HHS Public Access

Author manuscript

Virology. Author manuscript; available in PMC 2018 January 15.

Published in final edited form as:

Virology. 2017 January 15; 501: 88–91. doi:10.1016/j.virol.2016.11.004.

Serological evidence for high prevalence of Influenza D Viruses in Cattle, Nebraska, United States, 2003–2004

Junrong Luo[1,2,1], **Lucas Ferguson**[2], **David R. Smith**[3], **Amelia R. Woolums**[3], **William B. Epperson**[3], and **Xiu-Feng Wan**[2,*]

[1]College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang 330045, China

[2]Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, United States

[3]Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, United States

Abstract

Influenza D virus (IDV), a new member of the influenza virus family, was first reported in 2011 in swine in Oklahoma, USA, and then soon found in cattle across North America and Eurasia. Earlier studies suggested cattle serve as natural reservoir for IDV. The goal of this study is to perform a retrospective study looking at sera collected from Nebraska beef herds in 2003–2004 and 2014 for evidence of IDV antibodies. Results showed that all 40 randomly selected farms (2003–2004) we tested contained IDV seropositive adult animals and that approximately 98% of newborn calves (2014) had high levels of maternal antibodies against IDV. This study suggested that IDV exposures were present in Nebraska beef cattle since at least 2003.

Keywords

Influenza D virus; Serological surveillance; seroprevalence; Nebraska; cattle; influenza virus

1. Introduction

Since its identification in 2011, influenza D virus (IDV) has been isolated from cattle and/or swine in the United States, China, France, Italy, and Japan, and serologic evidence suggests it may also be affecting small ruminants such as goats and sheep (Chiapponi et al., 2016; Collin et al., 2015; Ducatez et al., 2015; Ferguson et al., 2015; Hause et al., 2014; Hause et al., 2013; Jiang et al., 2014; Murakami et al., 2016; Quast et al., 2015). Laboratory studies demonstrated cattle, swine, ferrets, and guinea pigs are susceptible to IDV infection (Collin

*Correspondence to: Xiu-Feng Wan, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, 240 Wise Center Dr., Mississippi State, MS 39762, United States. wan@cvm.msstate.edu.

[1]JL and LF contributed equally to this study.

et al., 2015; Ferguson et al., 2016; Hause et al., 2014; Hause et al., 2013; Sreenivasan et al., 2015). Serologic assays in two independent studies showed that IDV could potentially infect humans, although seropositivity rates in the studies differed. One of the studies reported 91% seropositivity among 35 persons working with cattle (White et al., 2016), and the other reported only 1% seropositivity among 741 persons with suspected high exposure to IDV (Eckard, 2016). Nevertheless, epidemiologic, serologic, and pathologic studies have suggested cattle are the primary natural reservoir for IDV (Collin et al., 2015; Ducatez et al., 2015; Ferguson et al., 2015; Ferguson et al., 2016; Hause et al., 2014; Jiang et al., 2014).

A previous study in young calves reported that 94% of newborn calves had high levels of maternal antibodies against IDV, which decreased in the next six months, leading to the increasing susceptibility to IDV (Ferguson et al., 2015). Laboratory studies suggested that IDV can be efficiently transmitted in cattle, with viral replications in the upper and lower respiratory tracts (Ferguson et al., 2016). An earlier study suggested that IDV was detected at higher frequency in cattle with bovine respiratory disease (BRD) than healthy cattle (Ferguson et al., 2015), which was consistent with the findings in two metagenomic studies (Mitra et al., 2016; Ng et al., 2015).

Although cattle are proposed as the natural reservoir for IDV, the natural history of IDV and the extent of IDV prevalence in bovine population is not yet clear. Evolutionary analyses of five gene segments suggested that IDV could have diverged from those in influenza C virus, another member in the *Orthomyxoviridae* family, from approximately 300 to over 1,200 years ago (Sheng et al., 2014). A serological study reported that IDV was circulating in Mississippi beef cattle as early as 2004. In this study, we aim to investigate the seroprevalence of IDV among randomly selected beef cattle farms in Nebraska between 2003 and 2004.

2. Materials and Methods

2.1. Viruses

D/bovine/Mississippi/C00013N/2014 (D/13N) and D/bovine/Mississippi/C00046N/2014 (D/46N) used in serological assays were genetically separated into two reported clusters of IDVs, which were also antigenically different (Collin et al., 2015; Ferguson et al., 2015).

