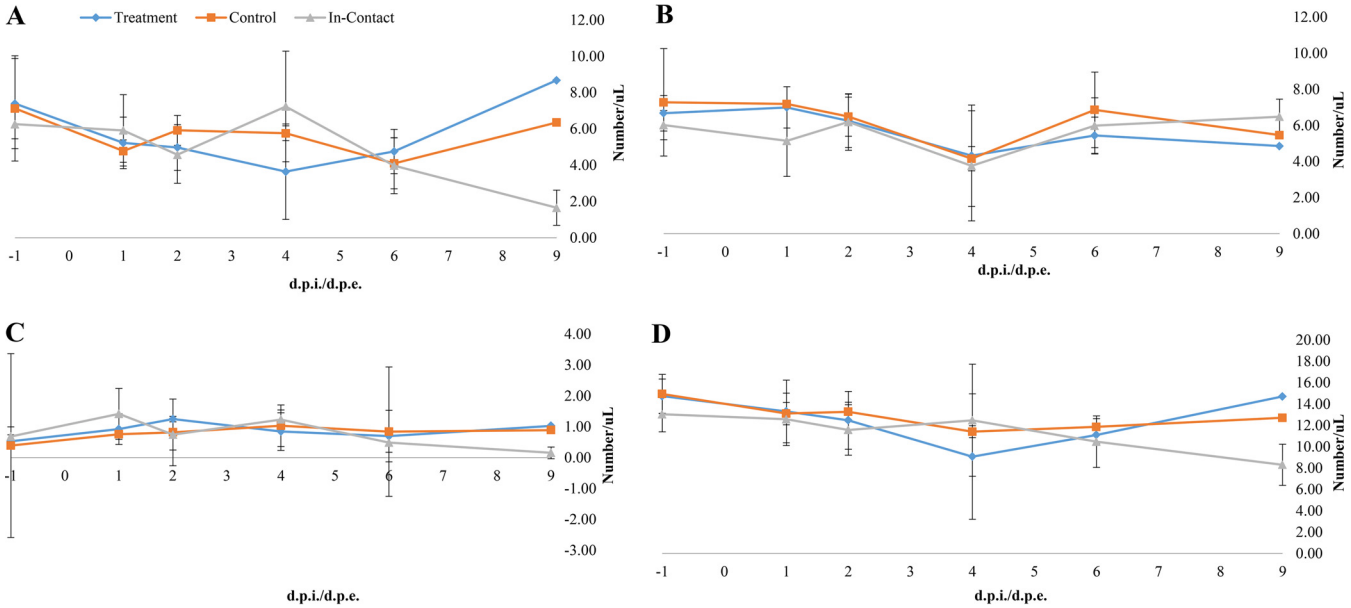

FIG 1 Complete blood count data for experimental calves. (A) Neutrophils. (B) Lymphocytes. (C) Monocytes. (D) Total white blood cells. Each data point for each treatment group includes three calves, with only two treatment calves and two control calves remaining at 6 dpi and only one treatment calf and one control calf remaining at 9 dpi Each sample was analyzed in triplicate. The error bars represent 1 standard deviation from the replicates at each data point.

bronchus; however, no antigen was detected in any of the lung samples. All tissues from the 6-dpi treatment calf (calf 26) and control calves (calves 25 and 31) were IDV negative via IHC.

IDV was not transmitted from cattle to ferrets via fomites. To assess the threat of zoonotic transmission from IDV-infected calves, we exposed two ferrets to a fomite soaked with nasal discharge from the IDV-infected calves (at a time when shedding was evident). This setup was used to mimic possible exposure from a contaminated surface, as might be expected in an occupational setting (e.g., a milking shed). Despite the ferrets' interactions with the fomite, IDV was not detected in any of the ferret nasal washes, and no seroconversion was detected. The

