

Influenza D Virus in Animal Species in Guangdong Province, Southern China

Technical Appendix

RT-PCR method and subcloning protocol used in this study

A classic reverse transcription PCR (RT-PCR) method was developed using a pair of primers (HEF-F: 5'-AAC CRC ATC TTC TTG TTC TTC A-3' and HEF-R: 5'-TGC TTC TTC WGT GGC ATT ATC T-3') targeting at the partial hemagglutinin-esterase-fusion (HEF) gene (at positions 582–1077) to test the presence of IDVs and further define IDV genetic lineages in those samples. For RT-PCR, the primers were diluted to 10 μ M with ddH₂O.

A 25 μ L RT-PCR system was constructed using a one-step RT-PCR kit (Takara Bio Inc.), which contained 0.5 μ L of each primer, 0.5 μ L of enzyme mixture (including PrimeScript RTase, DNA polymerase, RNase inhibitor), 12.5 μ L of 2 \times buffer, 8.5 μ L of ddH₂O, and 2.5 μ L of viral RNA. RT-PCR was performed as follows: reverse transcription (RT) at 50°C for 30 min, 95°C for 5 min, followed by 35 PCR cycles (95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec), and a final extension at 72°C for 10 min. The positive PCR fragments (\approx 500 bp) were purified and cloned into the pGEM-T vector (TIANGEN, Inc., Beijing). Positive recombinant plasmids were purified according to the manufacturer's instructions (TIANGEN, Inc., Beijing) and sequenced using the Sanger sequencing method (Sangon Biotech Co., Ltd., Shanghai). RT-PCR detection data were analyzed by chi-square test (Ziyue software), and significance was set at $p < 0.05$.

Technical Appendix Table. Reference influenza D virus isolates with partial hemagglutinin-esterase-fusion sequences used in this study

Strain or isolate	Specimen origin of isolate	Country	Year	GenBank
				accession no.
D/bovine/Nebraska/9–5/2012	Bovine (nasal swab)	USA	2012	KM392471
D/bovine/Oklahoma/660/2013	Bovine (nasal swab)	USA	2013	KF425662
D/bovine/Mississippi/C00014N/2014	Bovine (nasal swab)	USA	2014	KT581417
D/bovine/Mississippi/C00013N/2014	Bovine (nasal swab)	USA	2014	KT581416
D/bovine/Kansas/11–8/2012	Bovine (nasal swab)	USA	2012	KM392506
D/bovine/Kansas/13–21/2012	Bovine (nasal swab)	USA	2012	KM392492
D/bovine/Texas/3–13/2011	Bovine (nasal swab)	USA	2011	KM392485
D/bovine/Mexico/S56/2015	Bovine (nasal swab)	Mexico	2015	KU171128
D/bovine/Mexico/S8/2015	Bovine (nasal swab)	Mexico	2015	KU171127
D/bovine/Mexico/S7/2015	Bovine (nasal swab)	Mexico	2015	KU171126
D/bovine/France/2986/2012	Bovine (nasal swab)	France	2012	LN559126
D/bovine/Kansas/1–35/2010	Bovine (nasal swab)	USA	2010	KM392478
D/bovine/Mexico/S62/2015	Bovine (nasal swab)	Mexico	2015	KU171129
D/swine/Oklahoma/1334/2011	Swine (nasal swab)	USA	2011	JQ922308
D/bovine/Mississippi/C00030P/2014	Bovine (nasopharyngeal swab)	USA	2014	KT581418
D/bovine/Mississippi/C00046N/2014	Bovine (nasal swab)	USA	2014	KT581412
D/bovine/Minnesota/729/2013	Bovine (nasal swab)	USA	2013	KF425669
D/bovine/Minnesota/628/2013	Bovine (nasal swab)	USA	2013	KF425655
D/bovine/Kansas/14–22/2012	Bovine (nasal swab)	USA	2012	KM392499
D/bovine/Shandong/Y125/2014	Bovine (nasal swab)*	China	2014	KM015494
D/bovine/Shandong/Y127/2014	Bovine (nasal swab)*	China	2014	KM015501
D/bovine/Italy/46484/2015	Bovine (nasal swab)	Italy	2015	KT592526
D/bovine/Italy/1/2014	Bovine (nasal swab)	Italy	2014	KT592522
D/bovine/Shandong/Y217/2014	Bovine (nasal swab)*	China	2014	KM015508
D/swine/Italy/199724–3/2015	Swine (nasal swab)	Italy	2015	KT592533
D/bovine/Ibaraki/7768/2016	Bovine (nasal swab)	Japan	2016	LC128433
D/swine/Guangdong/YS1/2016	Swine (lung)	China	2016	KY441104
D/swine/Guangdong/YS2/2016	Swine (lung)*	China	2016	KY441105
D/swine/Guangdong/P8/2016	Swine (lung)	China	2016	KY441106
D/swine/Guangdong/P14/2016	Swine (nasal swab)	China	2016	KY441107
D/swine/Guangdong/PS1/2016	Swine (nasal swab)*	China	2016	KY441108
D/swine/Guangdong/U1/2016	Swine (serum)	China	2016	KY441109
D/swine/Guangdong/U16/2016	Swine (nasal swab)*	China	2016	KY441110
D/bovine/Guangdong/LG2/2016	Bovine: dairy cow (serum)	China	2016	KY441111
D/bovine/Guangdong/LG5/2016	Bovine: dairy cow (nasal swab)	China	2016	KY441112