2.2. Bovine serum samples

From September 2003 to May 2004, a total of 15,402 bovine serum samples were collected from 73 beef cattle farms, in which the total number of cattle were 20,865, across 42 counties in Nebraska [(Smith et al., 2005), Figure 1]. All cattle were 2 years or older. Using these convenient samples, to evaluate the prevalence of IDV, we randomly selected 40 farms representing the 73 farms sampled (Figure 1). From each farm, we selected 4 to 10 samples for serological testing. A total of 293 serum samples were analyzed for the presence of IDV antibody. If at least one serum sample is positive for each farm, by assuming these herds to be representative of beef cattle farms in Nebraska at the time, we would have 95% confidence that the prevalence of seropositive herds was 91% to 100%.

To evaluate the contemporary situation of IDV in cattle of Nebraska, we collected sera from 242 calves from one farm in the spring of 2014. These sera were collected from the same animals at 1 week post-birth, and again at approximately 3 months later. Measurement of the presence of IDV antibody in these paired sera can help evaluate the status of maternal antibodies against IDV in these sera thus the status of IDV exposure in the bovine herds.

2.3. Hemagglutination (HA), Hemagglutination inhibition (HI) and neutralization inhibition (NI) assays

The HA and HI assays were performed against D/13N and D/46N using 0.5% turkey RBC as described elsewhere (Ferguson et al., 2015). The NI assays were performed against D/46N in HRT-18G cells. Simply, serial dilutions of a serum were prepared and mixed with an equal volume of 100 TCID50 influenza virus. Virus and diluted serum were incubated for 1 hour at 37°C, and 200μL of mixture were transferred to a 96-well cell culture plate of HRT-18G cells and incubated for 5 days at 37°C with 5% CO2. The viral titers were determined by HA assay as described elsewhere (Ferguson et al., 2015). The highest dilution of serum that prevents HA is called the NI titer of the serum. A serum sample was determined as seropositive when the HI or NI titer ≥ 1:40.

3. Results and Discussion

Results showed that 235 out of 293 (80.2%) bovine serum samples were seropositive against D/13N and that 237 out of 293 (80.9%) against D/46N (Table 1). Overall, there were 240 samples (81.9%) seropositive against D/13N, D/46N, or both. Among the samples we tested from each farm, the HI titers were as high as 1:1280 against at least one of the tested IDVs (Table 2). Interestingly, three samples were seropositive against D/13N but seronegative against D/46N whereas five samples were seropositive against D/46N but negative against D/13N. Among the 232 samples seropositive to both D/13N and D/46N, 80 samples had a higher titer against D/46N and 33 against D/13N, and 119 samples had the same titer. The $\log_2$ difference between the HI titers against D/13N and D/46N for those samples, which did not share the same titer (n = 113), was 1.09 (±0.29) (±standard deviation)], suggesting there were likely two antigenic clusters of IDV circulating in these Nebraska cattle herds (Collin et al., 2015).

Cattle from all of the 40 farms had evidence of IDV exposure in the period between September 2003 to May 2004, and these farms were geographically located across Nebraska (Figure 1). By assuming these herds to be representative of those in Nebraska at the time, we would have 95% confidence that the state prevalence of seropositive herds was 91% to 100%. The seropositive rates varied among farms from 16.7% to 100% against D/13N and from 33.3% to 100% against D/46N.

To confirm the HI titers, neutralization inhibition (NI) assays were performed against two serum samples from each farm, one with the highest HI titer and one with the lowest. Results showed that 44 out of 50 samples with an HI titer ≥ 1:40 also had a NI titer ≥ 1:40 and that only two samples with a HI titer ≥ 1:40 against D/13N and/or D/46N had a NI titer <1:10 against D/46N (Table 2). Results further confirmed the samples seronegative against D/13N and/or D/46N were seronegative against D/46N in NI assays.

To evaluate the current situation of IDV in cattle of Nebraska, we tested the presence of IDV antibody in paired sera from 242 calves in 2014. These sera were collected from the same animals at 1 week post-birth, and again at approximately 3 months later. HI results showed that 98% of sera samples collected from 1-week-old calves were seropositive against D/46N with a GMT of 1:648, ranging from <1:10 to 1:1280. Three months later, 76% calves remained seropositive against D/46N with a GMT of 1:127, ranging from <1:10 to 1:640. For most calves, the HI titers decreased between one week and three months. Only one calf had an increase in HI titer, which rose from 1:10 to 1:40. These results suggest that these newborn calves have high levels of maternal antibodies against IDV and that IDV is likely to still be prevalent in the beef cattle population in Nebraska.

