Repeated avian infectious bronchitis virus infections within a single chicken farm

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ABSTRACT. Genotyping of avian infectious bronchitis virus (IBV) was performed on trachea and kidney samples of six chickens obtained from a single farm in Japan. Using two primer sets targeting the spike (S) protein gene, the S1 and S2 regions of DNA fragments were amplified. Sequences of amplified S1 fragments extracted from both organs were identical among the six chickens, showing a JP-I genotype. Sequences of amplified S2 fragments differed between trachea and kidney samples. The kidney profile showed a group IV genotype, whereas the trachea profile showed an unclassified group. This result showed that two different IBVs infected the six chickens. The first IBV infection induced poor protective immunity in this farm, permitting a second IBV infection to occur.

KEY WORDS: coronavirus, infectious bronchitis virus, virus infection

Avian infectious bronchitis virus (IBV) is a highly contagious pathogen of chickens that replicates primarily in the upper respiratory tract. Secondary replication also occurs in the lower respiratory tract and non-respiratory epithelial cells of the alimentary tract, kidney, and gonads, suggesting various pathologies depend on the type of infected tissue [1]. IBV infection affects egg production of laying hens and growth of broiler chickens, leading to economic losses in the poultry industry that cannot be ignored [3]. IBV is spread by aerosol and is endemic in many countries where chickens are farmed.

IBV is a member of the coronaviruses in the genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*, order *Nidovirales*, and has a 27.6 kb positive sense non-segmented RNA genome [5]. Coronaviruses virions are commonly enveloped, pleomorphic, approximately 120 nm in diameter, and under electron microscopy viruses are characterized by a surface projection similar to a solar corona constructed by the viral spike (S) protein. The S protein cleaves to two subunits, the amino-terminal S1 and carboxy-terminal S2. The S1 and S2 subunits are responsible for the virus attaching to cells [11] and membrane fusion [4, 10], respectively. The S protein presents antigenic determinants that induce neutralizing antibodies [14] and triggers the cellular immunity [15]. There are dozens of IBV genotypes based on *S* gene classification utilizing the variable nucleotide region of the gene. The 671 bp-region in the 5′ of the *S1* gene is frequently used for the genotyping [9, 12], as is the 400 bp-region of the *S2* gene [6–8]. Many genotypes of IBVs are reported worldwide. As amino acid mutations in the S1 protein are known to cause poor protection by IBV vaccines, many live and killed vaccines against various genotypes have been developed to march the new genotypes [3]. It is useful to know the genotype of the prevailing IBV within the chicken farm before the vaccination in order to administer a suitable vaccine.

The present study performed genotyping of IBV extracted from the trachea and kidney samples of six dead laying hens (22–37 days old) kept in a single farm in the Kyusyu region of Japan using reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted and purified using the RNA tissue Kit SII (FUJIFILM Wako Pure Chemical Co., Tokyo, Japan) and QuickGene-Mini80 (FUJIFILM Wako Pure Chemical Co.) according to the manufacture's instructions. Using two primer sets for the 400 bp-length fragment in the S2 [6–8] and the 671bp-length fragment in the S1 [9] regions of IBV, DNA fragments were amplified using the one-step PrimeScript RT-PCR kit (Takara Bio Inc., Tokyo, Japan). The modified reverse primer for S2 (5′-ARYAARCCATTATAYTCWCGRGcac-3′, where underscores and small letters show modified nucleotides and added nucleotides, respectively) was used in this study in place of the original sequence (5′-AGCAAACCATTATATTCACGAG-3′) to adapt the nucleotide substitution from field IBVs. Primer sets and the purified RNA were pre-incubated at 94°C for 2 min and chilled on ice. The reaction mixture was then added and the reverse transcriptions were performed at 45°C for 30 min following heat inactivation at 94°C for 2 min. The DNA fragments were then amplified for 40 cycles at three step temperatures comprising 94°C for 15 sec for denaturation, 50°C, for 30 sec for annealing, and 72°C for 30 sec for extension. The length and amount of

