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**Research Note—**

**Genetic and Pathologic Characterization of a Novel Recombinant TC07-2-Type Avian Infectious Bronchitis Virus**

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**SUMMARY.** Avian infectious bronchitis viruses (IBVs) with the TC07-2 genotype have spread rapidly in East Asia since they were first reported in China in 2007. In 2015, an IBV with the TC07-2 genotype (designated KrD1515) was isolated from layer chickens with severe respiratory symptoms in Korea. In the present study, the full-length open reading frames of the spike (S) and nucleocapsid (N) genes of the virus were sequenced and analyzed. S1 gene phylogenetic analysis revealed that the KrD1515 virus clustered with viruses with the TC07-2 genotype, whereas N gene phylogenetic analysis revealed that the KrD1515 virus clustered with Korean IBVs, but not with Chinese TC07-2 IBV. When 7-day-old specific-pathogen-free chickens were inoculated with the KrD1515 virus, they developed severe respiratory symptoms and tracheal lesions. However, there were no other clinical symptoms or pathologic lesions in other tissues. The virus was shed from the trachea for at least a week and from the cloaca for only a day. Our findings suggest that the KrD1515 virus is a recombinant virus between a Chinese TC07-2 IBV and a non-TC07-2 Korean IBV and engages in respiratory tropism in chickens.

**RESUMEN.** La bronquitis infecciosa aviar (IBA) es una enfermedad de los aves que es causada por el virus de la bronquitis infecciosa (IBV). El genotipo TC07-2 de IBV se ha extendido rápidamente en Asia Oriental desde que fue reportado por primera vez en China en 2007. En 2015, se aisló un virus de la bronquitis infecciosa con el genotipo TC07-2 (designado KrD1515) de gallinas ponedoras con signos respiratorios severos en Corea. En el presente estudio, los genes completos de lectura continua de los genes de la espícula (S) y de la nucleocápside (N) del virus fueron secuestrados y analizados. La filogenia del gen S1 reveló que el virus KrD1515 se agrupaba con virus con el genotipo TC07-2, mientras que el análisis filogenético del gen N reveló que el virus KrD1515 se agrupaba con virus de la bronquitis coreanos, pero no con el virus de China TC07-2. Pollos libres de patógenos específicos de diez días de edad fueron inoculados con el virus KrD1515 y desarrollaron signos respiratorios severos y lesiones truquerales. Sin embargo, no hubo otros signos clínicos o lesiones patológicas en otros tejidos. El virus fue eliminado de la tráquea por al menos una semana y de la cloaca por solo un día. Estos hallazgos sugieren que el virus KrD1515 es un virus recombinante entre un virus de la bronquitis infecciosa de China TC07-2 y un virus coreano distinto a la cepa TC07-2 y que presenta tropismo respiratorio en pollos.

**Keywords:** avian infectious bronchitis virus, phylogenetic analysis, TC07-2-like genotype, pathogenicity

**Abbreviations:** aa = amino acid; IB = infectious bronchitis; IBV= infectious bronchitis virus; EID50 = 50% egg infective dose; N = nucleocapsid; nt = nucleotide; ORF = open reading frame; pc = postchallenge; RT-PCR = reverse transcriptase–PCR; S = spike; SPF = specific-pathogen-free

Infectious bronchitis virus (IBV) is a *Coronavirus* species of the genus *Gammacoronavirus* in the family *Coronaviridae*. IBV has a single-stranded positive-sense RNA genome that is approximately 28 kb in size. It encodes four structural proteins, namely, the spike (S) glycoprotein, the membrane protein, the envelope, and the phosphorylated nucleocapsid (N) protein (7). Of these proteins, the S and N proteins harbor the B cell and T cell epitopes, respectively, that generate protective immunity and to act as a major determinant of tissue tropism. The S protein is cleaved by a cellular protein convertase into two subunits, namely, S1 and S2 (2,20). The S1 subunit contains hypervariable regions carrying epitopes for serotype-specific and virus-neutralizing antibodies.

IBV causes infectious bronchitis (IB) in chickens, which results in severe economic losses in the poultry industry each year despite the widespread use of live attenuated and inactivated vaccines. IBV is ubiquitous in most parts of the world where poultry are reared.

