

# Influenza C Virus in Cattle with Respiratory Disease, United States, 2016–2018

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We identified influenza C virus (ICV) in samples from US cattle with bovine respiratory disease through real-time PCR testing and sequencing. Bovine ICV isolates had high nucleotide identities ( $\approx 98\%$ ) with each other and were closely related to human ICV strains ( $\approx 95\%$ ). Further research is needed to determine bovine ICV's zoonotic potential.

Influenza viruses are contagious zoonotic pathogens that belong to the *Orthomyxoviridae* family, which consists of 4 genera: *Alphainfluenzavirus* (influenza A virus), *Betafluenzavirus* (influenza B virus), *Gammafluenzavirus* (influenza C virus [ICV]), and *Deltafluenzavirus* (influenza D virus) (1–4). Classification of influenza viruses is based on the antigenic differences in the nucleoprotein and matrix protein and supported by intergenomic homologies of 20%–30% and intragenomic homologies  $>85\%$  (3).

The most common influenza pathogen is influenza A virus, which can infect humans, pigs, cattle, birds, as well as other animals (2,4). ICV was first identified in humans in 1947. This group of influenza viruses was initially thought to exclusively infect humans until isolates were identified in pigs in China (5,6) and Japan (7). Antigenic and genetic analyses suggest that ICV might transmit between humans and pigs in nature (8); however, interspecies transmission has not been confirmed experimentally. In 2011, an influenza C–like virus was identified in swine and cattle in the United States (9); this virus was initially proposed to be an ICV subtype but was later identified as influenza D virus (3) because the virus had  $\approx 50\%$  overall amino acid identity with human ICV strains, a level of divergence similar to that between influenza A and influenza B viruses.

Although influenza viruses of other genera can infect cattle, the potential for ICV infection in cattle has not been previously investigated. The objective of this study

was to determine if ICV can be found in specimens from cattle with bovine respiratory disease and, if so, determine the prevalence.

## The Study

Bovine respiratory disease complex (BRDC) is one of the most common causes of death in livestock in US feedlots and feedlots worldwide (10). During October 2016–January 2018, we collected 1,525 samples (mainly nasal swab and lung tissue specimens) from cattle in the Midwest of the United States and submitted them to Kansas State Veterinary Diagnostic Laboratory (Manhattan, Kansas, USA) for BRDC diagnostic testing. We screened samples for ICV by real-time reverse transcription PCR, as well as for 10 other BRDC-associated pathogens (*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Bibersteinia trehalosi*, *Mycoplasma bovis*, bovine viral diarrhoea virus, bovine respiratory syncytial virus, bovine respiratory coronavirus, bovine herpesvirus 1, and influenza D virus; online Technical Appendix, <https://wwwnc.cdc.gov/EID/article/24/10/18-0589-Techapp1.pdf>). We sequenced a 590-bp fragment of the matrix gene from 12 ICV-positive samples (GenBank accession nos. MH421865–73; online Technical Appendix Table) to confirm the PCR results and perform a phylogenetic analysis. We selected 1 isolate (*C/bovine/Montana/12/2016*) for complete genome sequencing (GenBank accession nos. MH348113–9).

Of 1,525 samples, 64 (4.20%) were positive for ICV: 38 samples with a cycle threshold ( $C_t$ )  $<36$  and 26 with a  $C_t$  36–39. The most common pathogens were bovine respiratory coronavirus (34.98%), *M. bovis* (32.27%), and *M. haemolytica* (17.04%). The remaining BRDC pathogens were present but less prevalent: *P. multocida* (13.42%), *H. somni* (12.58%), influenza D virus (11.93%), bovine respiratory syncytial virus (9.19%), bovine viral diarrhoea virus (7.05%), *B. trehalosi* (3.47%), and bovine herpesvirus 1 (2.95%).

