

Detection of infectious bronchitis virus strains similar to Japan in Taiwan

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ABSTRACT. A total of 1,320 tracheal samples from 66 broiler flocks sent to slaughterhouses and 42 tracheal samples from 42 flocks of local chickens in the field were collected for infectious bronchitis virus (IBV) gene detection by reverse transcription polymerase chain reaction using nucleocapsid-specific primers and spike-specific primers. Prevalence in broiler flocks was 39.4% (26/66) and in local chicken flocks was 11.9% (5/42). Several IBVs similar to Japan were detected in Taiwan. One-direction neutralization revealed that the reference antisera did not offer protection against the IBVs similar to those from Japan.

KEY WORDS: infectious bronchitis, Japan, prevalence, Taiwan

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Avian infectious bronchitis virus (IBV) belongs to genus *Gammacoronavirus*, and its genome encodes four structure proteins, spike glycoprotein (S), and membrane, envelope and nucleocapsid (N) proteins [2, 11]. New variant IBV strains in chickens continually emerge worldwide owing to the high mutation rate of its genome [4]. Various vaccines have been developed but the disease still occurs, because different serotypes of the virus do not cross-protect and vaccine failures are often associated with the emergence of antigenic variants that differ from the vaccine viruses [9, 14].

IBVs in Taiwan can be divided into two groups, Taiwan Group I (TW-I) and Taiwan Group II (TW-II) [16]. In order to control this disease, our laboratory has developed two live IB vaccines from the TW-I and TW-II IBV strains [9]. They can effectively protect chickens against homologous challenges [9]. They have been approved by the Taiwan government and will be used in the field in the poultry industry in Taiwan. The objective of this study was to investigate the IBV status in the field in order to prevent this disease in Taiwan.

In order to investigate the IBV prevalence and types in the field, a total of 66 broiler flocks from slaughterhouses were sampled in different seasons in 2013: 17 flocks in spring, 13 in summer, 15 in fall and 21 in winter. The broilers were at the market ages of 5 to 6 weeks old and sampled throughout Taiwan. The samples from slaughterhouses were named S before sampling numbers. Twenty tracheas were taken from each flock, and five tracheas were pooled into one tube for IBV gene detection using reverse transcription polymerase chain reaction (RT-PCR). The pooled tracheas were homogenized with tryptose phosphate broth (TPB), and the nucleic acids were extracted using a commercial viral nucleic acid

extraction kit (Geneaid Biotech. Ltd., Taipei, Taiwan). In addition, 42 tracheal samples from 42 local chicken (LC) flocks [3] (one trachea per flock) were collected in the field in Southern Taiwan and named TC1 to TC42.

The presence of IBV in the trachea was evaluated by RT-PCR using the N-specific primers NP1/NP2 [8]. The primer sequences for this gene are as follows: NP1: 5'-GGTA G (C/T) GG (C/T) GTTCCTGATA A-3' (26029–26048, H120 numbering, GenBank accession number: FJ888351) and NP2: 5'-TCATCTTGTC (A/G) TCAC CAAAA-3' (26647–26628). The RT-PCR was performed according to a previously described method [8]. The RT-PCR products produced with this primer set were sequenced and compared. The RT-PCR products were sequenced at least twice from at least two RT-PCR products to make sure that the sequences were identical.

The samples that were IBV positive with the N-specific primer were subjected to RT-PCR and sequencing using the S-specific primer, oligo5/rC3-r for genotyping [17]. The sequences of these primers are as follows: oligo5: 5'-AAACT GAACA AAAGA CAGAC TTAG-3' (20251–20274, H120 numbering, GenBank accession number: FJ888351) and rC3-r: 5'-(A/G) CAAT GTGTA ACAA (T/C) ACT-3' (20655–20637). The same RT-PCR procedure was performed, except the annealing temperature for this primer set was 52°C. For LC field cases, one trachea from each flock was subjected to RT-PCR using the same method described for broilers. The prevalence differences among different seasons or chicken types were analyzed using chi-square tests or Fisher's exact tests when any cell having expected value less than 5 [12]. The level of a significant difference was set at α 0.05.

