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Serological evidence for the presence of influenza D virus in small ruminants

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Abstract

Influenza D virus (FLUDV) was isolated from diseased pigs with respiratory disease symptoms in 2011, and since then the new virus has also been spread to cattle. Little is known about the susceptibility of other agricultural animals and poultry to FLUDV. This study was designed to determine if other farm animals such as goats, sheep, chickens, and turkey are possible hosts to this newly emerging influenza virus. 648 goat and sheep serum samples and 250 chicken and turkey serum samples were collected from 141 small ruminant and 25 poultry farms from different geographical locations in the United States and Canada. Serum samples were examined using the hemagglutination inhibition (HI) assay and the sheep and goat samples were further analyzed using the serum neutralization assay. Results of this study showed FLUDV antibodies were detected in 13.5% (17/126) of the sampled sheep farms, and 5.2% (29/557) of tested sheep serum samples were positive for FLUDV antibodies. For the goat results, the FLUDV antibodies were detected in 13.3% (2/15) of the sampled farms, and 8.8% (8/91) of the tested goat serum samples were positive for FLUDV antibodies. Furthermore, all tested poultry serum samples were negative for FLUDV antibodies. Our data demonstrated that sheep and goat are susceptible to FLUDV virus and multiple states in U.S. have this virus infection already in these two species. This new finding highlights a need for future surveillance of FLUDV virus in small ruminants toward better understanding both the origin and natural reservoir of this new virus.

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Keywords

Influenza virus; type D; serology; sheep; goats; chicken; turkey

1. Introduction

Influenza viruses are divided into three types: A, B, and C, and this classification is based on their antigenic differences in the matrix and nucleoprotein (Palese and Shaw, 2007; Treanor, 2000). We recently identified a novel influenza virus with bovine as a primary reservoir. Phylogenetic analysis suggests that it is most closely related to influenza C (FLUCV), rather than to influenza A (FLUAV) and influenza B (FLUBV) viruses. However, the distance between the new virus and FLUCV is similar to the differences between FLUAV and FLUBV for most of the genomic segments (Hause et al., 2013). In addition to bovine, this new virus is also isolated from U.S. swine exhibiting severe influenza-like illness as well as from diseased cattle in France and China (Ducatez et al., 2015; Jiang et al., 2014). Its intercontinental transmission and prevalence in both cattle and swine highlight its potential threat to other agricultural animals and humans. We recently proposed that this new group of viruses represents a new genus, designated influenza D, in *Orthomyxoviridae* family. Henceforth, we refer to this virus as influenza D virus (FLUDV) (Hause et al., 2014).

Since FLUDV was discovered, pigs, cows, ferrets, and guinea pigs have been found susceptible to the virus (Hause et al., 2014; Hause et al., 2013). Humans and multiple animal species are susceptible to influenza virus; therefore, other potential hosts of this virus need to be determined. The primary objective of this study is to investigate the seroprevalence of FLUDV in agricultural animals such as small ruminants (sheep and goats) and poultry (chicken and turkey) by conducting a serological survey.

2. Materials and Methods

2.1. Cell Culture and Virus Production

Swine Testicular (ST) cells (ATCC CRL-1746) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (PAA Laboratories Inc., Dartmouth, MA, USA) and 1% penicillin and streptomycin (Life Technologies, Carlsbad, CA, USA). Influenza D/bovine/Oklahoma/660/2013 (D/660) and D/swine/Oklahoma/1334/2011 (D/OK) were previously isolated from bovine or swine with respiratory disease symptoms. The virus was grown on ST cells at 0.01 multiplicity of infection (MOI) and incubated at 37°C with ~5% CO₂ for 5 days. For virus growth/maintenance media, DMEM with 0.1 µg/mL exogenous tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin (Sigma, St. Louis, MO, USA) was used. Virus titer was determined using Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) according to Reed and Meunch's method (Reed and Meunch, 1938).

2.2. Serology

The hemagglutination inhibition (HI) and the microneutralization (MN) assays were performed as described in the WHO standard manual (W.H.O., 2011). Turkey red blood

cells (Lampire Biological Laboratories, Pipersville, PA, USA) were used for the HI assay, while MDCK cells were employed for the MN assay. For the HI assay, an antibody titer of 40 was used as a threshold, i.e., a sample with a titer of less than 40 was judged as negative, and those with a titer equal to or higher than 40 were viewed as positive. For the HI and MN assays, serial 2-fold dilutions of serum sample were tested in duplicate. HI or MN titers were expressed as the reciprocal of the highest dilution of serum that gave complete hemagglutination or 50% neutralization, respectively. All samples were assayed in three separate experiments and the mean antibody titers were calculated from these triplicate data.