Co-infections with  $\geq 1$  pathogen are common in BRDC cases. ICV-positive samples were also found to be positive for  $\geq 1$  bovine respiratory disease pathogen ( $n = 12$ , Table 1), the most common being *M. bovis* (9/12), followed by *H. somni* (7/12), and *M. haemolytica* (6/12). Among the ICV-positive samples, ICV12 was strongly positive ( $C_t$  15.81); this sample was also positive for *M. haemolytica* and *P. multocida*, both bacterial pathogens commonly associated with secondary infections. Other BRDC pathogens associated

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**Table 1.** Cycle thresholds for ICV and other bovine respiratory pathogens in 12 ICV strong positive samples from cattle with respiratory disease, United States, October 2016–January 2018\*

ID no.	State	ICV	BVDV	BHV-1	BRSV	BCoV	IDV	<i>Mycoplasma bovis</i>	<i>Mannheimia haemolytica</i>	<i>Pasteurella multocida</i>	<i>Histophilus somni</i>	<i>Bibersteinia trehalosi</i>
ICV1†	TX	29.95	–	–	–	–	36.77	39.41	–	–	–	–
ICV2†	OK	23.92	–	–	–	–	24.25	29.17	31.30	–	31.32	–
ICV3†	OK	21.02	–	–	38.93	27.00	29.15	31.40	NT	NT	NT	NT
ICV4‡	OK	29.98	–	–	–	–	–	24.54	31.33	–	31.05	–
ICV5†	MO	24.47	–	34.98	–	–	–	30.12	–	28.00	24.40	34.00
ICV6†	CO	26.91	–	–	–	–	–	30.25	32.78	29.60	30.00	35.00
ICV12†	MT	15.81	–	–	–	–	–	–	22.87	25.70	–	–
ICV16‡	NE	27.18	16.44	–	–	–	–	28.47	–	–	–	–
ICV18†	MN	30.58	–	–	–	–	–	–	35.06	–	–	–
ICV20‡	KS	27.92	–	–	–	–	–	–	–	–	23.49	–
ICV21‡	KS	26.72	–	–	35.59	–	–	25.67	30.95	–	28.47	–
ICV22†	MT	25.08	–	–	–	–	20.24	35.58	–	–	35.64	–

\*BCoV, bovine respiratory corona virus; BHV-1, bovine herpesvirus 1; BRSV, bovine respiratory syncytial virus; BVDV, bovine viral diarrhea virus; ICV, influenza C virus; ID, identification; IDV, influenza D virus; NT, not tested (sample used up); –, negative.

†Nasal swab sample used in analysis.

‡Lung sample used in analysis.

with secondary infections (*M. bovis*, bovine viral diarrhea virus, and *H. somni*) were also detected in samples ICV4, ICV16, ICV18, and ICV20 (11–13). These results suggest that ICV is associated with bovine respiratory disease in cattle.

We further evaluated 12 strong positive ( $C_t < 31$ ) samples by sequencing a 590-bp fragment of their matrix gene. Alignment of the partial matrix gene sequences indicated that the isolates in 3 samples (ICV2, ICV3, and ICV4) obtained from different cattle on the same farm in Oklahoma were identical. Because these 3 influenza viruses were most likely the same strain, the virus in just 1 sample (ICV2) was used for phylogenetic analysis. The matrix gene sequence in sample ICV5 from Missouri (GenBank accession no. MH421866) was identical to that in ICV6 from Colorado (GenBank accession no. MH421867).

Phylogenetic analysis indicated that the bovine ICV isolates are closely related to the porcine and human ICV isolates, and the bovine ICV isolates are more closely related to each other (Table 2; Figure). The bovine ICV isolates' partial matrix gene sequences shared high nucleotide identities ( $\approx 98\%$ ). For both partial matrix gene sequences and the whole genome sequence (7 segments), the nucleotide identity between bovine and human isolates was  $\approx 95\%$ . The full genome sequence of C/bovine/Montana/12/2016