All the tracheas from the slaughterhouses and from LC flocks were free of obviously gross lesions. Sixty-six flocks were sampled in the study period. Twenty tracheal samples per flock were taken for a total of 1,320 tracheal samples. Five trachea samples were pooled into one tube for IBV gene detection, resulting in four tubes from 20 tracheal samples per flock. A flock was considered positive, if any one tube showed positive. Among the 66 flocks, 26 (39.4%) were positive for IBV infection. The prevalence in each season

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was 29.4% (5/17, positive flock number/tested flock number) in spring, 38.5% (5/13) in summer, 13.3% (2/15) in fall and 66.7% (14/21) in winter. The prevalence in winter was higher than that in other seasons ($P < 0.05$). Among LC flocks, the presence of IBV was lower than that in broiler chickens, with a flock prevalence of 12% (5/42) ($P < 0.05$). The lower prevalence in LC flocks might be due to sampling differences, because 20 tracheas were sampled from each broiler flock but only one trachea was sampled from each LC flock.

The present investigation might have under-estimated the actual prevalence, because we only sampled tracheas for virus detection. IBV can be detected in the trachea during the first 3 to 5 days post-infection. After this period, the virus titer falls quickly in the second week [6]. We may have missed the acute infection stage when sampling slaughterhouses due to the elapsed time between the beginning of the infection and sampling. Indeed, the present prevalence is lower than that reported in previous studies in the United Kingdom and Jordan [10, 13]. The prevalence in those studies reached about 60%, because the sampled chickens exhibited respiratory signs of infection or IB was suspected during sampling. In the present investigation, the broilers may have been healthy, because they reached market age for the slaughterhouses.

Sequences were compiled and aligned using the Lasergene software package (DNASTAR, Madison, WI, U.S.A.). Phylogenetic trees were constructed with the neighbor-Joining method using MegAlign for the N gene and S gene to evaluate the relationships of the sampled IBVs with reference strains. The reference IBV strains that were used and their accession numbers are as follows: Armidale: DQ490205, Vic: DQ490221, CK/CH/LAH/99I: DQ167129, CK/CH/LGD/96I: DQ167136, CK/CH/LHN/00I: DQ167143, CK/CH/LSC/99I: DQ167147, CK/CH/LSHH/03II: DQ167150, J2: AF286303, LX4: AY338732, Q1: AF286302, QXIBV: AF193423, AF199412, SAIBK: DQ288927, T3: AF227438, JP8127: AY296744, JP8147: AY363966, JP8443: AY296745, AY363967, JP9758: AY296746, AY363968, JP/Fukui/2000 (JP-III): AB120646, JP/Toyama/2000 (JP-I): AB120644, JP/Yamanashi/93 (JP-II): AB120638, K234-02: AY790352, K281/01: AY257062, K40/09: HM486957, K514-03: AY790354, K748-01: AY790347, KM91: FJ807946, JQ977698, H120: KF188436, AY028296, 2296/95: AY606321, AY606334, 2575/98: AY606314, AY606327, 3374/05: EU822337, 3382/06: GQ229232, THA361052: GU111582, THA40151: GQ503612 and 4/91: AF093793, AF093794, EU780081.

The phylogenetic tree constructed using the N gene sequences is shown in Fig. 1. The Taiwanese strain could be distinguished from the other IBVs using this N gene. However, this segment could not be used to separate the Taiwan strain into its different groups, TW-I and TW-II. In order to distinguish TW-I from TW-II, we used primers located at the 5' terminus of the S gene, and the resulting phylogenetic tree could distinguish between TW-I and TW-II (Fig. 2). There were more TW-II samples than TW-I samples in this investigation. The S1 and S7 isolates might be from the 4/91 vac-

cine strain, because they were very similar (Figs. 1 and 2). The S36, S75, S76 and S77 isolates were from another vaccine, H120, commonly used in Taiwan.