(A) Sequences of amplified S1 region

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Trachea     1 GATGTTGGTGAAGTCACTGTTTATAGTGACTCTTTTGTTTGCACTATGTAGTGCAATTCT      60
Kidney      1 GATGTTGGTGAAGTCACTGTTTATAGTGACTCTTTTGTTTGCACTATGTAGTGCAATTCT      60
              ************************************************************
Trachea    61 CTATGATAATAATACTTATGTTTACTACTATCAGAGTGCCTTCAGGCCACCGTTTGGTTG     120
Kidney     61 CTATGATAATAATACTTATGTTTACTACTATCAGAGTGCCTTCAGGCCACCGTTTGGTTG     120
              ************************************************************
Trachea   121 GCATGTACATGGAGGTGCTTATGCAGTAGTTAATGTGAGTCAGGAAACTAATAATGCTGG     180
Kidney    121 GCATGTACATGGAGGTGCTTATGCAGTAGTTAATGTGAGTCAGGAAACTAATAATGCTGG     180
              ************************************************************
Trachea   181 CAGTGCTTCTGAATGTACTGCTGGCGCTATTCATTGGAGTAAGAATTTTAGTGCAGCCTC     240
Kidney    181 CAGTGCTTCTGAATGTACTGCTGGCGCTATTCATTGGAGTAAGAATTTTAGTGCAGCCTC     240
              ************************************************************
Trachea   241 TGTAGCCATGACTACACCATTGAGTGGTATGAATTGGTCAACTTCACAATTTTGTACGGC     300
Kidney    241 TGTAGCCATGACTACACCATTGAGTGGTATGAATTGGTCAACTTCACAATTTTGTACGGC     300
              ************************************************************
Trachea   301 TCACTGTAATTTTTCCAATATTGTAGTGTTTGTTACACACTGCTATAAACACGGCCCTCA     360
Kidney    301 TCACTGTAATTTTTCCAATATTGTAGTGTTTGTTACACACTGCTATAAACACGGCCCTCA     360
              ************************************************************
Trachea   361 ATTTTGCCCATTAACAGGTTTTATTGAAAAGGGCCATATTCGTATTTCTGCTATGAAAAA     420
Kidney    361 ATTTTGCCCATTAACAGGTTTTATTGAAAAGGGCCATATTCGTATTTCTGCTATGAAAAA     420
              ************************************************************
Trachea   421 TGTAGGTAGTGGACCTGCAGACTTGTTTTATAACTTAACAGTTCCTGTGACTAAATATTC     480
Kidney    421 TGTAGGTAGTGGACCTGCAGACTTGTTTTATAACTTAACAGTTCCTGTGACTAAATATTC     480
              ************************************************************
Trachea   481 TAAATTTAGATCACTTCAATGTGTTAATAATCAAACATCTGTGTACTTAAATGGAGATCT     540
Kidney    481 TAAATTTAGATCACTTCAATGTGTTAATAATCAAACATCTGTGTACTTAAATGGAGATCT     540
              ************************************************************
Trachea   541 TGTTTTTACTTCTAATGAGACTACAGATGTTGTAGGAGCAGGTGTTTCTTTTAAAGCTGG     600
Kidney    541 TGTTTTTACTTCTAATGAGACTACAGATGTTGTAGGAGCAGGTGTTTCTTTTAAAGCTGG     600
              ************************************************************
Trachea   601 TGGACCTATAACTTATAAAGTTATGAGAGAGGTAAAAGCCCTAGCTTATTTTA           653
Kidney    601 TGGACCTATAACTTATAAAGTTATGAGAGAGGTAAAAGCCCTAGCTTATTTTA           653
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(B) Sequences of amplified S2 region