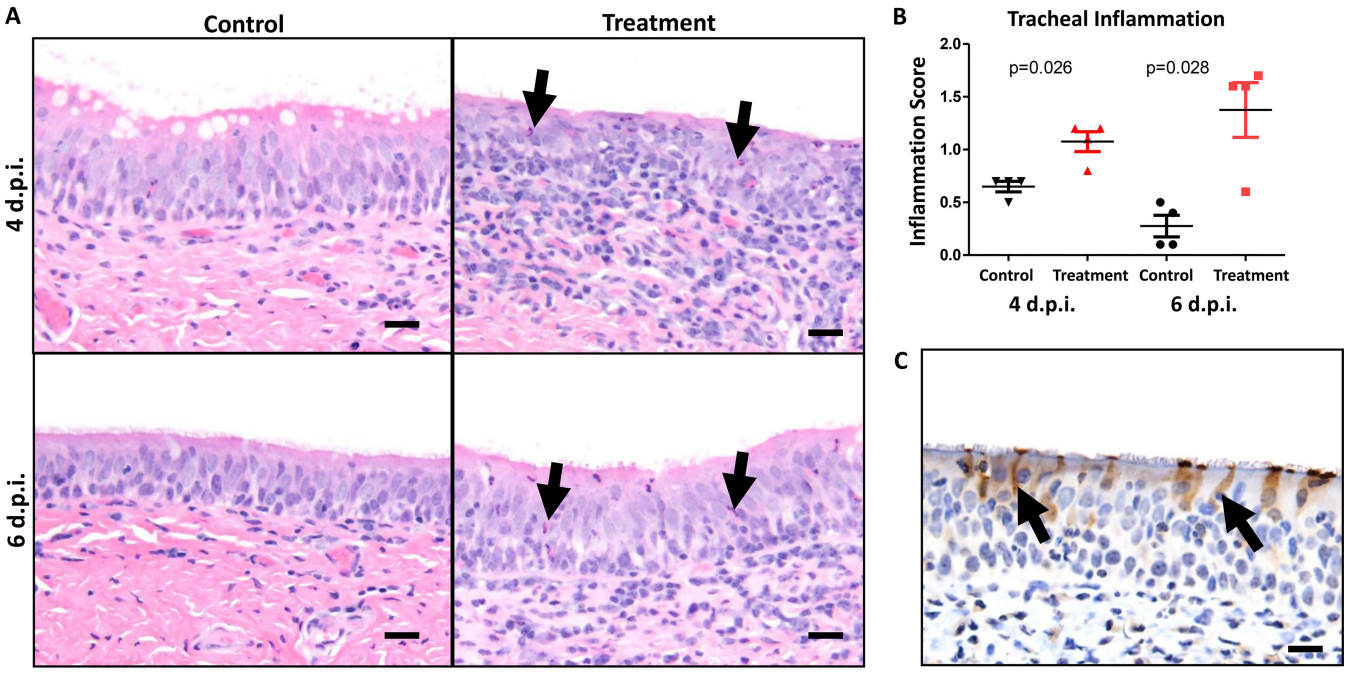

FIG 2 (A) H&E-stained tracheal sections demonstrating neutrophil migration (arrows) within the epithelium and submucosa at 4 and 6 dpi. (B) Tracheal neutrophil inflammation scores were significantly higher in tracheal sections from the treatment calf than in those from the contact calf at both 4 and 6 dpi. (C) Detection of IDV antigen at 4 dpi by immunohistochemistry demonstrating that multifocal tracheal epithelial cells are immunoreactive (brown staining; arrows). The horizontal line and error bar denote the mean and standard deviation of inflammation scores. Bars, 25 μm.

two ferrets in the control group were also negative for IDV on serology and viral titration.

DISCUSSION

The goal of this study was to understand the pathogenesis of IDV in IDV-seronegative calves and to better understand our previous findings that IDV was more likely to be detected in symptomatic animals in a field setting. Our data clearly demonstrate that IDV can readily infect cattle and can be transmitted efficiently between cattle through direct contact. Although the resulting disease was mild, the infected animals did show signs of respiratory disease and associated inflammation in their respiratory tracts. IDV infection in IDV-seronegative calves induced multifocal mild tracheal epithelial attenuation and neutrophil infiltration. It has been shown that neutrophils are important for guiding influenza virus-specific $CD8^+$ T cells in the airway (10). IDV was detected in nasal turbinate, trachea, bronchus, and right medial lung lobe tissues collected at 4 dpi, and IHC confirmed the presence of IDV antigen in all the tissues except RMD. In other animal models, IDV was shown to replicate within the nasal turbinate; could be detected in nasal washes from infected swine, ferret, and guinea pig; and could also be transmitted through direct contact among members of the same species (1, 11). We were able to detect IDV in the right medial lung lobe at 4 dpi via both quantitative RT-PCR and $TCID_{50}$, but no antigen was seen via IHC and there was no evidence of pulmonary pathology, again suggestive of an upper respiratory tract infection. Our results are different from those of another challenge study of IDV in 3-day-old gnotobiotic calves, which showed a high titer of IDV in the lungs with notable histopathological findings (S.-K. Welch, U. S. patent application no. PCT/US2013/050982). The age of cattle used in the experiments could account for such a discrepancy, and it is also notable that our calves were naturally born and given thawed colostrum at birth. On the other hand, our data are similar to swine and ferret IDV experimental infection models but unlike an IDV guinea pig model in which the highest viral titer was found in lung tissue despite intranasal inoculation (11). Structural modeling suggested that, similar to ICV, IDV binds to 9-*O*-acetyl sialic acid (1). It is likely that the distributions of this receptor in the various model species are different and that this is, at least in part, responsible for the differences in viral load and distribution.