In addition to the state of Mississippi (MS) (Ferguson et al., 2015) and Oklahoma (OK) (Hause et al., 2013), IDV seems to be present across a number of states in the United States. A serological surveillance study using 141 bovine sera from 8 different farms, from each of which 11 to 27 serum samples were collected, from South Dakota (SD), Vermont (VT), Pennsylvania (PA), Idaho (ID), and California (CA) in the United States, reported 7 out of 8 tested farms were IDV positive (Hause et al., 2014). In addition, IDVs were detected using quantitative PCR in nasal swabs, pharyngeal swabs, or lung tissue samples collected from sick cattle in Kansas (KS), Nebraska (NE), and Texas (TX) (Collin et al., 2015). In addition to beef cattle (Ferguson et al., 2015), using metagenomic approaches, IDV was detected in 62% of 50 samples collected from California dairy calves between 27 and 60 days of age (Ng et al., 2015). This study found that 100% of the beef cattle farms tested in Nebraska had IDV exposure, further demonstrating the high prevalence of IDV infections in the bovine herds in the United States.

Similar to a previous study in Mississippi beef cattle (Ferguson et al., 2015), this study confirmed that Nebraskan beef calves had high levels of maternal antibodies against IDV, which gradually diminished with age. It seems likely that cattle are often seronegative by six months of age, the age when beef cattle are traded and transported to order-buyer facilities or feedlots. The waning of maternal antibodies can allow for a susceptible population of young beef cattle creating a permissive environment for IDV infection at a critical time. Thus, a susceptible population of young beef cattle in the order-buyer facilities or feedlots would allow active IDV transmission and generate the infection cycle and ecology for IDV infection. However, there is still a lack of knowledge regarding the transmission within the cattle herds on individual farms. The virus detection rate using quantitative PCR on the cattle with signs of respiratory disease from individual farms (208 samples from 12 states, primarily those in the Midwest; age unknown) was 4.8% (Collin et al., 2015), which is much lower than in order-buyer facilities (up to 23.8%), where cattle aged 6 to 9 months were sampled (Ferguson et al., 2015).

4. Conclusions

This study demonstrates that IDV was prevalent in Nebraskan cattle herds as early as 2003 and continues to circulate in Nebraska cattle. An earlier report of IDV was able to document the presence of IDV as far back as 2004 in two herds of Mississippi beef cattle, this study demonstrated serologic evidence of IDV in all 40 Nebraska beef herds tested as far back as

2003. Further risk assessment is needed to confirm the impacts of ubiquitous IDV exposure in bovine production, given the fact that bovine respiratory disease is the leading cause of economic loss to the beef industry in the United States, and to clarify what, if any, the potential risk IDV may pose to public health.

Acknowledgments

We are grateful to Kaijian Luo and Hailiang Sun for technical supports. We are also grateful to Liesel Schneider, Kathleen Barton, Bianca Quade, and Hui Wang for helping procure the archived samples. Lucas Ferguson is supported by NIH NIAID 1R15AI107702A-01.

References

Chiapponi C, Faccini S, De Mattia A, Baioni L, Barbieri I, Rosignoli C, Nigrelli A, Foni E. Detection of Influenza D Virus among Swine and Cattle, Italy. Emerg Infect Dis. 2016; 22:352–354. [PubMed: 26812282]

Collin EA, Sheng Z, Lang Y, Ma W, Hause BM, Li F. Cocirculation of two distinct genetic and antigenic lineages of proposed influenza D virus in cattle. J Virol. 2015; 89:1036–1042. [PubMed: 25355894]

Ducatez MF, Pelletier C, Meyer G. Influenza D virus in cattle, France, 2011–2014. Emerg Infect Dis. 2015; 21:368–371. [PubMed: 25628038]

Eckard, LE. Assessment of the Zoonotic Potential of a Novel Bovine Influenza Virus. University of Tennessee Health Science Center; 2016. p. 88