Strain or isolate	Specimen origin of isolate	Country	Year	GenBank
				accession no.
D/bovine/Guangdong/LG9/2016	Bovine: dairy cow (nasal swab)*	China	2016	KY441113
D/bovine/Guangdong/QQ1/2016	Bovine: dairy cow (nasal swab)	China	2016	KY441114
D/bovine/Guangdong/QQ4/2016	Bovine: dairy cow (serum)	China	2016	KY441115
D/bovine/Guangdong/QQ7/2016	Bovine: dairy cow (serum)	China	2016	KY441116
D/bovine/Guangdong/QQ12/2016	Bovine: dairy cow (nasal swab)*	China	2016	KY441117
D/bovine/Guangdong/NS1/2016	Bovine: buffalo (serum)	China	2016	KY441118
D/bovine/Guangdong/RS1/2016	Bovine: yellow cattle (nasal swab)	China	2016	KY441119
D/bovine/Guangdong/RS4/2016	Bovine: yellow cattle (nasal swab)*	China	2016	KY441120
D/bovine/Guangdong/PY1/2016	Bovine: buffalo (serum)	China	2016	KY441121
D/caprine/Guangdong/JK1/2016	Caprine: goat (rectal swab)	China	2016	KY441122

*Healthy.

Majority	AACCGCATCTTCTGTCTTCAAGCTGGATGAAAAGCCCGTTGTGGTATGCAGAACTCTCTGTTAATCCTGGAGCTAAACCTCAAGTTTGTGGGACTGAG										
	10	20	30	40	50	60	70	80	90	100	
JK1.seq	100
LG2.seq	100
LG5.seq	100
LG9.seq	100
NS1.seq	100
P14.seq	100
P8.seq	100
PS1.seq	100
PY1.seq	100
QQ1.seq	100
QQ12.seq	100
QQ4.seq	100
QQ7.seq	100
RS1.seq	100
RS4.seq	100
U1.seq	100
U16.seq	100
YS1.seq	100
YS2.seq	100
Y125.seq	100
Y127.seq	100
Y217.seq	100
628.seq	100
729.seq	100
1-35.seq	100
C00030P.seq	100
14-22.seq	100
C00046N.seq	100
1334.seq	100
S62.seq	100
46484.seq	100
1.seq	100
199724-3.seq	100
S8.seq	100
S7.seq	100
S56.seq	100
13-21.seq	100
11-8.seq	100
9-5.seq	100
660.seq	100
C00014N.seq	100
C00013N.seq	100
3-13.seq	100
2986.seq	100
7768.seq	100

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Majority	CAATCGGCAACTTTTACTTTGCCGACAAGCTTCGGAAATTTACA AATGCAACAAGCATGTAGTGCAGCTTTGTTACTTTGTGTACGAAAACAAAACACAT										
	110	120	130	140	150	160	170	180	190	200	
JK1.seq										G	200
LG2.seq											200
LG5.seq											200
LG9.seq											200
NS1.seq											200
P14.seq											200
P8.seq											200
PS1.seq											200
PY1.seq											200
QQ1.seq											200
QQ12.seq				G							200
QQ4.seq											200
QQ7.seq											200
RS1.seq											200
RS4.seq											200
U1.seq											200
U16.seq				G			G				200
YS1.seq											200
YS2.seq				G							200
Y125.seq											200
Y127.seq											200
Y217.seq											200
628.seq					C						200
729.seq				C							200
1-35.seq			T								200
C00030P.seq											200
14-22.seq								A			200
C00046N.seq				A	TAC						200
1334.seq									G	A	200
S62.seq									G	A	200
46484.seq									G		200
1.seq			C						G		200
199724-3.seq									G		200
S8.seq	A			T			A		T	G	200
S7.seq	A			T			A		T	G	200
S56.seq	A			T			A		T	G	200
13-21.seq	A			T			A		T	G	200
11-8.seq	A						A		T	G	200
9-5.seq	A		T				A		T	G	200
660.seq	A		T				A		T	GM	200
C00014N.seq	A		T				A		T	G	200
C00013N.seq	A		T				A		T	G	200
3-13.seq	A						A		T	G	200
2986.seq	A	T							T	G	200
7768.seq	G				T				G	G	200