Numerous variants and serotypes of IBV, generated via point mutations, insertions, deletions, and genetic recombination, occur in poultry flocks worldwide. This has complicated the control of IB in the field in many countries (6,17).

Many IBV genotypes have been reported in Korea since IBV was first described in 1986 (9,12). Some genotypes (e.g., Mass, QX, and TC-07) occur in many countries while others (e.g., K1, KM91, and K3) are restricted to Korea. Currently, Mass-type and KM91-type (K2) vaccines (live attenuated and killed) are widely used to control the disease in poultry in Korea.

In 2007, a novel genotype of IBV that was designated TC07-2-like was isolated from broilers with respiratory illness (isolate TC07-2) in Guangdong, China (10). Two years later, the same genotype was also isolated in other far eastern countries, including Japan (designated JP-IV) (14) and Korea (designated new cluster 2) (11). Although QX-type IBVs are still the main type of IBV in Korea, there has been a recent increase in the frequency with which TC07-2-type IBVs are being isolated from vaccinated broilers and layers. Despite these changes, the genetic characteristics and tissue tropism of TC07-2-type IBVs in Korea remain poorly understood. In 2015,
a TC07-2-type IBV that was designated KrD1515 was isolated from a layer flock with severe respiratory illness in the Gyeonggi Province of the Republic of Korea. In the present study, we subjected this field isolate to genetic and pathologic investigations.

**MATERIALS AND METHODS**

**IBV isolate.** The IBV field isolate KrD1515, which is kept in the laboratory of the Avian Disease Research Division of the Animal and Plant Quarantine Agency of Korea, was used in the study. The virus was isolated in 2015 from the tracheas of 42-day-old layer chickens with severe respiratory signs in the Gyeonggi Province of the Republic of Korea. Virus isolation and identification were performed using egg inoculation in combination with the reverse transcriptase–PCR (RT-PCR) assay, as previously described (1,3). The KrD1515 virus was propagated in specific-pathogen-free (SPF) embryonated chicken eggs (Valo BioMedia, Adel, IA) as described previously (3). The IBV-containing allantoic fluids were harvested and stored at −70 C until use. The virus was titrated using the end-point dilution method with SPF chicken eggs, and the titers were expressed as 50% egg infective dose (EID$_{50}$) by the Reed and Muench method (16).

**RT-PCR and sequencing.** IBV RNAs were extracted from the allantoic fluids using a QIAamp viral RNA mini-kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RT-PCR was performed using an Invitrogen SuperScript III one-step RT-PCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The IBV23859R (5'-CCA TTA AAC AGA CTT TTT AGG TCT G-3') and oligo(dT)$_2$ (enclosed in the kit) were used to synthesize the complementary DNAs encoding the S and N genes of IBV, respectively. Thereafter, the IBV20311F (5'-CGG AAC AAA AGA CMG ACT TAG T-3') and IBV23859R primers were used to amplify cDNAs encoding the complete open reading frame (ORF) of the S gene while the IBV_full NF (5'-GAG CAA TAG CAA GAA AAG C-3') and IBV_full NR (5'-CTA CAT GCC TAT CTX CCT TCT T-3') primers were used to amplify the cDNAs encoding the complete ORF of the N gene. PCR conditions were 94 C for 3 min, 35 cycles at 94 C for 30 sec, 53 C for 30 sec, 68 C for 4 min, and a final step at 68 C for 5 min for the S gene, and 94 C for 3 min, 35 cycles at 94 C for 30 sec, 50 C for 30 sec, and 68 C for 2 min, and a final step at 68 C for 5 min for the N gene. Each PCR amplicon was cloned into the pGEM-T easy vector system (Promega, Madison, WI). All sequencing work was performed using the custom sequencing service provided by Cosmo Genetech Co., Ltd., (Seoul, Korea).

**Phylogenetic analysis.** The sequences of the S and N genes of representative IBV strains that were retrieved from the GenBank database system were used for sequence comparison and phylogenetic analysis. Editing and sequence analysis were performed using CLC Genomic Workbench 6.7.2 (CLC Bio, Aarhus, Denmark). Multiple amino acid (aa) sequences encoded by the S and N genes were aligned using the BioEdit sequence alignment editor. Phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replications; for this, MEGA 7.0 software was employed (19). The nucleotide sequence data reported here were deposited in the GenBank database system (MF176212 and MF176213).