2.3. Serum sample collection

250 chicken and turkey serum samples were acquired from Minnesota Poultry Testing Laboratory in Willmar, Minnesota and were taken from 25 poultry farms in Minnesota and Iowa in April 2014. Among them, 100 samples were chickens and 150 samples were turkeys. A total of 499 serum samples from small ruminants (27 from goats and 472 from sheep) were collected through Animal Disease Research and Diagnostic Laboratory at South Dakota State University (SDSU) from March to September 2014. Goat and sheep farms are located in the Midwest region including South Dakota (SD), Minnesota (MN), Iowa (IA), Nebraska (NE), Missouri (MO), and North Dakota (ND). Washington State University (WSU) at Pullman, Washington, provided an additional 64 goat serum samples and 85 sheep serum samples for this study. Serum samples from WSU were collected from various age groups and breeds of animals from 2001 to 2007, and farms that derived these goat and sheep samples are located in Canada and various states of the United States including California (CA), Montana (MT), North Dakota (ND), Missouri (MO), Illinois (IL), Texas (TX), North Carolina (NC), South Carolina (SC), Maryland (MD), New York (NY), Massachusetts (MA), and Maine (ME).

3. Results and Discussion

Avian species are known hosts and reservoirs of FLUAV. To determine if chickens and turkeys are susceptible to FLUDV, we analyzed 100 serum samples from chickens and 150 from turkeys for the presence of virus-specific antibodies by the standard HI assay. Poultry farms that derived serum samples are located in the Midwest region where we frequently isolated FLUDV from bovine herds. All 250 samples had titers <10 for FLUDV antibody in the HI assay (data not shown). Absence of FLUDV-specific antibody indicated that the tested population was not exposed previously to the virus. On the other hand, it can be envisioned that both chicken and turkey may not be susceptible to this newly emerged influenza virus. Increased serum sample size from poultry with a wide geographical representation should be incorporated into future study if a definitive conclusion is to be drawn.

In addition to cattle, goats and sheep are two major species of domestic ruminants. Considering that FLUDV is widespread in cattle, we hypothesize that goats and sheep are susceptible to FLUDV. To test this hypothesis, we collected 472 serum samples from sheep with various ages and breeds from 111 farms located in multiple Midwest states and tested them in the HI assay. We scored any sera with HI antibody titers equal to or greater than 40

as positive (Trombetta et al., 2014). As seen in Table 1, the HI assay showed that serum specimens from 17 out of 111 (17/111; 15.3%) sheep farms were positive for antibodies to FLUDV, and a total of 29 serum samples (29/472; 6.1%) collected from the 17 positive farms tested positive (HI titers for ≥ 40) for FLUDV specific antibodies. Our data identified that 12 out of 29 (12/29; 41.4%) positive samples had HI antibody titers of 80 to 320. In addition to sheep farms, we surveyed 5 goat farms located in South Dakota and Montana (Table 1). From these 5 farms, 7 out of 27 serum samples were seropositive for FLUDV (7/27; 25.9%).

Despite the overall relatively low seroprevalence of FLUDV in small ruminants especially sheep (6.1%) in this study, serum samples collected from a larger proportion of sheep farms across the Midwest region tested positive for FLUDV antibodies. Moreover, an extremely high percentage of animals with high antibody titers were found in certain farms in the region where FLUDV was frequently isolated from cattle farms. This finding indicates that sheep and goats are susceptible to FLUDV. Also serological evidence indicates that the virus is present in U.S. sheep and goats.

Sheep and goat serum samples used for the above study were confined to the Midwest region and were collected between March and September of 2014. To investigate whether sheep and goats in the other geographical areas of U.S. were exposed to FLUDV and also to estimate the time when these animals were infected, we took advantage of 64 goat samples and 85 sheep samples from farms with more diverse geographical locations including Canada, which were collected and archived by Washington State University. These samples that represented sheep and goat farms with various locations across the west (California) to the east coast (Maine) were collected from 2001 and 2007 (Table 2). Analysis of these archived samples by the HI assay revealed that these samples were all seronegative for FLUDV with the exception of 1 serum sample derived from a Massachusetts-based farm. This positive sample with a HI antibody titer of 80 was collected in April 2002 (Table 2). Thus, this result suggested that FLUDV might have circulated in animals as early as 2002. Our previous results have shown that the new virus was present in U.S. swine and cattle herds in 2011 and 2013, respectively (Collin et al., 2014; Hause et al., 2014; Hause et al., 2013). Collection of more archived samples from various species susceptible to FLUDV is needed to reach a definite conclusion in terms of when FLUDV started to infect and transmitted among animals such as cows, pigs, goats, and sheep. On the other hand, the overall lack of FLUDV antibodies in sheep and goats before 2007 compared with positive animals readily found in 2014 appeared to indicate that FLUDV is widespread in recent years. The reason for escalated FLUDV infection over time, if true, clearly merits further investigation.