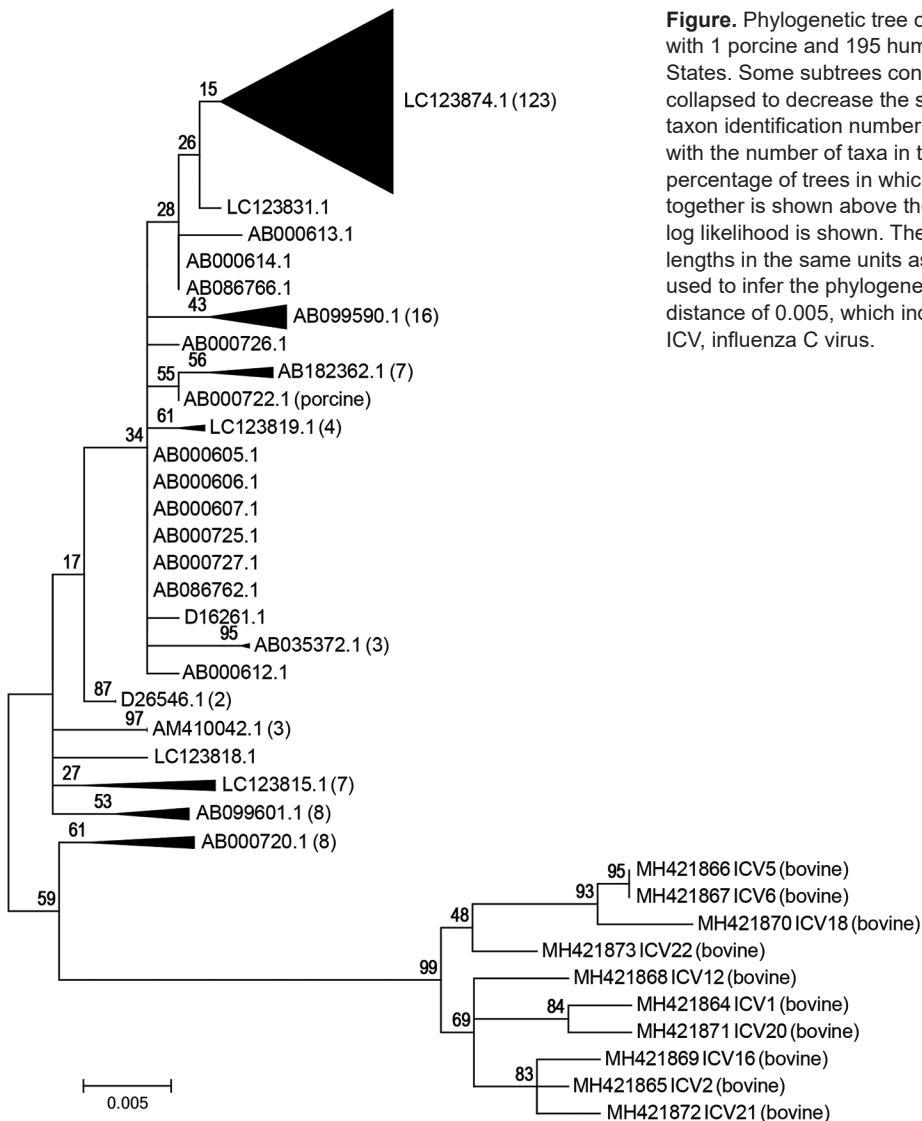
from sample ICV12 had high nucleotide identity to C/Mississippi/80 (and several other human ICV strains), with an overall identity of 97.1%. Nucleotide identities between these 2 isolates were also high for each gene: 97.0% for polymerase basic 2, 97.7% for polymerase basic 1, 97.5% for polymerase 3, 96.2% for hemagglutinin esterase, 96.8% for nucleoprotein, 96.8% for matrix, and 97.6% for non-structural protein. The only porcine ICV isolate available was more closely related to human ( $\approx 98\%$  identity) isolates than bovine ( $\approx 95\%$  identity) isolates; the porcine ICV isolate had nearly the same identity that the human ICV isolates had among each other (Table 2).

The phylogenetic tree of the partial matrix gene sequences (Figure) further demonstrates the relationship between bovine and human ICV isolates. All bovine ICVs formed a separate clade on the phylogenetic tree, with a 99% bootstrap value. Of the 195 partial matrix gene sequences from human ICVs, the 10 corresponding sequences from bovine ICVs had the highest identities (average 96.70%) to those from C/Mississippi/80 (GenBank no. AB000720.1), C/Nara/82 (GenBank no. AB000723), and C/Kyoto/41/82 (GenBank no. AB000724) and the lowest identities (average 94.21%) to those from C/Yamagata/30/2014 (GenBank no. LC123874) and C/Yamagata/32/2014 (GenBank no. LC123875).

**Table 2.** Average nucleotide identities among bovine, porcine, and human ICV strains, United States\*

Gene sequence	Bovine ICV, %	Human ICV, %	Bovine ICV vs. human ICV, %	Bovine ICV vs. porcine ICV, %	Human ICV vs. porcine ICV, %
Matrix, partial	98.43	98.47	95.54	96.06	98.84
Polymerase basic 2	NA	97.76	94.97	95.00	98.34
Polymerase basic 1, full length	NA	97.59	94.69	94.70	98.08
Polymerase 3	NA	97.79	96.36	95.50	97.31
Hemagglutinin esterase	NA	95.44	90.83	91.10	95.97
Nucleoprotein	NA	97.67	95.48	95.30	97.85
Matrix, complete	NA	98.33	95.51	95.80	98.82
Nonstructural protein	NA	98.32	95.67	95.80	98.36
Entire genome		97.56	94.79	94.74	97.82

\*ICV, influenza C virus; NA, not applicable.