Consistent with the mild symptoms displayed, epithelial damage was minimal in IDV-infected calves. This result conflicts somewhat with our surveillance findings and those of others (3, 4, 6, 7) showing that IDV associates with disease in the field. We hypothesize that the minimal amounts of damage seen in experimentally infected calves might induce inflammation (IDV infection induced neutrophil extravasation and migration into tracheal epithelium in our infected animals) that could facilitate coinfections with other bovine pathogens. IDV would thus be an important part of BRD but not alone sufficient to cause disease in the absence of secondary or concurrent infections. It is well known that BRD is a multifactorial respiratory infection marked by primary viral pathogens followed by bacterial colonization in the upper and lower respiratory tract. Similarly, a recent metagenomic study showed that bovine adenovirus 3, bovine rhinitis A virus, and IDV were the top three viruses associated with BRD, either alone or in coinfections, in dairy calves in California, which would be consistent with a hypothesis of IDV predisposing to or exacerbating disease (5). Of course, it is also likely that other conditions, such as inclement weather, transportation, and close housing, might contribute to the more severe IDV-induced disease in the field.

Other factors contributing to differences in disease might be infection routes and doses. We did note that calves infected through contact had slightly enhanced clinical signs compared to the directly inoculated animals. Although these animals were in direct contact, it is possible that they were infected through aerosol droplets or contaminated fomites. Further experimental infections will shed light on this. It is also possible that the virus gained host adaptations upon transmission that could have facilitated more efficient replication. Phenotypic changes in influenza viruses due to adaptation to the host have been shown in IAV studies in feral swine, domestic swine, and mice and have also been demonstrated for IDV in the guinea pig transmission model (11–14). Future studies will characterize the pathogenesis in the contact calves and potentially identify mutations in the isolates recovered from the contact calves.

Like the other three influenza viruses (IAV, IBV, and ICV), IDV can infect both swine and ferrets (1). Ferrets have a respiratory system similar to that of humans and are widely used in influenza studies (15). Because IDV was first identified only in 2011, little is known about the virus despite its cellular and host tropisms (1, 2, 8, 11). Studies have highlighted the need for understanding the host tropism and the zoonotic potential of IDV. The ferret model in this experiment suggests that IDV cannot be readily transmitted between cattle and ferrets through fomites, and therefore, zoonotic risk from cattle is likely to be minimal. However, a limitation of this study was that the fomite IDV titers and environmental viability of IDV on the fomite were not measured.

In summary, this study demonstrated that IDV caused upper respiratory tract lesions and mild respiratory disease in cattle and that IDV can be transmitted effectively between cattle, but not from cattle to ferrets through fomite exposure. These findings highlight the need for continued surveillance and risk assessment of this emerging virus.

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REFERENCES

1. **Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, Armien A, Kaplan B, Chakravarty S, Hoppe AD, Webby RJ, Simonson RR, 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. http://dx.doi.org/10.1371/journal.ppat.1003176.

