

**Table S1.** Summary of HA and virus titers, and length of open reading frame determined by a sequence analysis and GenBank accession number of three bovine influenza D viruses isolated from this study.

| Strains |                                 | HA<br>Titers<br>(HAU/<br>50 L) | Virus<br>Titers<br>(TCID <sub>50</sub> /<br>mL) | Length of ORF (Nucleotide) and GenBank Accession Number |                  |                  |                  |                  |                  |                 |
|---------|---------------------------------|--------------------------------|-------------------------------------------------|---------------------------------------------------------|------------------|------------------|------------------|------------------|------------------|-----------------|
|         |                                 |                                |                                                 | PB2                                                     | PB1              | P3               | HEF              | NP               | M                | NS              |
| HKD1    | D/bovine/Hokkaido/<br>HKD1/2018 | 64                             | 8.0                                             | 2280<br>LC565467                                        | 2238<br>LC565470 | 2133<br>LC565473 | 1980<br>LC565476 | 1659<br>LC565479 | 1128<br>LC565482 | 820<br>LC565485 |
| HKD2    | D/bovine/Hokkaido/<br>HKD2/2019 | 256                            | 8.1                                             | 2286<br>LC565468                                        | 2250<br>LC565471 | 2118<br>LC565474 | 1986<br>LC565477 | 1659<br>LC565480 | 1155<br>LC565483 | 820<br>LC565486 |
| HKD3    | D/bovine/Hokkaido/<br>HKD3/2020 | N.T.                           | 7.8                                             | 2241<br>LC565469                                        | 2238<br>LC565472 | 2100<br>LC565475 | 1965<br>LC565478 | 1659<br>LC565481 | 1125<br>LC565484 | 820<br>LC565487 |

N.T.: not tested

**Table S2.** A set of primers for genomic sequence determination of seven RNA segments from bovine influenza D virus, which originally designed in reference to other influenza D viruses available in GenBank.

| RNA Segment       | Full-Length*<br>(Length of ORF) | Forward Primer             | Position* | Reverse Primer                | Position* |
|-------------------|---------------------------------|----------------------------|-----------|-------------------------------|-----------|
| PB2 (5' terminus) | 2364                            | GCATAAGCAGAGGATGTCCTAC     | 2–24      | GCCTTACCCATTTTAGCAGCAC        | 1350–1371 |
| PB2 (3' terminus) | (2319)                          | GAAGGTCTGAAGGCCTAGATTG     | 1171–1192 | GCAGTAGCAAGAGGATTTTTTCAATGTGC | 2335–2363 |
| PB1 (5' terminus) | 2330                            | GGCATAAGCAGAGGATTTTATAAC   | 1–24      | CAGTTCCTTCTGGCCAATGCTTC       | 1313–1335 |
| PB1 (3' terminus) | (2262)                          | GAACCTTACATGGATGGAGAGTGC   | 1205–1227 | AGCAGTAGCAAGAGGATTTTTTCTG     | 2307–2330 |
| P3 (5' terminus)  | 2195                            | GGCATAAGCAGGAGATTTAGAA     | 1–22      | CATTAAACCATGTTGGGAACCCCC      | 1149–1172 |
| P3 (3' terminus)  | (2133)                          | GCCTCCAAGAAAATACAGGAGG     | 1064–1085 | GCAGTAGCAAGGAGATTTTAAAC       | 2172–2194 |
| HEF (5' terminus) | 2049                            | GCATAAGCAGGAGATTTTCAAAG    | 2–24      | CCATGGTTGTCATCTGCTTCTCCT      | 1128–1151 |
| HEF (3' terminus) | (1995)                          | CTGAGTGGTCAGCTTCACGAAGAT   | 1031–1054 | GCAGTAGCAAGGAGATTTTTTCTAAG    | 2023–2048 |
| NP (5' terminus)  | 1775                            | GCATAAGCAGGAGATTATTAAGCA   | 2–25      | CTGCATCTCCCACAATCCCTT         | 994–1015  |
| NP (3' terminus)  | (1659)                          | CTGGAGTAAGAGCCTTTATGGC     | 932–953   | GCAGTAGCAAGGAGATTTTTTG        | 1753–1774 |
| M                 | 1219<br>(1164)                  | GCATAAGCAGAGGATATTTTTGACGC | 2–27      | GCAGTAGCAAGAGGATTTTTTCGCG     | 1194–1218 |
| NS1               | 868<br>(820)                    | GCATAAGCAGGGGTGTACAATTTC   | 2–25      | GCAGTAGCAAGGGGTTTTTTCATAC     | 843–867   |

\*Full-length of each RNA segment and position represents in reference to D/bovine/Ibaraki/7768/2016 (GenBank accession number LC128433-LC128439).