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                                                    1  2 3  4  5
Trachea     1 TCCAAATTAATTGCCTTCAATATGTTTGCGGCAATTCTTTGGAGTGTAGAAAATTGTTTC      60
Kidney      1 TCCAAATTAATTGCCTTCAATATGTTTGTGGCAATTCTATTGAATGCAGAAAATTGTTTC      60
              ****************************  *********  *  **  **  *************
                6         7  8    9                 10          11 12
Trachea    61 AACAATATGGTCCGGTTTGTGATAACATATTGTCTGTTGTAAATAGTGTTGGTCAAAAAG     120
Kidney     61 AGCAATATGGTCCTGTCTGTGACAACATATTGTCTGTAGTAAATAGTGTTGGTCAGAGAG     120
              *  ***********  **  *****  ***************  ****************** *  **
                13                     141516   17  18 1920     21
Trachea   121 AAGATATGGAACTTTTAAGTTTCTATTCTTCTACTAAACCAGCTGGTTATAATGCACCTG     180
Kidney    121 AAGACATGGAACTTTTAAGTTTCTATTCTTCTACTAAACCAAAAGGTTTTGACACACCAG     180
              ****  ***********************************     ****  *  *     ****  *
                22 23      24        26          27        28              29
Trachea   181 TTTTTAGTAATATTAGTACTGGTGATTTTAATATCTCTCTTTATTAACACCACCTAGCA     240
Kidney    181 TTCTGAGTAATGTTAGTACTGGTGCTTTTAATATCTCACTTTTGTTAACACCACCTAGTA     240
              **  *  *******  ************  ***********  *****  ************** *
                30      31   32
Trachea   241 GTCCTAGCGGTCGTTCTTTTATAGAAGATTTGTTATTTACAAGTGTTGAAACAGTTGGTT     300
Kidney    241 GTACTAGTGGGCGTTCTTTTATAGAAGATTTGTTATTTACAAGTGTTGAAACAGTTGGGT     300
              **  ****  *****************************************  *
                                              34          35        36
Trachea   301 TGCCAACTGATGCTGAATATAAAAAATGCACAGCAGGACCTTTAGGTACTCTTAAGGATC     360
Kidney    301 TGCCAACTGATGCTGAATATAAAAAATGCACAGCGGGACCTTTGGGTACTCTTAAAGATC     360
              **********************************  ********  ***********  ****
Trachea   361 TTATCTGTGCTCGTGAATA           379
Kidney    361 TTATCTGTGCTCGTGAATA           379
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Fig. 1. Partial sequence of the *S* gene of IBV. Samples isolated from tracheas and kidneys of six chickens were amplified by RT-PCR either with the S1 primer pair (A) or S2 primer pair (B). DNA fragments read from both termini by the dideoxy-chain-termination method were aligned. Asterisks show the nucleotides that are identical among the samples. Blanks show the nucleotides that differ between tracheas and kidneys. The 653 bp-regions of S1 (A) and the 379 bp-regions of S2 (B) are shown. A: Open circles indicated on the nucleotides shows the minor nucleotide changes found in one of six chickens (A63G, T86C and C246T). B: Numbers (1–36) shown on the nucleotides in the blank space corresponds to those in Fig. 2.

amplified DNA fragments were confirmed using the 2.0% (w/v) agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer. Finally, the DNA fragments were purified using a NucleoSpin Gel and PCR Clean-Up kit (Machery-Nagel Takara, Tokyo, Japan). DNA sequencing was determined by the commercial service (Fasmac Co., Ltd., Atsugi, Japan).