Ferguson L, Eckard L, Epperson WB, Long LP, Smith D, Huston C, Genova S, Webby R, Wan XF. Influenza D virus infection in Mississippi beef cattle. Virology. 2015; 486:28–34. [PubMed: 26386554]

Ferguson L, Olivier AK, Genova S, Epperson WB, Smith DR, Schneider L, Barton K, McCuan K, Webby RJ, Wan XF. Pathogenesis of Influenza D Virus in Cattle. J Virol. 2016; 90:5636–5642. [PubMed: 27030270]

Hause BM, Collin EA, Liu R, Huang B, Sheng Z, Lu W, Wang D, Nelson EA, Li F. Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family. MBio. 2014; 5:e00031–00014. [PubMed: 24595369]

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. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. PLoS Pathog. 2013; 9:e1003176. [PubMed: 23408893]

Jiang WM, Wang SC, Peng C, Yu JM, Zhuang QY, Hou GY, Liu S, Li JP, Chen JM. Identification of a potential novel type of influenza virus in Bovine in China. Virus Genes. 2014; 49:493–496. [PubMed: 25142163]

Mitra N, Cernicchiaro N, Torres S, Li F, Hause BM. 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. 2016

Murakami S, Endoh M, Kobayashi T, Takenaka-Uema A, Chambers JK, Uchida K, Nishihara M, Hause B, Horimoto T. Influenza D Virus Infection in Herd of Cattle, Japan. Emerg Infect Dis. 2016; 22:1517–1519. [PubMed: 27434213]

Ng TF, Kondov NO, Deng X, Van Eenennaam A, Neibergs HL, Delwart E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. J Virol. 2015; 89:5340–5349. [PubMed: 25740998]

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. Serological evidence for the presence of influenza D virus in small ruminants. Vet Microbiol. 2015; 180:281–285. [PubMed: 26414999]

Sheng Z, Ran Z, Wang D, Hoppe AD, Simonson R, Chakravarty S, Hause BM, Li F. Genomic and evolutionary characterization of a novel influenza-C-like virus from swine. Arch Virol. 2014; 159:249–255. [PubMed: 23942954]

Smith, DR.; Schomer, TJ.; Hinkley, S.; Clowser, S.; Galeota, JA.; Weiss, JC.; Akin, KJ. An estimate of the proportion of beef cattle herds with Mycobacterium avium spp. paratuberculosis infected cattle and associated risk factors. Proceedings, 38th Annual Convention of the American Association of Bovine Practitioners; Salk Lake City, UT. 2005. p. 214

Song H, Qi J, Khedri Z, Diaz S, Yu H, Chen X, Varki A, Shi Y, Gao GF. An Open Receptor-Binding Cavity of Hemagglutinin-Esterase-Fusion Glycoprotein from Newly-Identified Influenza D Virus: Basis for Its Broad Cell Tropism. PLoS Pathog. 2016; 12:e1005411. [PubMed: 26816272]

Sreenivasan C, Thomas M, Sheng Z, Hause BM, Collin EA, Knudsen DE, Pillatzki A, Nelson E, Wang D, Kaushik RS, Li F. Replication and Transmission of the Novel Bovine Influenza D Virus in a Guinea Pig Model. J Virol. 2015; 89:11990–12001. [PubMed: 26378161]

White SK, Ma W, McDaniel CJ, Gray GC, Lednicky JA. Serologic evidence of exposure to influenza D virus among persons with occupational contact with cattle. J Clin Virol. 2016; 81:31–33. [PubMed: 27294672]

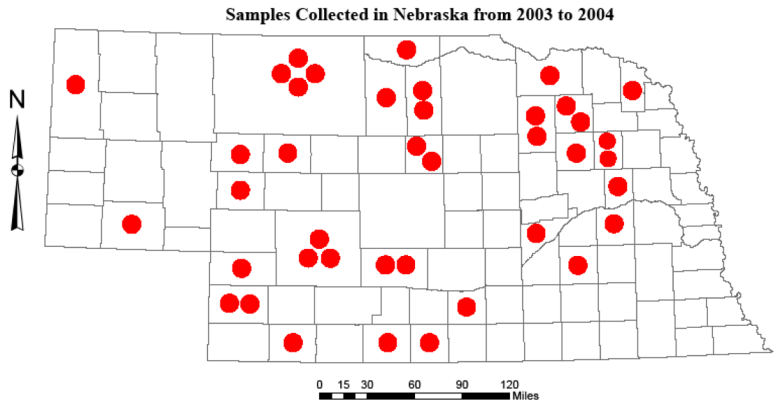

Figure 1.
Geographic distribution of the 40 Nebraska farms where the testing samples were collected (2003–2004).