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Majority	TTAACACTTTTGGCTGTGGAGATTATTACCAAAATTACTATGATGGGAATGGAAACCTGATACGGGGAATGGATAACAGAGTGGCAGCATACAGAGGAAT									
	210	220	230	240	250	260	270	280	290	300
JK1.seq	.	.	.G.	.	.	.C.	.	.	.	300
LG2.seq	.	.	.G.	.	.C.	.	A.	.	.	300
LG5.seq	.	.	.G.	.	.C.	.	.	.G.	.	300
LG9.seq	.	.	.G.	.	.C.	300
NS1.seq	.	.	.G.	.	.C.	300
P14.seq	.	.	.G.	.	.C.	300
P8.seq	.	.	.G.	.	.C.	300
PS1.seq	.	.	.GG.	.	.C.	300
PY1.seq	.	.	.G.	.	.C.	300
QQ1.seq	.	.	.G.	.	.C.	.	A.	.	.	300
QQ12.seq	.	.	.G.G.	.	.C.	300
QQ4.seq	.	.A.	.G.	.	.C.	300
QQ7.seq	.	.	.G.	.	.C.	.	A.	.	.	300
RS1.seq	.	.	.G.	.	.C.	300
RS4.seq	.	.	.G.	.	.C.	.	A.	.	.	300
U1.seq	.	.	.G.	.	.C.	300
U16.seq	.	.	.G.G.	.	.C.	300
YS1.seq	.	.	.G.G.	.	.C.	.	A.	.	.	300
YS2.seq	.	.	.G.G.	.	.C.	.	.G.	.	.	300
Y125.seq	.	.	.G.	.	.C.	300
Y127.seqC.	300
Y217.seqC.	.	.	.T.	.	300
628.seq	.	.G.	.T.GC	300
729.seq	.	.G.	.T.GC	300
1-35.seqA.	.	.C.	.	.	300
C00030P.seq	300
14-22.seq	300
C00046N.seq	300
1334.seq	300
S62.seq	300
46484.seq	300
1.seq	300
199724-3.seq	.	.C.A.	.	.	300
88.seq	.C.C.	.	.G.	.C.	.G.	300
87.seq	.C.C.	.	.G.	.C.	.G.	300
S56.seq	.C.	.T.	.	.	.C.	.	.G.	.C.	.	300
13-21.seq	.C.C.	.	.G.	.C.	.	300
11-8.seq	.C.	.A.	.	.	.C.	.	.G.	.	.	300
9-5.seq	.C.C.	.	.G.	.R.	.	300
660.seq	.C.C.	.	.G.	.	.	300
C00014N.seq	.C.C.	.	.G.	.A.	.	300
C00013N.seq	.C.C.	.	.G.	.A.	.	300
3-13.seq	.C.C.	.	.G.	.	.	300
2986.seqG.	.	.	300
7768.seq	.G.	300

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Majority	AGCAAACGCTGGAGTTAAABATTGAATGTCCTTCCAAAATCTTGAACCCCTGGGACTTACAGCATTAGATCAACACCAAGATTCCCTTCTAGTACCAAAAAGG									
	310	320	330	340	350	360	370	380	390	400
JK1.seq	400
LG2.seq	.A.....	400
LG5.seq	400
LG9.seq	400
NS1.seq	400
P14.seq	400
P8.seq	400
PS1.seq	400
PY1.seq	400
QQ1.seq	400
QQ12.seq	400
QQ4.seq	400
QQ7.seq	400
RS1.seq	400
RS4.seq	400
U1.seq	400
U16.seq	400
YS1.seq	400
YS2.seq	400
Y125.seq	400
Y127.seq	400
Y217.seq	400
628.seqG.....	400
729.seqG.....	400
1-35.seqA.....	400
C00030P.seqA.....	400
14-22.seqC.....	400
C00046N.seq	400
1334.seqA.....	400
S62.seqA.....	400
46484.seqT.....	400
1.seqT.....	400
199724-3.seqT.....T.....	400
S8.seq	...GG.T.....	.G.....A.....C.A.....	.G.....	400
S7.seq	...GG.T.....	.G.....A.....C.A.....	.G.....	400
S56.seq	...GG.T.....	.G.....A.....C.....C.A.....	.G.....	400
13-21.seq	...GG.T.....	.G.....A.....C.....A.....	.G.....	400
11-8.seq	...GG.T.....	.G.....A.....A.....	.G.....	400
9-5.seq	...GG.T.....	.G.....A.....R.....A.....	.G.....	400
660.seq	...GG.T.....	.G.....A.....A.....	.G.....	400
C00014N.seq	...GG.T.....	.G.....A.....A.....	.G.....	400
C00013N.seq	...GG.T.....	.G.....A.....A.....	.G.....	400
3-13.seq	...GG.T...R.....	.G.....A.....A.....	.G.G.G.....	400
2986.seq	...GGT...C.....	.G.....T.....A.....G.A.....	.G.....	400
7768.seq	...G...A.....	.G.....G.A.....	.G.....	400