The striking finding in this investigation was the appearance of IBVs similar to those reported in Japan, including the S14, S41, S78 and S79 isolates and a field case, TC3. Sixteen percent (5/31) of IBVs detected were similar to those isolates in Japan. The S14, S41, S78 and S79 isolates were sampled from Changhua (Central Taiwan), Chiayi (South-central Taiwan), Hsinchu (North-central Taiwan) and Taoyuan (Northern Taiwan), respectively. The TC3 field case was from Southern Taiwan. All the IBV strains that were similar to Japanese ones were from different locations, indicating that these IBVs might be widespread in Taiwan. The sequence similarity among them reached 95.38% to 100%.

Eight tracheal samples positive for the IBV gene were selected for virus isolation, including S14, S41, S78, TC3, TC4, TC5, TC17 and TC38, using specific-pathogen-free (SPF) chicken embryos (Animal Health Research Institute, Chiding, Taiwan). Tracheal samples were frozen at -20°C . After thawing, the organs were homogenized in sterile TPB in a 1:10 ratio. The homogenate was then centrifuged at 1,500 rpm for 5 min. The supernatant was treated with a 1% mixture of ampicillin, streptomycin and amphotericin (Biological Industries, Kibbutz Beit-Haemek, Israel) for 1 hr at room temperature and then inoculated into the allantoic cavity of five 9-to-11-day-old SPF embryonated eggs. The eggs were incubated for 5 days and candled each day. After 5 days of incubation, the eggs were chilled at 4°C , and the allantoic fluid was collected. Each sample was given two blind passages before being considered negative. The presence of IBV in the allantoic fluid was detected using RT-PCR with NP1/NP2 primers [8]. Only two IBVs, S78 and TC3, were isolated. The IBVs were confirmed as IBV using sequencing. The IBV isolates were then used for one-direction neutralization tests with reference anti-IBV antisera.

The one-direction neutralization tests were performed using a constant virus-constant antiserum procedure as described previously [15]. The antisera used for this test included anti-TW-I, anti-TW-II and anti-Mass (Charles River Lab, North Franklin, CT, U.S.A.) [4]. Briefly, 100 μl of virus (containing 100 50% viral embryo infectious doses) was incubated with 100 μl of antiserum (containing 20 units) at room temperature for 1 hr. The infectivity of the virus-antiserum mixture was assayed in 10 9-to-11-day-old SPF chicken embryos. After 1 week, embryos were evaluated for IBV infection by observing for death, dwarfing and urate deposition as described for virus titration. In the control groups, antisera were replaced with PBS. A virus was considered to match the serotype of the antiserum, if the antiserum protected five or more of the embryos. The results showed that the two new IBV isolates similar to Japan, S78 and TC3, were not neutralized completely with the reference antisera. The protection rates for S78 by anti-TW-I, anti-TW-II and anti-Mass were 0/10 (numbers protected/ numbers tested), 0/10 and 0/10, respectively. Those for TC3 were 1/10, 4/10 and 0/10, respectively. The results indicated that both viruses

