Veterinary Sciences and Medicine

SHORT COMMUNICATION

# Antigenic Variation among Bovine Influenza D Viruses in Japan

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## Abstract

Influenza D virus, a potential causative agent of bovine respiratory disease, is genetically classified into 3 clusters, namely D/OK-, D/660-, and D/Japan-lineages. The antigenic heterogeneity of the hemagglutinin-esterase-fusion (HEF) protein among the lineages of influenza D viruses has not been elucidated in detail. Herein, to evaluate the antigenic heterogeneity of influenza D viruses in Japan, we compared the hemagglutination inhibition-antibody titers to each lineage strain using bovine sera collected at two geographically different regions in Japan. In western Japan, antibody titers to the D/Japan-lineage strain were higher than those to the other clustered strains in a majority of the samples; whereas in eastern Japan, the antibody titers to the D/OK-lineage strain were higher than those to the others. These findings suggest that influenza D viruses with HEF antigenic variation are circulating and are distributed regionally in Japan.

**Key words:** Antigenicity, Cattle, Influenza D virus, Serology

## Introduction

The influenza D virus was first isolated from pigs with respiratory illness in Oklahoma, USA, in 2011 [1]. Epidemiological analyses have indicated that cattle are the major reservoirs of this virus [2-4], and that the influenza D virus is potentially involved in the pathogenesis of the bovine respiratory disease complex [5-7]. The high morbidity and mortality of this disease in feedlot cattle is partly due to co-infection with several viruses and bacteria. In addition to pigs and cattle, influenza D viruses have also been detected in small ruminants, camelids, and horses in several countries, which suggests that these viruses are globally distributed [8-18]. Phylogenetic analyses have revealed 3 clusters of the hemagglutinin-esterase-fusion (*HEF*) gene among influenza D viruses: D/swine/Oklahoma/1334/2011 (D/OK)-, D/bovine/Oklahoma/660/2013 (D/660)-, and D/bovine/Ibaraki/7768/2016 (D/Ibaraki; also called D/Japan)-lineages [19]. Antigenic differences in the HEF protein have also been identified among the clusters using serology [20]. In this study, we evaluated the HEF antigenicity of influenza D viruses in Japan by utilizing one strain from each phylogenetic cluster as antigens for serological testing of serum samples collected from Japanese cattle.

## Materials and Methods

### Test samples

Serum samples (a total of 1,810) were collected from Holstein and Japanese Black beef cattle in the eastern region (n = 435), including Iwate, Miyagi, Tochigi, Gunma, Nagano, Saitama, Tokyo, Kanagawa, and Yamanashi Prefectures, and the western region (n = 1,375), including Osaka, Tottori, Yamaguchi, Fukuoka, and Miyazaki Prefectures of Japan. The two regions are at least 300 kilometers apart. These samples were collected from apparently healthy animals in 2013-2017 and were stored at -20°C. There were no records of their clinical histories. The permit to carry out our work was obtained from The Committee of Animal Experiments at the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

### Viruses

Three influenza D strains, D/OK [1] (as D/OK-lineage virus), D/bovine/Nebraska/9-5/2013 [20] (D/NE; as D/660-lineage virus), and D/Yamagata/10710/2016 [21] (D/Yamagata; as D/Japan-lineage virus) were used for the test. D/OK and D/NE were provided by Dr. Ben Hause (Kansas State University). They were inoculated in swine testis (ST) cell culture (ATCC™ CRL-1746), which was grown at 37°C in Dulbecco's modified Eagle's medium (WAKO, Osaka, Japan) with 10% fetal bovine serum, and then propagated in serum-free medium in the presence of TPCK (N-tosyl-L-phenylalanine chloromethyl ketone)-trypsin (1 µg/mL) (Worthington, Lakewood, NJ, U.S.A).

### Hemagglutination (HA)-inhibition (HI) test

The HI test was performed as per the World Health Organization manual on animal influenza diagnosis and surveillance, in order to detect the anti-influenza virus antibodies in the

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serum samples [22]. The samples were treated with receptor-destroying enzyme (RDEII; DENKA SEIKEN, Tokyo, Japan) at 37°C for 16 h, followed by heat inactivation at 56°C for 30 min. Serially diluted samples were then treated with 4 hemagglutinating units (HAUs) of each of the influenza D strains for 30 min at 25°C. This was followed by incubation with 0.6% suspension of turkey red blood cells (NIPPON BIO-TEST, Saitama, Japan) for 30 min at 25°C before the result was read. The HI titer of each sample was expressed as the reciprocal of the highest sample dilution that completely inhibited HA. The samples showing an HI titer ≥40 were considered to be positive as an HI titer of 40 is commonly used as the threshold for a seropositive result in influenza D virus surveillance [2, 19, 16]. This threshold authenticates reaction specificity in the HI test [23].

### Statistical analysis

The HI titer data between the three virus antigens was compared by the non-parametric Wilcoxon's signed rank test using EZR software [24].

## Results and Discussion

To evaluate antigenic variation of influenza D viruses in Japan, we conducted the HI test using bovine serum samples against one virus antigen from each phylogenetic cluster. The results showed a total positive rate of 37.3% to D/Yamagata, 32.3% to D/OK, and 24.9% to D/NE, respectively (Table 1). Interestingly, the differences in positive rates were found between two geographical regions of Japan; in the eastern region, the positive rate to D/OK was over two-fold greater than those to the other clustered strains, whereas in the western region, that to D/Yamagata was considerably higher than those to the other clustered strains, suggesting that the viruses with different HEF antigenicities were circulating in these two regions.

