A Novel Genotype of Avian Infectious Bronchitis Virus Isolated in Japan in 2009

Masaji MASE1,2,*, Nahoko KAWANISHI3, Yoshiko OOTANI4, Kazunori MURAYAMA5, Ayako KARINO6, Toshikazu INOUE1 and Junko KAWAKAMI3


(Received 25 February 2010 / Accepted 17 April 2010 / Published online in J-STAGE 1 May 2010)

ABSTRACT. Avian infectious bronchitis viruses (IBVs) isolated from commercial layer flocks kept in Ibaraki Prefecture in 2009 were genet-

ically and serologically characterized. Reverse transcription-PCR coupled with direct nucleotide sequencing and GenBank BLAST database analysis of the hypervariable region of the S1 subunit of the virus spike gene showed that these isolates are genetically very different from the previously known IBV genotypes in Japan. Furthermore, none of the antisera used in this study neutralized the index isolate (JP/Ibaraki/168–1/2009) in virus neutralization tests. These results suggest that the isolates are a novel IBV genotype in Japan (designated JP-IV).

KEY WORDS: genotype, infectious bronchitis virus, phylogenetic analysis, serotype.

By phylogenetic analysis of mainly hypervariable regions of the S1 glycoprotein genes [8, 9]. Among them, comparatively newly confirmed genotypes JP-II, JP-III and 4/91 were confirmed in the 1980s, 1990s and 2000s. Therefore, continuous molecular epidemiology of the IBV strains is important for understanding the introduction or emergence of novel genotypes in poultry flocks in Japan.

We report here on our confirmation, in 2009, of a novel genotype of IBV in chickens in Ibaraki Prefecture, located in eastern Japan, by phylogenetic analysis of the S1 gene.

MATERIALS AND METHODS

Background information: One-hundred 30-day-old commercial layer chickens kept in Ibaraki Prefecture were sampled on September 3, 2009 for diagnosis of IBV infection because of failure to start laying eggs. Respiratory signs and increased mortality were not observed. The trachea, kidney and rectums were collected from four chickens and homogenized for virus isolation. By histopathological examination of the affected chickens, typical changes caused by IBV were not confirmed. No bacteria related with this symptom were isolated from major organs of affected chickens.

Isolation and identification of the virus: The IBV specimens were isolated by two passages using 10-day-old embryonated chicken eggs. The presence of IBV in the inoculated embryos was initially determined by IBV-specific reverse transcriptase polymerase chain reaction (RT-PCR) as described previously [8].

Genetic analysis: Viral RNA was extracted from infected allantoic fluids using a QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.). Reverse transcription (RT), PCR amplification, sequencing, phylogenetic analysis and RFLP analysis were performed as described previously [8, 9].
PCR-restriction fragment length polymorphism (PCR-RFLP) analysis: The generated PCR products were employed in restriction endonuclease analysis to develop a simple and rapid classification method for IBV strains in Japan. After comparing all the sequences obtained in this study (GENETYX-Mac, ver. 13, Software Development Corp., Tokyo, Japan), we found that Eco T22I (TaKaRa, Tokyo, Japan) worked best for differentiating between the new identified genotype and the other IBV genotypes in Japan.

Virus neutralizing (VN) test: The VN test was performed according to the method described previously [12]. Briefly, IB virus was diluted (10-fold) with PBS containing BSA (0.1 mg/ml). Each dilution was added to an equal volume of anti-chicken serum, and these samples were incubated at 37°C for 1 hr. After incubation, 0.1 ml of each sample was inoculated into four 10-day-old SPF eggs via the allantoic cavity, and the eggs were then incubated for 8 days at 37°C. Embryos were examined for dwarfing and death on the final day, and virus titers (EID\textsubscript{50}) were calculated by the method of Reed and Muench [10]. VN titer was determined as follows: VN titer against IBV = \log (EID\textsubscript{50} against control serum) – \log (EID\textsubscript{50} against serum).