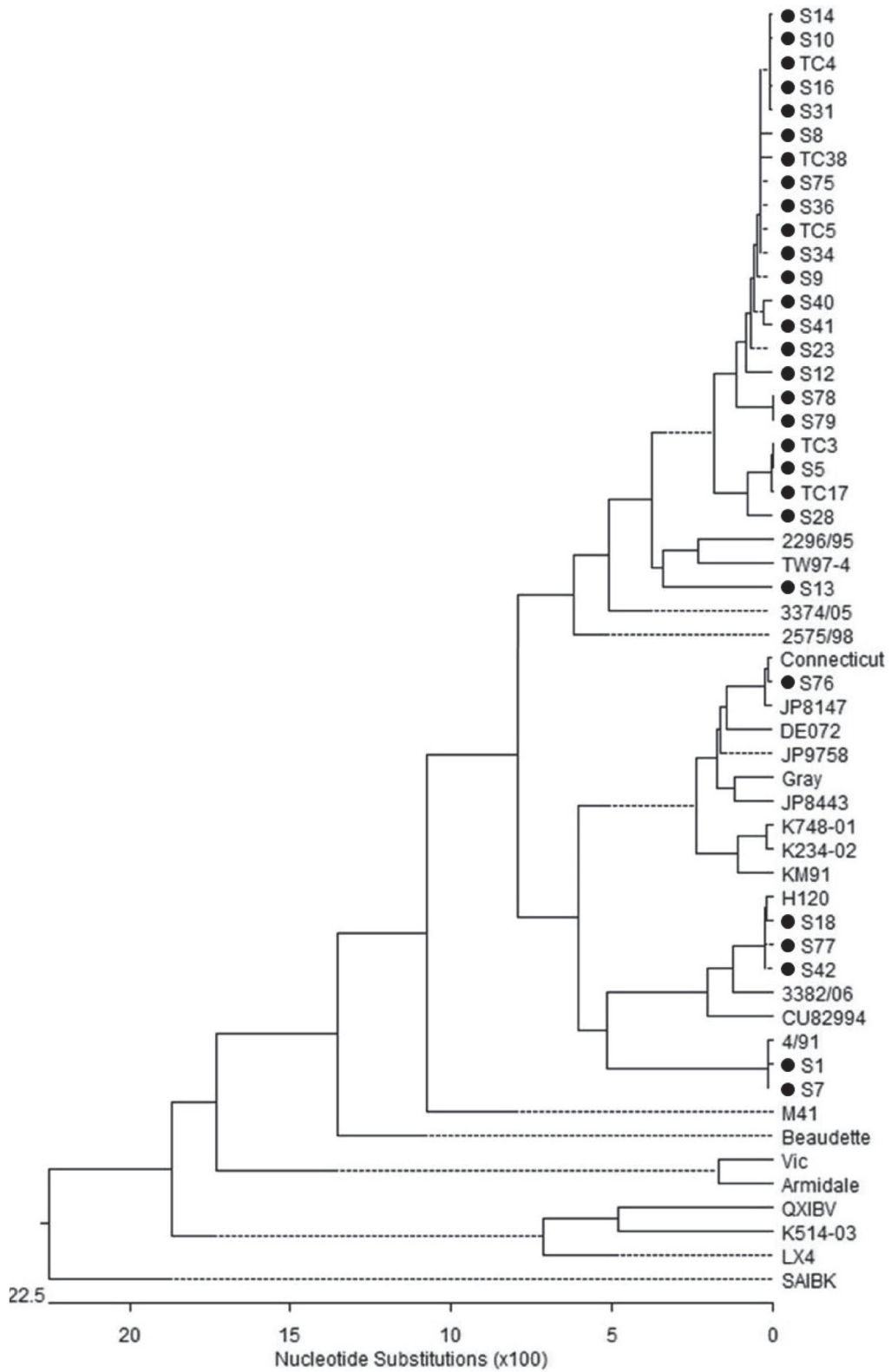


Fig. 1. Phylogenetic tree based on the partial IBV N gene (nucleotides 174–690). ● indicates IBVs identified in the present study.