When we compared positive HI titers in the eastern area (Figure 1A), those to D/OK were significantly higher ($p < 0.001$) than those to the other clustered strains in a majority of the samples, supporting a higher positive rate to D/OK. These findings indicate that viruses having similar HEF antigenicity to D/OK are extensively circulating in the eastern region, although a virus of the D/OK-lineage has not yet been isolated in Japan. Notably, the D/Yamagata used in this study was isolated from cattle in this area, which suggests that viruses with antigenic variation may be concomitantly circulating there and antibodies to D/OK possess a weak cross-reactivity to D/Japan as well as D/NE. Since a previous study indicated that substitution of a single amino acid at position 212 in the HEF protein resulted in its antigenic difference [20], some

**Table 1:** HI positive rates (%) of bovine serum samples from different regions of Japan against 3 influenza D viruses; D/Yamagata: D/Yamagata/10710/2016, D/OK: D/swine/Oklahoma/1334/2011, and D/NE: D/bovine/Nebraska/9-5/2013.

| Virus | Region of Japan (no. of samples) | | |
|---|---|---|---|
| | Eastern (n = 435) | Western (n = 1,375) | Total (n = 1,810) |
| D/Yamagata | 15.2 | 44.3 | 37.3 |
| D/OK | 33.6 | 31.9 | 32.3 |
| D/NE | 8.0 | 30.2 | 24.9 |

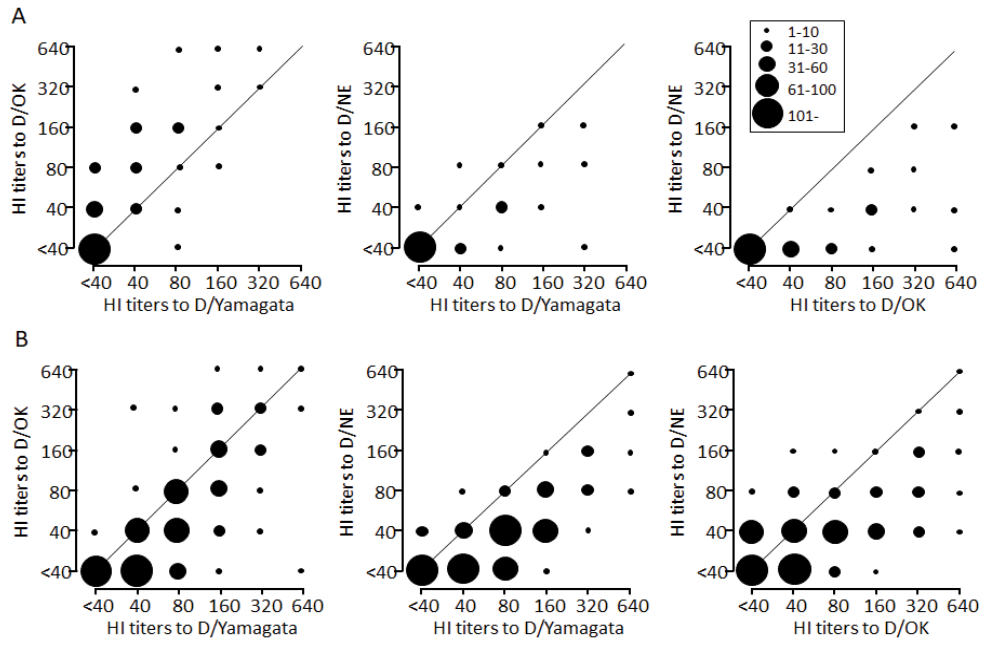


**Figure 1:** Comparison of HI titers of bovine serum samples against 3 influenza D strains. Samples were collected from cattle in eastern (A) and western (B) regions of Japan. Ranges in numbers of positive samples are represented by sizes of circles in titers to D/Yamagata versus D/OK (left panel), D/Yamagata versus D/NE (middle panel), and D/OK versus D/NE (right panel).

antigenic variants could likely naturally occur, even in the same lineage.

In contrast to the eastern region, the positive titers to D/Yamagata were significantly higher ($p < 0.001$) than those to the other clustered strains in a majority of the samples from the western region (Figure 1B). This indicates that viruses having similar HEF antigenicity to D/Yamagata are extensively circulating in this area. This observation was strongly supported by a report showing that the HEF amino acid sequence of D/bovine/Miyazaki/B22/2016, which was detected in the western area [25], exhibited a highly similar identity (99.9%) to that of D/Yamagata. In addition, HI positive rates and titers to each lineage strain in the western region suggest that antibodies to D/Yamagata might cross-react to D/OK better than to D/NE.

Collectively, the results of this study demonstrate the presence of antigenic variants of influenza D viruses in Japan. It could not be determined if the antigenic variants arose from the D/Japan-lineage virus or if they were D/OK-lineage viruses. An extensive survey of influenza D viruses is required to further clarify this distinction. Elucidation of antigenic heterogeneity among influenza D viruses will contribute toward the development of efficient influenza D vaccines by assisting in selection of vaccine strains for each region.

## Conclusion

We serologically detected antigenic variation in the HEF protein among bovine influenza D viruses in Japan, emphasizing that a larger antigenic heterogeneity could exist among influenza D viruses globally. Therefore, in epidemiological surveys for influenza D viruses, we recommend the use of multiple strains of different clusters as antigens for serological tests to obtain reliable results.

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