Firstly, the 653 bp sequences of S1 were confirmed by duplicate readings from both termini. One minor nucleotide change was found in the kidney sample of one chicken (T to C at the 86th nucleotide, T86C) and in the trachea samples of two chickens (A63G and C246T). Within these changes, smaller but apparent signals showing the nucleotide those are same to other five chicken samples were found in every sequence output (data not shown). Therefore, it was considered that viruses that infected into tracheas and kidneys of these six chickens possessed essentially identical 653 bp sequences of S1 (Fig. 1A). Phylogenetic analysis was then applied using the unweighted pair group method with arithmetic mean (UPGMA) and bootstrap values calculated from 1,000 replications (GENETYX-MAC, GENETYX Co., Tokyo, Japan). The S1-based genotype of IBV was classified into Far East
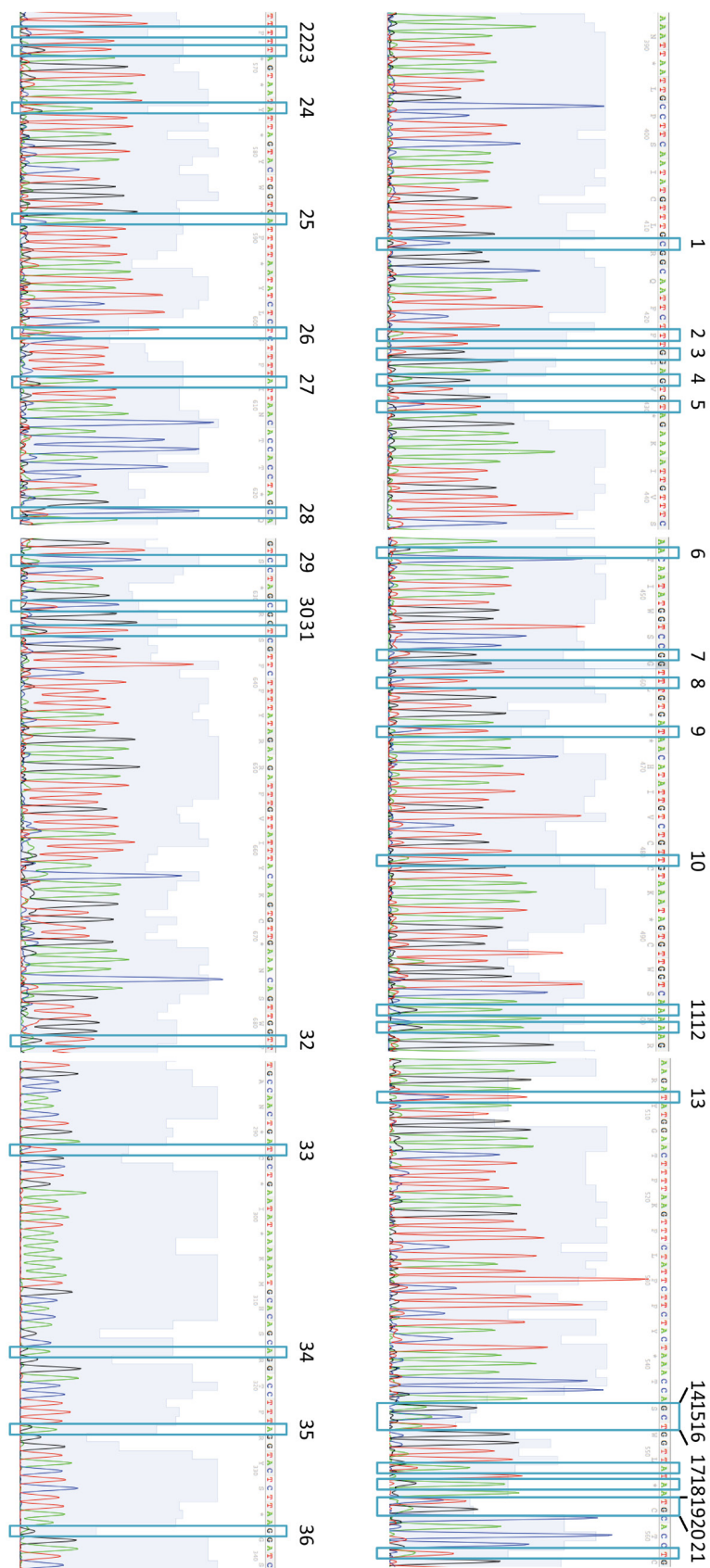