To confirm the results of the HI assay, the MN assay was performed for a subset of FLUDV positive sera. These positive sera included 8 goat and 7 sheep serum samples (Table 3). Also, FLUDV negative samples were tested which included 14 goat serum samples and 14 sheep serum samples (Table 3). One of the positive goat sera collected in 2002 from a Massachusetts-based farm was also included in MN-based validation. As demonstrated in Table 3, MN assay results further confirmed the positive serum findings from the HI assay. For MN assay, we used a neutralizing antibody titer threshold of ≥ 40 to be considered as

positive (Trombetta et al., 2014). The MN assay appeared to be more sensitive than the HI assay, since the neutralizing antibody titers of positive samples were higher than those obtained with the HI assay. In addition, some of the samples tested negative by HI, showed measurable antibody titer by MN assay. Out of the 28 samples tested negative by HI, 5 samples showed measurable neutralizing antibody titers to be considered positive in the MN assay. This inconsistency is likely due to more sensitive nature the MN assay offers in the antibody detection. Another possibility is that the MN assay could detect other functional antibodies such as those blocking virus-cell fusion that could not be detected by HI assay (Stephenson et al., 2009). In summary, results of the MN assay confirmed the results of the HI assay with the positive samples, but indicated varied results for the negative samples. Based on these MN results, the HI seroprevalence results may be slightly underestimated. Data obtained from the MN assay further demonstrated that FLUDV-specific antibodies were detected in U.S. sheep and goat farms. The data also suggested that FLUDV infection of goats could be traced back as early as in 2002.

Since FLUDV-specific antibodies were found in U.S. sheep and goat farms, we wanted to determine if these species were susceptible to another lineage of FLUDV represented by D/swine/Oklahoma/1334/2011 (D/OK). We analyzed a subset of the original samples (293 out of total 648 serum samples) against D/OK. 293 serum samples that were analyzed for D/OK antibodies included i) a panel of animal serum samples (64 goat and 85 sheep samples) provided by Washington State University (WSU) that was collected from 2001 to 2007 from farms located in Canada and various states of the United States and ii) a panel of animal serum samples (27 goat and 117 sheep samples) collected from the Mid-west region from March-September 2014. It should be noted that all samples tested positive for D/660 (29 sheep and 8 goat samples) were included in this newly added experiment. Interestingly, our results demonstrated that 8/29 sheep tested positive for D/660 antibodies also possessed antibodies against D/OK, while none of the negative sheep samples (for D/660) were positive for D/OK antibodies (data not shown). For goat samples, none of the D/660-positive goat samples had antibodies against D/OK, but two D/660-negative goat samples were positive for D/OK antibodies (data not shown). These results indicate that D/OK or D/OK-like virus, another lineage of influenza D virus, is present in small ruminants. Sheep seem to possess antibodies to both lineages. Furthermore, it also suggests that D/660 is more prevalent than D/OK in small ruminants, which is in good agreement with our bovine data in that D/660 has become widespread. Considering that antibody titers to both lineages in positive animals were similar, it is possible that these animals were previously exposed to an influenza D virus that can induce antibodies, which are cross-reactive to both lineages (D/660 and D/OK).

In summary, we presented serological evidence that small ruminants, sheep and goats, are susceptible to FLUDV. Based on this new observation, these two animal species should be added into host range of this newly emerged influenza virus that include pigs and bovines already. As such, future surveillance for FLUDV in sheep and goats is needed and should be of great interest to investigate both the origin and natural reservoir of this new virus.

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Highlights

- Antibodies to newly emerged influenza D virus were found in sheep and goats.
- Small ruminants are susceptible to influenza D virus infection.
- Sheep and goat farms in multiple states in U.S. have this virus infection.
- This finding suggests that sheep and goat are new hosts of influenza D virus.

Table 1

Summary of the serological survey from the goats and sheep farms in the Midwest by the HI assay.

Animal	Farm	Location	No. of Seropositive ^a for FLUDV ^b	HI Titer Range	Date of Sample Taken
Goat	A	Montana	0/7	0	March 2014
	B	South Dakota	0/2	0	March 2014
	C	South Dakota	0/1	0	March 2014
	D	South Dakota	0/3	0	May 2014
	E	South Dakota	7/14 (77.1) ^c	40-80	May 2014
Sheep ^d	F	South Dakota	1/1 (60)	60	July 2014
	G	South Dakota	1/4 (80)	80	July 2014
	H	Iowa	1/2 (40)	40	July 2014
	I	South Dakota	4/19 (50)	40-80	July 2014
	J	North Dakota	1/1 (40)	40	July 2014
	K	South Dakota	1/3 (40)	40	July 2014
	L	South Dakota	1/2 (160)	160	July 2014
	M	South Dakota	1/1 (80)	80	August 2014
	N	South Dakota	3/20 (60)	40-80	August 2014
	O	South Dakota	1/16 (40)	40	August 2014
	P	South Dakota	1/7 (240)	240	August 2014
	Q	Minnesota	1/5 (320)	320	August 2014
	R	South Dakota	4/4 (65)	40-80	August 2014
	S	South Dakota	2/20 (50)	40-80	August 2014
	T	South Dakota	1/15 (80)	80	August 2014
	U	North Dakota	1/5 (320)	320	September 2014
	V	South Dakota	4/17 (50)	40-80	September 2014