Virology. Author manuscript; available in PMC 2018 January 15.

Table 1

Cross-reactive antibody responses against D/13N and D/46N in the bovine sera samples from Nebraska (2003–2004).

Farm	Sampling date	D/13N		D/46N		Overall
		GMT(lowest titer-highest titer) [b]	Seropositive (%)[c]	GMT (lowest titer-highest titer)[b]	Seropositive (%)[c]	
J5(10)	10/17/03	160 (40–640)	90.0	246 (160–640)	80.0	90.0
J6(10)	10/17/03	160 (80–320)	80.0	103 (40–160)	80.0	80.0
J7(6)	10/17/03	226 (160–320)	66.7	160 (160–160)	66.7	66.7
J8(6)	10/21/03	160 (160–160)	16.7	113 (40–320)	33.3	33.3
J9(6)	10/21/03	80 (40–160)	66.7	80 (40–160)	50.0	66.7
J10(8)	10/22/03	160 (80–320)	50.0	121 (40–320)	62.5	62.5
J14(10)	10/28/03	160 (80–320)	80.0	146 (40–320)	80.0	80.0
J17(10)	10/31/03	177 (80–320)	70.0	190 (40–320)	80.0	80.0
J18(10)	10/31/03	243 (160–320)	100.0	211 (160–320)	100.0	100.0
J19(8)	10/31/03	215 (80–640)	87.5	215 (80–640)	87.5	87.5
J20(8)	10/31/03	269 (160–320)	100.0	226 (160–320)	100.0	100.0
J23(7)	11/4/03	177 (40–320)	100.0	160 (40–320)	100.0	100.0
J25(10)	11/6/03	173 (40–1280)	90.0	235 (80–640)	90.0	90.0
J26(10)	11/7/03	147 (80–640)	80.0	320 (160–640)	80.0	80.0
J28(7)	11/10/03	143 (80–320)	85.7	180 (80–320)	85.7	85.7
J31(10)	12/5/03	147 (40–320)	80.0	190 (40–320)	80.0	80.0
J33(5)	11/19/03	95 (80–160)	80.0	190.3 (160–320)	80.0	80.0
J34(10)	11/19/03	195 (80–320)	70.0	238 (160–320)	70.0	70.0
J35(10)	12/3/03	226 (40–640)	80.0	177 (80–320)	70.0	70.0
J36(10)	11/21/03	139 (80–320)	50.0	160 (80–320)	50.0	50.0
J37(8)	11/21/03	160 (80–320)	87.5	131 (80–320)	87.5	87.5
J38(10)	11/24/03	215 (80–320)	70.0	173 (40–320)	90.0	90.0
J39(5)	11/26/03	226 (160–320)	80.0	381 (320–640)	80.0	80.0
J45(4)	12/4/03	113 (40–160)	100.0	226 (80–320)	100.0	100.0
J48(9)	12/8/03	173 (40–320)	100.0	173 (40–320)	100.0	100.0
J54(7)	12/22/03	184 (40–640)	71.4	279 (40–640)	71.4	71.4
J55(6)	12/31/03	184 (40–640)	83.3	279 (80–640)	83.3	83.3

Farm	Sampling date	D/13N		D/46N		Overall
		GMT(lowest titer-highest titer) [b]	Seropositive (%)[c]	GMT (lowest titer-highest titer)[b]	Seropositive (%)[c]	
J56(5)	10/23/03	113 (80–160)	80.0	226 (160–320)	80.0	80.0
J57(7)	1/22/04	95 (40–320)	57.1	113 (80–320)	57.1	57.1
J60(6)	2/25/04	320 (320–320)	83.3	368 (320–640)	83.3	83.3
J62(8)	2/27/04	190 (40–640)	100.0	269 (80–640)	100.0	100.0
J63(5)	3/1/04	121 (40–640)	100.0	211 (40–640)	100.0	100.0
J64(6)	3/8/04	243 (160–640)	83.3	243 (160–640)	83.3	83.3
J66(5)	3/12/04	226 (160–320)	80.0	320 (320–320)	80.0	80.0
J67(6)	3/31/04	160 (80–320)	100.0	285 (160–320)	100.0	100.0
J68(5)	4/14/04	269 (160–640)	80.0	381 (320–640)	80.0	80.0
J69(5)	4/22/04	320 (160–640)	80.0	538 (320–640)	80.0	80.0
J70(5)	4/26/04	190 (40–320)	80.0	269 (80–640)	80.0	80.0
J71(5)	5/4/04	279 (80–640)	100.0	320 (160–640)	100.0	100.0
J72(5)	5/21/04	135 (40–320)	80.0	135 (40–320)	80.0	80.0