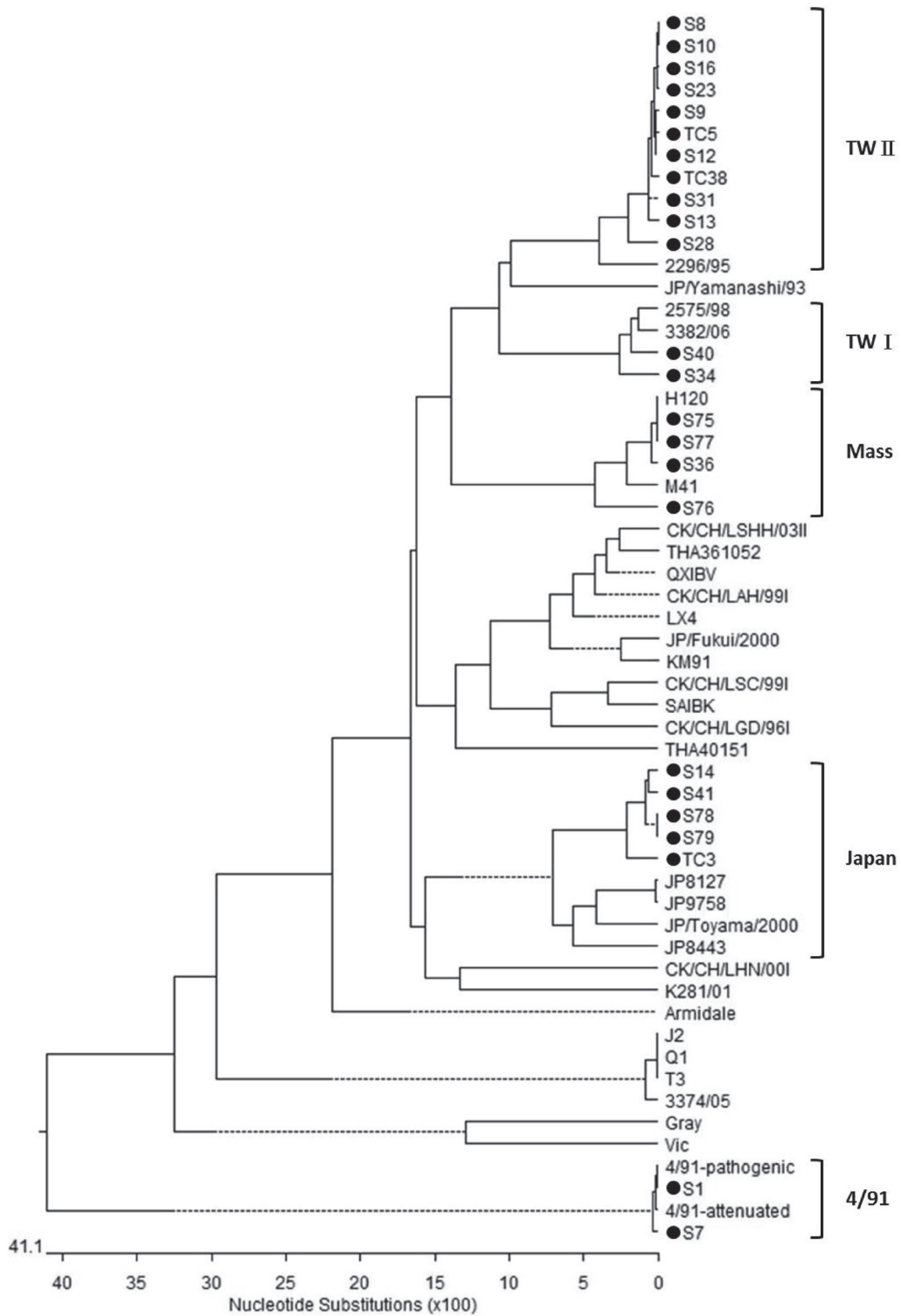


Fig. 2. Phylogenetic tree based on the partial IBVs 5' S gene (nucleotides 1-313). ● indicates IBVs identified in the present study.

could not be neutralized by reference antisera, since their protection rates were less than 50%.

Despite extensive vaccination, IB has occurred occasionally, especially in cold seasons. Most IBVs present in Taiwan are TW-I or TW-II. Surprisingly, in the present study, TW-II was more predominant than TW-I, contrary to previous reports [5, 7–9]. Furthermore, a challenging aspect of this investigation was the emergence of new types, such as the IBVs similar to strains isolated in Japan, which were found to be widespread in Taiwan since they were identified in different locations and different chicken breeds. A previous study revealed that five genotypes existed in Japan in 2010, JP-I, JP-II, JP-III, 4/91 and Mass [1]. In the present study, the IBVs that were similar to Japanese strains showed the highest similarity to JP-I. However, the origin of these isolates is unknown and needs further investigation. The current vaccine strains might not protect against these new isolates, since the one-direction neutralization tests showed less than 50% protection in chicken embryos.

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