**Animal experiment.** All animal experimental procedures were approved and supervised by the Institutional Animal Care & Use Committee of the Animal and Plant Quarantine Agency of Korea. Forty-four 7-day-old SPF chickens were purchased from a local company (Namduk SPF, Gyeonggi, Korea) and randomly divided into two groups that either underwent IBV challenge (n = 22) or served as mock-infected controls (n = 22). Each group was housed in a separate isolator equipped with an air filter (Three-shine, Daejeon, Korea). The challenged birds were inoculated via the ocularonasal route with the KrD1515 virus (10$^{0.5}$ EID$_{50}$ per 0.1 ml per dose), whereas the mock-infected birds were sham challenged with a phosphate-buffered saline solution in the same manner. The clinical signs of the birds were observed daily and recorded for 21 days after the challenge. Oropharyngeal and cloacal swabs were collected on day 0, 3, 5, 7, 9, 14, and 21 postchallenge (pc). Three birds in each group were randomly selected and humanely sacrificed on day 0, 3, 7, and 14 pc. The remaining 10 birds in each group were humanely euthanized on day 21 pc. Organs and tissues, namely the trachea, lungs, kidneys, proventriculus, and cecal tonsils, were collected carefully.

**Virus detection in the birds.** IBV in the swab and tissue samples was detected using an IBV-specific RT-PCR assay. Thus, the Maxime RT-PCR PreMix kit (nLRON Biotechnology, Gyeonggi, Korea) was used, according to the manufacturer’s instruction. The UTR1 and UTR2 primers, which target the 3’ untranslated region of the IBV genome, were used for the RT-PCR assay as described previously (1). The amplified RT-PCR products were subjected to electrophoresis on a 1.5% agarose gel.

**Tracheal ciliary activity test in the birds.** To measure the tracheal ciliary activity of each sacrificed bird, three, four, and three tracheal rings were cut from the upper, middle, and lower part of the dissected trachea, respectively. The rings were then placed in minimal essential medium (Gibco’, Grand Island, NY) in a 96-well plate and examined under light microscopy. The degree of ciliary activity was scored from 4 (100% activity) to 0 (no activity) as described previously (3).

**Virolologic and pathologic examination of the birds.** Half of the trachea, lung, kidney, proventriculus, and cecal tonsil tissues from the sacrificed birds were used to detect the virus with RT-PCR. The remaining half was fixed in 10% neutral buffered formalin, processed routinely, sectioned into 0.15 mm-thick sections, and stained with hematoxylin and eosin as described previously (3). The pathologic lesions were examined under a light microscope. The lesions were scored from 0 (no lesion) to 3 (severe lesion) as described previously (8).

**Statistical analysis.** The challenged and mock-infected birds were compared in terms of tracheal ciliary activity scores and tracheal lesion scores on days 0, 3, 7, and 14 pc using ANOVA and a Tukey-Kramer test. P values < 0.05 were considered to indicate statistically significant differences.

**RESULTS**

**Sequence analysis of the S gene and genotyping.** The complete ORF of the S gene of KrD1515 was amplified by RT-PCR, cloned into the pGEM-T easy vector, and then fully sequenced, which revealed an ORF of 3,516 nucleotides (nt) (1,186 aa). A GenBank BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the S gene sequence was most similar (97.1% nt identity) to that of the Chinese TC07-2-like IBV strain CK/CH/SD09/005 (accession no. KF668605). By contrast, the S gene of KrD1515 was much less similar (70.05–71.31%) to those of viruses used in locally available live IBV vaccines (K2 and Mass types). The S protein cleavage site in KrD1515 (HRRKR) was located between aa residues 542–546 and has the same sequence as that of the CK/CH/SD09/005 strain. A comparison of the complete ORF of the S gene of KrD1515 with that of the S gene of the CK/CH/SD09/005 strain revealed no deletions or insertions. Phylogenetic analysis using the S gene of KrD1515 and those of 31 reference IBV strains widely used for genotyping IBV isolates (Fig. 1) revealed that KrD1515 clustered among those with the TC07-2 genotype, including TC07-2, CK/CH/SD09/005, CK/CH/GD/KP10, and CK/CH/GD/NC10. TC07-2-type IBVs exhibited the greatest evolutionary distances from all other genotypes such as KM91 (major Korean genotype in 1990s), QX, TW (mainly found in Taiwan), Mass, K1 (Korean genotype 1), and K3 (Korean genotype 3). Thus, our results indicate
that KrD1515 belongs to IBVs with the TC07-2 genotype. Simplot analysis (13), revealed that KrD1515 shares high sequence similarity (95%) with the Chinese TC07-2 genotype but low sequence similarity (84%) with other genotypes, such as KM91 and QX over the entire S gene (data not shown).