Fig. 2. Minor peaks in the S2 region of trachea samples. Representative nucleotide sequencing peaks of the S2 region of trachea samples are shown. Green, black, blue, and red lines show the four nucleotide bases, adenine, guanine, cytosine, and thymine, respectively. Thirty-six nucleotides that differed between the samples obtained from tracheas and kidneys are marked and boxed. Under the main nucleotide peak, a smaller peak is present, but its color is identical to that in the kidney sample.
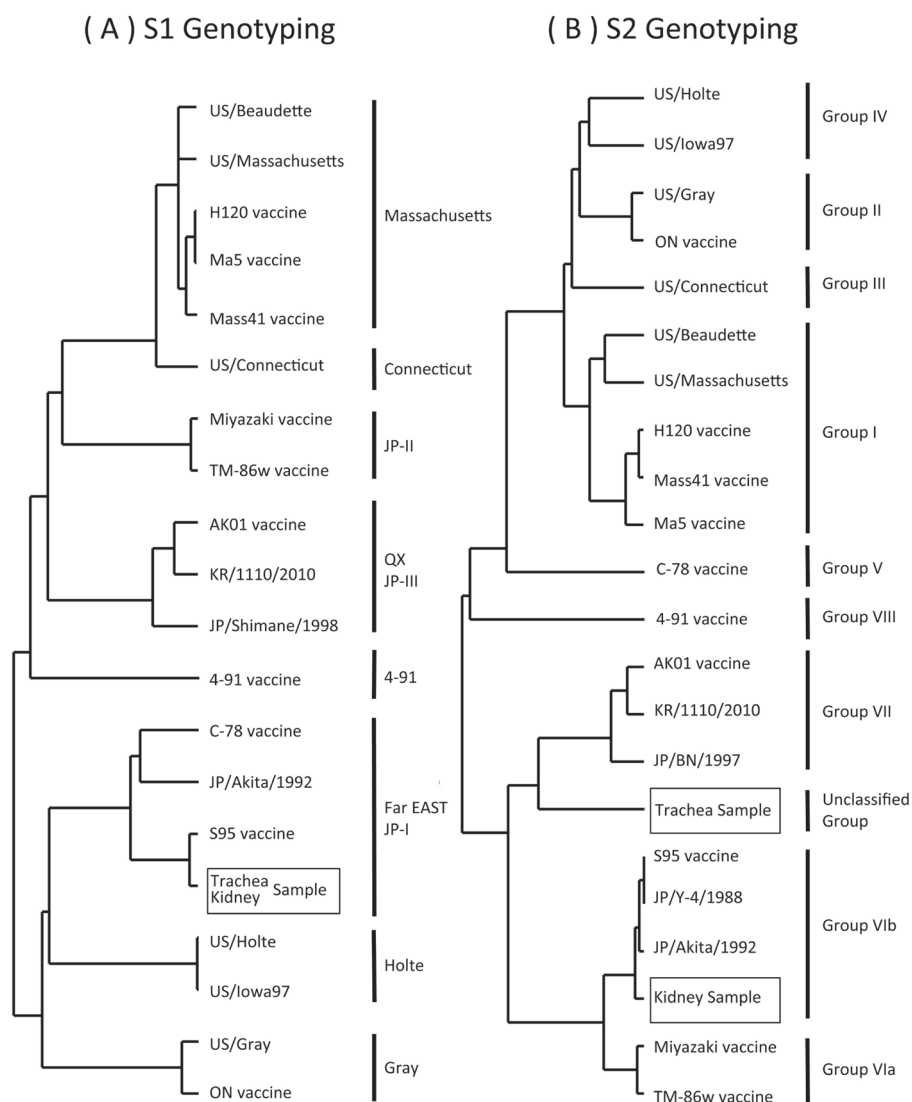
Fig. 3. Phylogenetic tree based on the partial *S* gene sequences. Phylogenetic tree analysis based on the nucleotide sequences of amplified S1 (A) and S2 (B) regions were performed using the unweighted pair group method with arithmetic mean (UPGMA) method. Naming of groups followed previous publications [9–13]. Sequences of representative IBV were either downloaded through NCBI, PubMed, Nucleotide-searching site, or obtained by our own sequencing.