**Table S3.** Summary of 960 serum sample collected between 2009 and 2018 used in retrospective surveillance.

| <b>Collection<br/>Year</b> | <b>Age [Months]</b> |              |              |               |                |                |                | <b>Total<br/>Number</b> |
|----------------------------|---------------------|--------------|--------------|---------------|----------------|----------------|----------------|-------------------------|
|                            | <b>24–47</b>        | <b>48–71</b> | <b>72–95</b> | <b>96–119</b> | <b>120–143</b> | <b>144–167</b> | <b>168–191</b> |                         |
| <b>2009</b>                | 72                  | 20           | 4            | 0             | 0              | 0              | 0              | 96                      |
| <b>2010</b>                | 88                  | 3            | 5            | 0             | 0              | 0              | 0              | 96                      |
| <b>2011</b>                | 78                  | 18           | 0            | 0             | 0              | 0              | 0              | 96                      |
| <b>2012</b>                | 77                  | 15           | 3            | 1             | 0              | 0              | 0              | 96                      |
| <b>2013</b>                | 73                  | 19           | 2            | 0             | 0              | 0              | 2              | 96                      |
| <b>2014</b>                | 71                  | 21           | 3            | 1             | 0              | 0              | 0              | 96                      |
| <b>2015</b>                | 80                  | 15           | 1            | 0             | 0              | 0              | 0              | 96                      |
| <b>2016</b>                | 75                  | 15           | 6            | 0             | 0              | 0              | 0              | 96                      |
| <b>2017</b>                | 80                  | 14           | 2            | 0             | 0              | 0              | 0              | 96                      |
| <b>2018</b>                | 82                  | 12           | 1            | 1             | 0              | 0              | 0              | 96                      |
| <b>Total Number</b>        | 776                 | 152          | 27           | 3             | 0              | 0              | 2              | 960                     |

**Table S4.** Open reading frame (ORF) nucleotide sequence identities among genotypes on individual RNA segments of influenza D viruses.

| <b>PB2</b>      | <b>ORF Nucleotide Sequence Identities (%)</b> |           |           |           |           |
|-----------------|-----------------------------------------------|-----------|-----------|-----------|-----------|
| <b>Genotype</b> | 1                                             | 2         | 3         | 4         | 5         |
| 1 (n = 28)      | 97.9–100                                      | 96.0–98.1 | 96.9–98.2 | 94.8–96.1 | 94.6–95.6 |
| 2 (n = 28)      |                                               | 97.5–100  | 96.4–98.4 | 94.6–96.4 | 94.2–95.7 |
| 3 (n = 4)       |                                               |           | 97.8–99.8 | 95.2–96.2 | 94.9–95.6 |
| 4 (n = 4)       |                                               |           |           | 98.7–99.8 | 96.7–97.4 |
| 5 (n = 2)       |                                               |           |           |           | 98.8      |

| <b>PB1</b>      | <b>ORF Nucleotide Sequence Identities (%)</b> |           |           |           |
|-----------------|-----------------------------------------------|-----------|-----------|-----------|
| <b>Genotype</b> | 1                                             | 2         | 3         | 4         |
| 1 (n = 50)      | 96.9–100                                      | 96.1–97.8 | 94.6–96.4 | 95.0–96.2 |
| 2 (n = 3)       |                                               | 99.6–99.8 | 95.3–96.1 | 95.4–95.6 |
| 3 (n = 5)       |                                               |           | 97.4–99.8 | 95.9–96.5 |
| 4 (n = 1)       |                                               |           |           |           |

| <b>P3</b>       | <b>ORF Nucleotide Sequence Identities (%)</b> |           |           |           |           |
|-----------------|-----------------------------------------------|-----------|-----------|-----------|-----------|
| <b>Genotype</b> | 1                                             | 2         | 3         | 4         | 5         |
| 1 (n = 57)      | 97.5–100                                      | 96.5–97.8 | 97.1–98.1 | 95.1–96.3 | 95.0–96.4 |
| 2 (n = 4)       |                                               | 98.5–100  | 97.4–97.6 | 95.1–95.6 | 95.1–95.4 |
| 3 (n = 1)       |                                               |           |           | 95.6–95.9 | 95.5–95.8 |
| 4 (n = 4)       |                                               |           |           | 99.3–99.8 | 97.7–97.8 |
| 5 (n = 2)       |                                               |           |           |           | 98.7      |