Sequence analysis and phylogenetic analysis of the N gene. The complete ORF of the N gene of the KrD1515 virus was also amplified by RT-PCR, cloned into the pGEM-T easy vector, and then fully sequenced. The ORF of the N gene comprised 1,230 nt (409 aa). A GenBank BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the N gene was most similar (94.80% nt identity) to that of the Korean IBV strain K281-01 (accession no. AY790345) but was less similar to that of the Chinese TC07-2 type-IBV strain CK/CH/SD09/005 (86.83% nt identity). Deletions and insertions were not detected in the N protein of KrD1515.

Phylogenetic analysis using the N gene of KrD1515 and those of 28 reference IBV strains showed that Korean IBV strain K281-01 (accession no. AY790345) was less similar to that of the Chinese TC07-2 type-IBV strain CK/CH/S009/005 (86.83% nt identity). Deletions and insertions were not detected in the N protein of KrD1515. Phylogenetic analysis using the N gene of KrD1515 and those of 28 reference IBV strains showed that Korean IBV reference IBVs formed two phylogenetic clusters, namely KR (mainly found in Korea) and CH (predominant type in China) (Fig. 2). Most (17/19) of the Korean reference IBVs clustered in the KR cluster regardless of the genotype defined by the S1 gene. The KrD1515 virus also occurred in the KR cluster. By contrast, the Chinese TC07-2 IBV CK/CH/SD09/005, to which KrD1515 exhibited a close relationship using the S1 gene, was classified separately from the other groups. In Simplot analysis, KrD1515 shared high sequence similarity (>92%) with the SNU8067 strain (KR cluster) throughout the entire N gene but shared low sequence similarity (<80%) in the region from nt 400 to 850 with Chinese TC07-2 IBV (CK/CH/SD09/005) (data not shown).

Pathogenicity for SPF chickens. The pathogenicity of the KrD1515 virus was assessed by inoculating 7-day-old SPF chickens oculonasally with the virus or by mock infecting them. None of the mock-infected birds exhibited any signs of disease. By contrast, all challenged birds exhibited respiratory signs starting on day 5 pc. The signs continued until day 14 pc, after which all birds recovered. Other clinical signs such as diarrhea were not observed at any time point. The pathologic results of the tracheas of the challenged and mock-infected birds are summarized in Table 1. On days 3 and 7, the tracheal ciliary activity of the challenged birds was almost completely inhibited compared with that of birds in the control group (P < 0.05). The tracheal ciliary activity was almost completely restored on day 14 pc. None of the mock-infected birds had tracheal ciliary activity scores that exceeded 1. Histology showed that the challenged birds had severe tracheal lesions on days 3 and 7 pc compared with the control group (P < 0.05) (Table 1). The tracheal lesions involved the loss of cilia and epithelial cells, epithelial cell degeneration, epithelial cell hyperplasia, and infiltration of inflammatory cells in the surface and lamina propria. On day
7 pc, some of the birds exhibited regeneration of the damaged tracheal epithelia. By day 14 pc, there was marked regeneration of the tracheal lesions. The trachea had recovered completely by day 21 pc. None of the mock-infected birds exhibited obvious tracheal lesions during the experiment. None of the challenged or mock-infected birds exhibited any lesions on other tissues or organs.

**Virus shedding and tissue distribution.** The virologic results of the challenged and mock-infected birds are summarized in Table 2. Analysis of oropharyngeal and cloacal swabs taken at regular intervals showed that the challenge virus was shed from the oral cavity of the challenged birds between days 3 and 9 pc and from the cloaca from two of three birds on day 7 pc only. Moreover, analysis of the organs and tissues of the challenged birds showed that the challenge virus was present in all tested tissues, namely the trachea, lung, kidney, proventriculus, and cecal tonsils. The viruses were detected in the trachea, lung, kidney, and proventriculus but not in the cecal tonsils on day 3 pc, in all tissues on day 7 pc, and in the trachea, lung, kidney, and cecal tonsils but not the proventriculus on day 14 pc. On day 21 pc, the virus was still detected in the lung, kidney, and cecal tonsils of some challenged birds. Virus was not detected in any of the swabs or tissues from the mock-infected birds at any time point.