**Figure.** Phylogenetic tree of 10 bovine ICV isolates compared with 1 porcine and 195 human ICV isolates (not labeled), United States. Some subtrees containing only human isolates were collapsed to decrease the size of the image. A representative taxon identification number for each collapsed subtree is shown with the number of taxa in the subtree in parentheses. The percentage of trees in which the associated taxa clustered together is shown above the branches. The tree with the highest log likelihood is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Scale bar indicates the relative distance of 0.005, which indicates a 0.5% sequence difference. ICV, influenza C virus.

## Conclusions

This study confirms the presence of ICV in US cattle with clinical signs of bovine respiratory disease. Although interspecies transmission of influenza viruses occurs between humans and other animals, we do not have data that indicates ICV is a zoonotic pathogen. However, the full genome sequence of *C/bovine/Montana/12/2016* has 97.1% nucleotide identity with the human isolate *C/Mississippi/80*, which is within the range of average identities among human isolates. More detailed investigations are needed to confirm if ICV is involved in bovine respiratory disease, to characterize the relationship between bovine and human ICV strains, and to determine the zoonotic potential of bovine ICV isolates to cause human disease.

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## About the Author

Mr. Zhang is a joint doctoral student candidate at the Chinese Academy of Agricultural Sciences in Changchun, China, and Kansas State University in Manhattan, Kansas, USA. His research interests are the development and validation of molecular diagnostic assays for animal and zoonotic pathogens.

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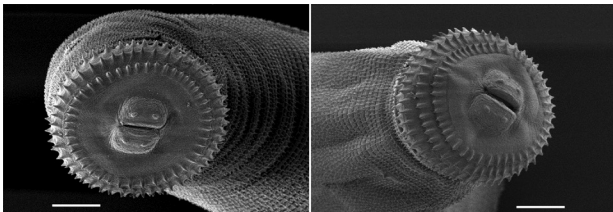
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- Distribution of Pandemic Influenza Vaccine and Reporting of Doses Administered, New York, New York, USA
- Regional Variation in Travel-related Illness acquired in Africa, March 1997–May 2011
- Underdiagnosis of Foodborne Hepatitis A, the Netherlands, 2008–2010
- Travel-associated Antimicrobial Drug-Resistant Nontyphoidal Salmonellae, 2004–2009
- Ciprofloxacin Resistance and Gonorrhoea Incidence Rates in 17 Cities, United States, 1991–2006
- Contact Investigation for Imported Case of Middle East Respiratory Syndrome, Germany
- Rapid Increase in Pertactin-deficient *Bordetella pertussis* Isolates, Australia
- *Gnathostoma* spp. in Live Asian Swamp Eels (*Monopterus* spp.) from Food Markets and Wild Populations, United States
- Epidemic of Mumps among Vaccinated Persons, the Netherlands, 2009–2012
- High Rates of Antimicrobial Drug Resistance Gene Acquisition after International Travel, the Netherlands
- Characteristics of Patients Infected with Norovirus GII.4 Sydney 2012, Hong Kong, China
- Pathology of US Porcine Epidemic Diarrhoea Virus Strain PC21A in Gnotobiotic Pigs
- Cetacean Morbillivirus in Coastal Indo-Pacific Bottlenose Dolphins, Western Australia