Note:

[a] The 40 farms listed were selected randomly from 73 farms we sampled in Nebraska;

[b] only those sera with a HI titer of 1:40 or more are used to calculate the GMT and the lowest and highest titers are measured from those sera with a HI titer of 1:40 or more;

[c] seropositive rate was calculated based on those samples whose HI titers ≥1:40 for either D/13N or D/46N;

[d] overall seropositive rates was calculated based on those samples whose HI titers ≥1:40 for D/13N, D/46N, or both.

Table 2

Neutralization assays for the highest and lowest HI titer samples.

Farm ID[a]	Sample ID[b]	HI titer[c]		NI titer[d]
		D/13N	D/46N	D/46N
J5(10)	150	640	640	80
	104	10	20	20
J6(10)	51	320	160	160
	110	20	20	10
J7(6)	23	320	160	40
	177	10	20	10
J8(6)	169	160	320	80
	30	<10	10	10
J9(6)	28	160	160	40
	1	10	20	10
J10(8)	229	320	320	160
	116	<10	<10	<10
J14(10)	85	320	320	160
	33	20	20	10
J17(10)	52	320	320	320
	94	<10	<10	<10
J18(10)	311	320	320	160
	127	160	160	80
J19(8)	156	640	640	160
	85	20	20	<10
J20(8)	143	640	320	160
	64	160	160	80
J23(7)	129	320	320	80
	71	40	40	<10
J25(10)	2	1280	640	320
	119	<10	<10	<10
J26(10)	98	640	640	160
	110	<10	<10	<10
J28(7)	116	320	320	80
	117	20	20	10
J31(10)	230	160	320	80
	217	<10	<10	20
J33(5)	128	80	320	80
	6	20	20	20
J34(10)	136	320	320	320
	179	<10	<10	<10
J35(10)	110	640	320	80
	59	<10	20	10

Farm ID[a]	Sample ID[b]	HI titer[c]		NI titer[d]
		D/13N	D/46N	D/46N
J36(10)	93	160	320	160
	26	<10	<10	<10
J37(8)	93	320	320	80
	122	<10	<10	<10
J38(10)	76	320	320	80
	26	20	40	<10
J39(5)	7	160	320	160
	11	10	20	20
J45(4)	70	160	320	160
	71	40	80	10
J48(9)	208	320	320	160
	180	40	40	20
J54(7)	275	640	640	160
	92	20	10	<10
J55(6)	39	640	640	160
	26	<10	<10	<10
J56(5)	19	160	320	80
	97	20	20	10
J57(7)	140	320	320	80
	23	<10	<10	<10
J60(6)	27	320	640	160
	55	10	20	<10
J62(8)	5	640	640	320
	33	40	80	20
J63(5)	133	640	640	80
	130	40	40	20
J64(6)	14	640	640	320
	101	10	20	20
J66(5)	316	320	320	80
	278	10	20	20
J67(6)	110	320	320	320
	88	80	160	160
J68(5)	9	640	640	160
	133	10	20	10
J69(5)	26	640	640	80
	28	20	20	20
J70(5)	82	320	640	160
	96	<10	<10	<10
J71(5)	72	640	640	320
	15	80	160	40
J72(5)	95	320	320	160

Farm ID[a]	Sample ID[b]	HI titer[c]		NI titer[d]
		D/13N	D/46N	D/46N
	274	<10	<10	<10

Note:

[a]The 46 farms listed were selected randomly from 73 farms in Nebraska;

[b]Two samples with the highest and lowest HI titers were selected for neutralization inhibition assays;

[c]HI assays were performed against two prototype viruses D/13N and D/46N using 0.5% turkey red blood cells;

[d]NI assays were performed in human rectal tumor (HRT-18G) cells against the prototype virus D/46N.