**Table 2. Shedding and tissue distribution of the virus in SPF chickens after challenge with the KrD1515 virus.**

<table>
<thead>
<tr>
<th>Sample tested</th>
<th>0 dpc</th>
<th>3 dpc</th>
<th>5 dpc</th>
<th>7 dpc</th>
<th>9 dpc</th>
<th>14 dpc</th>
<th>21 dpc</th>
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<td>NT</td>
<td>3/3</td>
<td>NT</td>
<td>1/3</td>
<td>0/10</td>
</tr>
<tr>
<td>Lungs</td>
<td>0/3</td>
<td>2/3</td>
<td>NT</td>
<td>3/3</td>
<td>NT</td>
<td>2/3</td>
<td>2/10</td>
</tr>
<tr>
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<td>NT</td>
<td>2/3</td>
<td>NT</td>
<td>1/3</td>
<td>2/10</td>
</tr>
<tr>
<td>Proventriculus</td>
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<td>3/3</td>
<td>NT</td>
<td>3/3</td>
<td>NT</td>
<td>0/3</td>
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<td>0/3</td>
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<td>0/10</td>
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<tr>
<td>Lungs</td>
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<td>NT</td>
<td>0/3</td>
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</table>

A dpc = days post-challenge; OP = oropharyngeal swab; NT = not tested.

The data are expressed as the no. positive/no. tested.

The emergence of recombinant variants of IBV, caused by gene mutations, such as insertions, deletions, and recombination, has been reported in many countries. In this study, KrD1515 was classified as a TC07-2-type IBV based on the results of phylogenetic analysis using the S1 gene. Conversely, KrD1515 clustered with the non-TC07-2-type Korean IBVs belonging to the KR cluster (major cluster in Korea) but not with the Chinese TC07-2-type IBVs, based on the results of phylogenetic analysis using the N gene, indicating that it is a recombinant virus belonging to the non-TC07-2-type Korean IBVs. The occurrence of recombination was supported by the results of the Simplot analysis. Recombination events have also been reported in non-TC07-2-type IBVs in Korea (4,12,15). This suggests that the KrD1515 acquired its N gene from a pre-existing Korean IBV or a pre-existing Korean IBV acquired the S gene of a Chinese TC07-2-type IBV via a recombination event.

IBV isolates exhibit various tissue tropisms, including respiratory, nephrotic, and gastrointestinal tropisms. Recombination events between different types of IBV in the field can lead to the emergence of novel variants with different tissue tropisms (6,18). TC07-2 IBV strains are known to exhibit respiratory tropism (10,11). In this study, KrD1515 caused severe respiratory symptoms and severe damage to the trachea of SPF chickens, but did not display other clinical signs or pathologic lesions, such as nephritis or enteritis, even though the virus was detectable in various organs and tissues. Our results indicate that despite the recombination event with a non-TC07-2-type IBV, the KrD1515 virus is respiratory in chickens. This may be in part explained by the fact that the acquired genes (e.g., N gene) have little effect on tissue tropism.

The possibility that recombinant IBVs may escape vaccine-induced immunity is supported by the sequence analysis of the KrD1515 virus; the S protein of KrD1515 shows strong sequence divergence from S proteins in viruses used in locally available IBV vaccines (K2 and Mass types), with an aa identity of <70%. This suggests that an antigenic mismatch might exist between Korean TC07-2-type IBVs and currently available IBV vaccines. This may not be particularly problematic at present because, although TC07-2-type IBVs are frequently isolated in Korean poultry farms, they are still not the main IBV type in Korea. However, should TC07-2-type
IBVs become more prevalent and result in substantial economic losses for poultry farmers, the development and use of TC07-2-type IBV vaccines should be considered. Moreover, to prevent serious economic losses, it is important to continuously subject IBVs in the field to intense surveillance.

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