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## Technical Appendix

### Real-Time Reverse Transcription PCR

Bovine clinical samples were homogenized, and the nucleic acids were extracted by using QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). Two sets of nonoverlapping real-time reverse transcription (RT) PCR primers and probes (labeled with the same dye) were designed to target the influenza C virus (ICV) matrix gene. The primers and probe of set 1 (amplicon size 144 bp): ICV1-F (5'-TCGGCAGATGGGAGAGATG-3'), ICV1-R (5'-GAATTGGTGAGTTGTCGGTTTC-3'), and ICV1-Pr (MAX-5'-CTCCCAGGTCAAGTCTCTCCCT-3'-IBFQ), and the primers and probe of set 2 (amplicon size 100 bp): ICV2-F (5'-TGGCCTTGGAGAAGAAGCA-3'), ICV2-R (5'-CAAGTGGGGTCTCATTATATTA TACTTCC-3'), and ICV2-Pr (MAX-3'-TGATTRCATAATATGGCCAACTTTCTG-5'-IBFQ) were synthesized from Integrated DNA Technologies, Inc. (Coralville, IA, USA). In silico analysis indicated that the 2 sets of assays covered 195 of 196 bp (99.5%) of the complete and near-complete ICV matrix gene sequences available. We performed real-time reverse RT-PCR tests using a 20- $\mu$ L reaction volume (0.4  $\mu$ M forward and reverse primers, 0.2  $\mu$ M probe, 3  $\mu$ L template nucleic acid, 2  $\mu$ L 10 $\times$  Multiplex Enzyme Mix, and 10  $\mu$ L 2 $\times$  Multiplex RT-PCR Buffer) and the Path-ID Multiplex One-Step RT-PCR Kit (Applied Biosystems, Grand Island, NY, USA). The thermocycling parameters included a RT step at 48°C for 10 min and RT inactivation and denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 45 sec. The cycle threshold was determined with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and analyzed by using Bio-Rad CFX Manager 3 software.



## **Partial Matrix Gene Sequencing of ICV-Positive Samples and Complete Genome Sequencing of Sample ICV12**

A pair of sequencing primers flanking the real-time PCR target were designed to amplify a 590-bp fragment of ICV matrix gene for Sanger sequencing confirmation of PCR-positive samples. The primer sequences were ICV-cF (5'-AAAGCCAGCACAGCAATGAA-3') and ICV-cR (5'-TCAAAAATACCATCATTGGAAAAAGG-3'). The ICV matrix gene fragment was amplified by using LA Taq PCR Kit (TaKaRa, Mountain View, CA, USA) from cDNA synthesized with the SuperScript III First-Strand Synthesis Supermix Kit (Invitrogen, Carlsbad, CA, USA). PCR products were sequenced by an outsourced sequencing facility (Genewiz, South Plainfield, NJ, USA). Sample ICV12 was selected for complete genome sequencing as previously described (1,2).

### **Phylogenetic Analyses**

A total of 195 complete and near-complete human ICV matrix segment sequences and 1 porcine ICV matrix segment sequence were obtained from GenBank (same as the sequences from Influenza Research Database). The analyses of 10 bovine and human ICV matrix gene sequences were carried out by using CLC Genomic Workbench version 9.0.1 (CLC bio, Boston, MA, USA) to generate a multiple sequence alignment. BioEdit version 7.2.5 (<https://www.bioedit.com/>) was used to generate a sequence identity table.

We constructed a phylogenetic tree with MEGA7 (3) using multiple alignments of the 10 bovine ICV matrix gene sequences generated in this study plus 195 complete and near-complete human ICV matrix gene sequences and 1 porcine ICV matrix gene sequence. The alignment length was trimmed to 559 bp. The evolutionary history was inferred by using the maximum likelihood method on the basis of the Jukes-Cantor model (4) with 1,000 bootstrap replicates (5). Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated by using the maximum composite likelihood approach and then selecting the topology with the superior log likelihood value.

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**Technical Appendix Table.** Sample and sequence information of bovine ICV isolates acquired from cattle with respiratory disease, United States, October 2016–January 2018\*

Sample ID	Sample source	Bovine ICV isolate	GenBank accession no.
ICV1	Nasal swab	C/bovine/Texas/1/2016	MH421864
ICV2	Nasal swab	C/bovine/Oklahoma/2/2017	MH421865
ICV3	Nasal swab	C/bovine/Oklahoma/3/2017	NA
ICV4	Lung	C/bovine/Oklahoma/4/2017	NA
ICV5	Nasal swab	C/bovine/Missouri/5/2017	MH421866
ICV6	Nasal swab	C/bovine/Colorado/6/2017	MH421867
ICV12	Nasal swab	C/bovine/Montana/12/2016	MH421868†
ICV16	Lung	C/bovine/Nebraska/16/2017	MH421869
ICV18	Nasal swab	C/bovine/Minnesota/18/2017	MH421870
ICV20	Lung	C/bovine/Kansas/20/2017	MH421871
ICV21	Lung	C/bovine/Kansas/21/2017	MH421872
ICV22	Nasal swab	C/bovine/Montana/22/2016	MH421873

\*ICV, influenza C virus; ID, identification; NA, not applicable.

†GenBank accession nos. for complete genome are MH348113–MH348119.