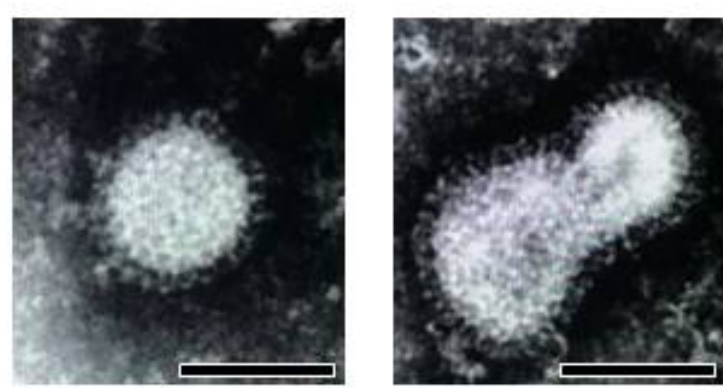
| <b>HEF</b>      | <b>ORF Nucleotide Sequence Identities (%)</b> |           |           |           |           |           |
|-----------------|-----------------------------------------------|-----------|-----------|-----------|-----------|-----------|
| <b>Genotype</b> | 1                                             | 2         | 3         | 4         | 5         | 6         |
| 1 (n = 34)      | 97.4–100                                      | 96.0–96.9 | 94.7–96.7 | 95.9–96.8 | 94.0–96.1 | 93.3–95.0 |
| 2 (n = 1)       |                                               |           | 95.9–97.0 | 96.7      | 95.9–96.7 | 94.3–95.0 |

|            |          |           |           |           |
|------------|----------|-----------|-----------|-----------|
| 3 (n = 20) | 97.4–100 | 97.2–98.0 | 93.5–95.9 | 93.0–94.7 |
| 4 (n = 1)  |          |           | 95.4–96.7 | 94.0–94.7 |
| 5 (n = 4)  |          |           | 98.2–99.9 | 92.5–94.3 |
| 6 (n = 2)  |          |           |           | 98.5      |

| NP         | ORF Nucleotide Sequence Identities (%) |           |           |           |           |           |           |
|------------|----------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Genotype   | 1                                      | 2         | 3         | 4         | 5         | 6         | 7         |
| 1 (n = 41) | 97.8–100                               | 96.8–98.6 | 96.6–97.4 | 96.4–97.5 | 96.6–97.6 | 94.3–95.6 | 94.3–95.5 |
| 2 (n = 13) |                                        | 97.6–100  | 96.2–98.0 | 95.8–97.8 | 96.2–97.7 | 94.1–96.3 | 94.3–96.3 |
| 3 (n = 1)  |                                        |           |           | 96.9–97.1 | 96.7–96.8 | 95.5–96.0 | 95.6–95.7 |
| 4 (n = 3)  |                                        |           |           | 99.6–99.8 | 96.2–96.4 | 94.7–95.3 | 94.9–95.1 |
| 5 (n = 3)  |                                        |           |           |           | 99.4–99.9 | 94.5–95.1 | 94.5–94.8 |
| 6 (n = 4)  |                                        |           |           |           |           | 99.1–99.9 | 97.4–98.0 |
| 7 (n = 2)  |                                        |           |           |           |           |           | 98.3      |

| P42        | ORF Nucleotide Sequence Identities (%) |           |           |           |
|------------|----------------------------------------|-----------|-----------|-----------|
| Genotype   | 1                                      | 2         | 3         | 4         |
| 1 (n = 30) | 96.8–100                               | 95.4–96.7 | 95.8–97.9 | 94.9–96.6 |
| 2 (n = 27) |                                        | 99.7–99.8 | 96.4–97.5 | 95.4–96.3 |
| 3 (n = 3)  |                                        |           | 97.4–100  | 95.8–97.3 |
| 4 (n = 6)  |                                        |           |           | 98.5–100  |

| NS         | ORF Nucleotide Sequence Identities (%) |           |           |           |           |
|------------|----------------------------------------|-----------|-----------|-----------|-----------|
| Genotype   | 1                                      | 2         | 3         | 4         | 5         |
| 1 (n = 54) | 98.1–100                               | 96.0–97.1 | 96.0–97.3 | 95.7–97.1 | 95.2–96.5 |
| 2 (n = 1)  |                                        |           | 96.9–97.3 | 96.0–96.3 | 96.0–96.3 |
| 3 (n = 3)  |                                        |           | 99.3–99.7 | 95.7–96.5 | 95.4–95.9 |
| 4 (n = 4)  |                                        |           |           | 99.2–100  | 97.1–97.6 |
| 5 (n = 2)  |                                        |           |           |           | 98.6      |



**Figure S1.** Image of BIDV virus, which isolated from HRT-18G cell culture, by transmission electron microscopy observation. The nasal swab sample collected from cattle with respiratory disorder was inoculated into HRT-18G cells, and cells were kept at 37 °C for a week. After a week, the supernatants were harvested from the cell culture which exhibited cytopathic effects, and then observed by transmission electron microscopy. Bar indicates 100 nm.