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Majority	TCATACTGCTTCGACACTGATGGAGGGTACCCTATACAAGTAGTTC AATCTGAGTGGTCAGCTTCACG AAGATCAGATAATGCCA T TAAAGAAGCA	
	410 420 430 440 450 460 470 480 490	
JK1.seqG.....	496
LG2.seqG.....T.....	496
LG5.seqG.....	496
LG9.seqG.....	496
NS1.seqG.....	496
P14.seqG.....	496
P8.seqG.....T.....	496
PS1.seqG.....	496
PY1.seqG.....	496
QQ1.seqG.....T.....	496
QQ12.seqC.....G.....	496
QQ4.seqG.....	496
QQ7.seqG.....G.....T.....	496
RS1.seqG.....	496
RS4.seqG.....	496
U1.seqG.....	496
U16.seqG.....	496
YS1.seqG.....	496
YS2.seqG.....	496
Y125.seqG.....	496
Y127.seqG.....G.....	496
Y217.seqG.....	496
628.seq	496
729.seq	496
1-35.seq	496
C00030P.seqG.....	496
14-22.seq	496
C00046N.seq	496
1334.seq	496
S62.seq	496
46484.seqA.....	496
1.seq	496
199724-3.seq	496
S8.seq	...T.....G.....T.....	496
S7.seq	...T.....G.....T.....	496
S56.seq	...T.....G.....T.....	496
13-21.seq	...T.....G.....T.....	496
11-8.seq	...T.....G.....TT.....	496
9-5.seq	...T.....G.....T.....	496
660.seq	...T.....G.....T.....	496
C00014N.seq	...T.....G.....T.....	496
C00013N.seq	...T.....G.....T.....	496
3-13.seq	...T.....G.....T.....	496
2986.seq	...T.....A.....T.....G	496
7768.seq	...T.....G.....C.....T.....	496

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Technical Appendix Figure 1. Multiple sequence alignment results of the current IDV nucleotide sequences (496 bp) and corresponding published reference sequences.

Majority	ANAGVKIECPKILNPGTYSIRSTPRFLLVPKRSYCFDITDGGYPIQVVQSEWSASRRSDNATEEA	
	110 120 130 140 150 160	
JK1.pro	165
LG2.pro	T.....	165
LG5.pro	165
LG9.pro	165
NS1.pro	165
P14.pro	165
P8.pro	165
PS1.pro	165
PY1.pro	165
QQ1.pro	165
QQ12.proH.....	165
QQ4.pro	165
QQ7.proR.....	165
RS1.pro	165
RS4.pro	165
U1.pro	165
U16.pro	165
YS1.pro	165
YS2.pro	165
Y125.pro	165
Y127.proG.....	165
Y217.pro	165
628.pro	165
729.pro	165
1-35.pro	.T.....	165
C00030P.pro	.T.....	165
14-22.pro	165
C00046N.pro	165
1334.proK.....	165
862.proK.....	165
46484.pro	.V.....	165
1.pro	.V.....	165
199724-3.pro	.V.....	165
S8.pro	.GS..R.....K.....	165
S7.pro	.GS..R.....K.....	165
S56.pro	.GS.....K.....	165
13-21.pro	.GS..R.....R.....K.....	165
11-8.pro	.GS.....K.....I.....	165
9-5.pro	.GS.....X.....K.....	165
660.pro	.GS.....K.....	165
C00014N.pro	.GS.....K.....	165
C00013N.pro	.GS.....K.....	165
3-13.pro	.GSX.....K.....	165
2986.pro	.G..A.....	165
7768.pro	.S.E.....S.....K.....	165

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Technical Appendix Figure 2. Multiple sequence alignment results of the current IDV amino acid sequences (165 aa) and corresponding published reference sequences.