[12] and JP-I genotype [15] (Fig. 3A). Although the farm from which the samples were obtained administered two doses of live attenuated IBV vaccine (AK-01 and C-78), the S1 sequences of vaccines strains were not identified from the samples.

Secondly, the 379 bp sequences of S2 were confirmed by duplicate readings from both termini. None of the S2 sequences derived from the IBV vaccines were found. Sequences of S2 fragments extracted from tracheas were identical among the six individuals and those extracted from kidneys were also identical among kidney samples. Surprisingly, the S2 sequence differed between trachea and kidney samples; 36 (9.5%) nucleotides changed between kidney and trachea samples (Fig. 1B). Within the sequencing signals of 36 nucleotides obtained from the trachea samples, lower but apparent signals identical to those obtained from kidney samples were found (see representative in Fig. 2). No such minor signals were found in the kidney samples, indicating that two different IBVs infected individual chickens sequentially. In brief, it was estimated that the first IBV propagated from the trachea to the internal organs, namely the kidneys, although some of the IBV still remained in the trachea, after which the second IBV superinfected to the trachea. The S2-based genotype of IBV in the kidney was classified into group VI (Fig. 3B). Interestingly, the majority of IBV in the trachea was not classified into group VI, but into an unclassified new group (Fig. 3B).

It is unclear why S2 sequences differed between the kidney and trachea samples of the six chickens, although the S1 sequences were similar (Fig. 1). One hypothesis is that the primer set for S2 can amplify a wider variety of IBVs than S1. Namely, the S1 primer pair amplified IBV (Far East, JP-I genotype) that is a unique population in kidneys and a minor population in the trachea, but may not amplify the major population of IBV co-infecting the trachea. However, the S1 primer pair, which we used, is synthesized on the conserved region of the S1 gene and is frequently used for IBV genotyping worldwide [1, 9]. Therefore, primer

mismatching is unlikely. Another hypothesis is recombination of the IBV genome. Coronaviruses are known to frequently undergo spontaneous recombination [3]. It is possible that the host's immune response against IBV promotes genome recombination to escape immunological pressure. IBV in the trachea may be a recombinant virus between the S1 and S2 regions of the *S* gene and thus IBV in the kidney and trachea can have the same S1 sequence, but different S2 sequences. Recently S2 subunits of IBV have been clearly identified as determinants of cellular tropism [2]. IBV with a specific S2 sequence may be superior for growth in chicken kidneys. Viruses isolated from trachea and kidney samples using primary chicken kidney (CK) cells or embryonated hen eggs would be useful to answer the above questions. However, a field IBV does not simply grow in CK cells or eggs without adaptation. Therefore, investigating virus isolation and sequencing of the entire *S* gene are potential future steps of our study.

Immunity against IBV by vaccination is useful to reduce economical loss in farms [5]. Even in vaccinated chickens, however, IBV infections are found, as demonstrated in this study. The present study revealed that the different S2 genotypes of IBVs were found in the trachea of six chickens in a single farm, although the S1 genotype was similar. Co-infections of two different IBVs, such as a field IBV and possibly the live attenuated IBV vaccine in the trachea, have been reported [13]. Our results indicate that two different field IBVs successively infected the chickens during the short period on the farm. It is suggested that the immunity raised by the vaccination and the IBV natural infection is not versatile, and allows for repeated IBV infection. To enhance the efficacy of vaccinations in the field, matching of prevailing IBV genotypes and vaccine genotypes is useful. Our study suggests that S1 genotyping alone is insufficient to select the appropriate IBV vaccines in the field, in which genomic recombination of IBVs frequently occurs. The combination of S1 and S2 genotyping may contribute to applying suitable vaccines against IBV infection.

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