^aData are the number of positive samples out of the total number tested.

^bD/bovine/Oklahoma/660/2013 was used.

^cNumbers in parentheses indicate mean HI titers for samples with a value of 40.

^dData shown are the only positive sheep serum samples.

Table 2

Summary of the serological data from sheep and goat farms with various locations in the U.S. and Canada by the HI assay.

Animal	Farm	Location	No. of Seropositive ^a for FLUDV ^b	Date of Sample Taken
Goat	AA	Canada	0/10	October 2002
	AB	California	0/12	March 2001
	AC	California	0/2	October 2001
	AD	Maine	0/3	July 2001
	AE	Texas	0/1	April 2002
	AF	Massachusetts	1/1 (80) ^c	April 2002
	AG	New York	0/1	October 2002
	AH	South Carolina	0/4	March 2001
	AI	New York	0/12	June 2003
	AJ	Texas	0/16	August 2006
	AK	Missouri	0/2	October 2003
Sheep	AL	California	0/2	July 2001 and August 2001
	AM	California	0/1	July 2002
	AN	New York	0/6	September 2002
	AO	California	0/2	February 2000 and 2003
	AP	Montana	0/9	October 2006
	AQ	Montana	0/27	April 2007
	AR	California	0/3	December 2001
	AS	North Carolina	0/5	March 2002
	AT	Maryland	0/2	December 2002
	AU	New York	0/1	November 2004
	AV	North Dakota	0/4	November 2001
	AW	Illinois	0/3	December 2003
	AX	North Carolina	0/9	March 2002
	AY	Canada	0/9	September 2002
	AZ	Missouri	0/2	October 2003

^aData are the number of positive samples out of the total number tested.

^bD/bovine/Oklahoma/660/2013 was used.

^cNumbers in parentheses indicate mean HI titers for samples with a value of ≥ 40.

Table 3

Comparison of neutralization and HI antibody titers against FLUDV for a subset of serum samples from the U.S. and Canada

Animal	Farm	Location	Antibody titer for FLUDV ^a	
			HI	SN
Goat	AF	Massachusetts	1:80	1:660
	AG	New York	0	0
	AH	South Carolina	0	0
	AI	New York	0	0
	AI	New York	0	0
	AI	New York	0	0
	AI	New York	0	0
	AI	New York	0	0
	E	South Dakota	1:80	1:960
	E	South Dakota	1:80	1:240
	E	South Dakota	1:80	1:1,280
	E	South Dakota	1:80	1:640
	E	South Dakota	1:60	1:320
	E	South Dakota	1:80	1:640
	E	South Dakota	1:80	1:320
	E	South Dakota	0	1:640
	B	South Dakota	0	0
	E	South Dakota	0	1:90
	B	South Dakota	0	0
	A	Montana	0	0
Sheep	AM	California	0	0
	AX	North Carolina	0	0
	AL	California	0	0
	AL	California	0	0
	F	South Dakota	1:80	1:50
	I	South Dakota	1:80	1:80
	G	South Dakota	1:80	1:80
	L	South Dakota	1:80	1:960
	M	South Dakota	1:80	1:240
	N	South Dakota	1:80	1:100
	R	South Dakota	1:80	1:1,280
	P	South Dakota	1:240	1:640
	T	South Dakota	1:80	1:320
	N/S ^b	Montana	0	1:200
	N/S	South Dakota	0	0
	N/S	South Dakota	0	0

Animal	Farm	Location	Antibody titer for FLUDV ^a	
			HI	SN
	N/S	South Dakota	0	0
	N/S	South Dakota	0	0
	N/S	South Dakota	0	1:80
	N/S	South Dakota	0	0
	N/S	South Dakota	0	1:40
	N/S	North Dakota	0	0
	N/S	North Dakota	0	0
	N/S	North Dakota	0	0

^aD/bovine/Oklahoma/660/2013 was used.

^bFarm label not stated (N/S).

Farms A-V: Archived serum samples from SDSU or the 2014 samples.

Farms AA-AZ: Archived serum samples from WSU or the archived samples.