2. **Hause BM, Collin EA, Liu R, Huang B, Sheng Z, Lu W, Wang D, Nelson EA, Li F.** 2014. Characterization of a novel influenza virus in cattle and swine: proposal for a new genus in the Orthomyxoviridae family. mBio **5:**e00031-00014. http://dx.doi.org/10.1128/mBio.00031-14.
3. **Collin EA, Sheng Z, Lang Y, Ma W, Hause BM, Li F.** 2015. Cocirculation of two distinct genetic and antigenic lineages of proposed influenza D virus in cattle. J Virol **89:**1036–1042. http://dx.doi.org/10.1128/JVI.02718-14.
4. **Ferguson L, Eckard L, Epperson WB, Long LP, Smith D, Huston C, Genova S, Webby R, Wan XF.** 2015. Influenza D virus infection in Mississippi beef cattle. Virology **486:**28–34. http://dx.doi.org/10.1016/j.virol.2015.08.030.
5. **Ng TF, Kondov NO, Deng X, Van Eenennaam A, Neibergs HL, Delwart E.** 2015. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. J Virol **89:**5340–5349. http://dx.doi.org/10.1128/JVI.00064-15.
6. **Ducatez MF, Pelletier C, Meyer G.** 2015. Influenza D virus in cattle, France, 2011-2014. Emerg Infect Dis **21:**368–371. http://dx.doi.org/10.3201/eid2102.141449.
7. **Jiang WM, Wang SC, Peng C, Yu JM, Zhuang QY, Hou GY, Liu S, Li JP, Chen JM.** 2014. Identification of a potential novel type of influenza virus in bovine in China. Virus Genes **49:**493–496. http://dx.doi.org/10.1007/s11262-014-1107-3.
8. **Quast M, Sreenivasan C, Sexton G, Nedland H, Singrey A, Fawcett L, Miller G, Lauer D, Voss S, Pollock S, Cunha CW, 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. http://dx.doi.org/10.1016/j.vetmic.2015.09.005.
9. **Reed LJ, Muench H.** 1938. A simple method of estimating fifty percent endpoints. Am J Epidemiol **27:**5.
10. **Lim K, Hyun YM, Lambert-Emo K, Capece T, Bae S, Miller R, Topham DJ, Kim M.** 2015. Neutrophil trails guide influenza-specific CD8(+) T cells in the airways. Science **349:**aaa4352. http://dx.doi.org/10.1126/science.aaa4352.
11. **Sreenivasan C, Thomas M, Sheng Z, Hause BM, Collin EA, Knudsen DE, Pillatzki A, Nelson E, Wang D, Kaushik RS, Li F.** 2015. Replication and transmission of the novel bovine influenza D virus in a guinea pig model. J Virol **89:**11990–12001. http://dx.doi.org/10.1128/JVI.01630-15.
12. **Sun H, Cunningham FL, Harris J, Xu Y, Long LP, Hanson-Dorr K, Baroch JA, Fioranelli P, Lutman MW, Li T, Pedersen K, Schmit BS, Cooley J, Lin X, Jarman RG, DeLiberto TJ, Wan XF.** 2015. Dynamics of virus shedding and antibody responses in influenza A virus-infected feral swine. J Gen Virol **96:**2569–2578. http://dx.doi.org/10.1099/jgv.0.000225.
13. **Mase M, Tanimura N, Imada T, Okamatsu M, Tsukamoto K, Yamaguchi S.** 2006. Recent H5N1 avian influenza A virus increases rapidly in virulence to mice after a single passage in mice. J Gen Virol **87:**3655–3659. http://dx.doi.org/10.1099/vir.0.81843-0.
14. **Wei K, Sun H, Sun Z, Sun Y, Kong W, Pu J, Ma G, Yin Y, Yang H, Guo X, Chang KC, Liu J.** 2014. Influenza A virus acquires enhanced pathogenicity and transmissibility after serial passages in swine. J Virol **88:**11981–11994. http://dx.doi.org/10.1128/JVI.01679-14.
15. **Belser JA, Katz JM, Tumpey TM.** 2011. The ferret as a model organism to study influenza A virus infection. Dis Model Mech **4:**575–579. http://dx.doi.org/10.1242/dmm.007823.
16. **Sission S, Grossman JD.** 1953. The anatomy of domestic animals, 4th ed. W. B. Saunders, Philadelphia, PA.
17. **Dyce KM, Sack WO, Wensing CJG.** 2002. Textbook of veterinary anatomy, 3rd ed. Elsevier Saunders, Philadelphia, PA.
18. **Cmielewski P, Farrow N, Donnelley M, McIntyre C, Penny-Dimri J, Kuchel T, Parsons D.** 2014. Transduction of ferret airway epithelia using a pre-treatment and lentiviral gene vector. BMC Pulm Med **14:**183. http://dx.doi.org/10.1186/1471-2466-14-183.
19. **Amiri MH, Gabella G.** 1988. Structure of the guinea-pig trachea at rest and in contraction. Anat Embryol **178:**389–397. http://dx.doi.org/10.1007/BF00306045.