RESULTS

Isolation and identification of IBV: The expected sizes of the DNA fragments by the previously reported PCR method, which amplified the S1 gene, were successfully obtained from allantoic fluids inoculated with trachea homogenates after first passage in the allantoic cavity. IBVs were isolated from two of the four chickens examined.

Sequence and phylogenetic analysis of the S1 gene of the isolated IBV: After determination of the sequences of the PCR product, two isolates were found to be genetically closely related to each other (99.7% identity). By GenBank BLAST database analysis, the obtained sequences of the S1 genes were closest to the TC07-2 strain isolated in south China (accession No. GQ265948). Phylogenetic trees for the S1 gene constructed by the neighbor-joining method [11] reinforced the viral RNA sequencing results suggesting that these isolates share an immediate ancestor with the TC07-2 strain isolated in south China (Fig. 1).

PCR-RFLP analysis: The new IBV genotype isolates were clearly distinguished by the Eco T22I restriction profiles (Fig. 2). The PCR products of these isolates were digested with 386 and 297 bp by Eco T22I, whereas this enzyme did not digest other genotypes.

Virus neutralization test and comparison between IBV genotypes in Japan based on the S1 gene: The results of the VNTs are shown in Table 1. None of the antiserum used in this study neutralized the JP/Ibaraki/168-1/2009 isolate. The low similarity of amino acids to the confirmed genotypes in Japan (almost <60%) based on the S1 gene including the hypervariable regions supported the results of the VNTs.

Fig. 1. Phylogenetic tree based on the S1 glycoprotein gene of IBV. Nucleotides 20,368–20,988 (621 bases) of the S1 gene of IBV Beaudette (GenBank Accession No. NC001451) were subjected to phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. The viruses employed in this study are shown in bold italics.
DISCUSSION

Three genotypes (JP-II, JP-III and 4/91) of IBV have emerged during the past 20 years in Japan [8, 9, 13]. The JP-II, JP-III and 4/91 genotypes were confirmed in the 1980s, 1990s and 2000s, respectively. The JP-III and 4/91 genotypes are genetically close to those prevalent in foreign countries [2, 6, 7], and thus, to understand the epidemiology of IBV in Japan, it is important to know how prevalent the viruses are worldwide.

Here, we isolated a novel genotype (designated JP-IV) of IBV in Japan based on analysis of the S1 gene. The introduction route of this genotype into Japan and its prevalence are unknown. This genotype of IBV was first isolated in China, but the prevalence of this genotype in China is also unknown. In China, many genotypes of IBV are prevalent, but information on this virus (TC07-2 strain) and the S1 gene sequence have only been deposited in GenBank and have not been published in any international academic journals at present. Therefore, we cannot determine whether this virus was isolated from chickens.

In previous studies, we reported the simple and rapid classification of genotypes of Japanese IBV strains using three restriction endonucleases, \( Hae \) II, \( Eco \) RI and \( Pst \) I [8, 9]. The newly identified genotype in this study, JP-IV, did not have sites for these three enzymes like the 4/91 genotype, so differentiation between the JP-IV and 4/91 types was difficult. Therefore, we selected another restriction endonuclease, \( Eco \) T22I, to differentiate between the JP-IV and 4/91 types. The strains belonging to the other genotypes identified in Japan do not have a restriction site for this enzyme, but the strains belonging to the JP-IV type do. These results revealed that differentiation of the Japanese IBV strains is possible at present by use of four restriction endonucleases.

By VNT, none of the antisera used in this study neutralized this novel genotype JP-IV of IBV, suggesting that the antigenicity of this isolate was different from others known in Japan. The low similarity of amino acids to the representative genotype (almost <60%) supported the results of the VNTs. If this novel genotype of IBV becomes prevalent and causes disease in poultry flocks, it will be necessary to develop a vaccine against it. The pathogenicity to the chicks of these isolates is still unknown. By pathological examination of affected birds, no IBV infection was suspected in the affected chickens, suggesting that this isolate was not directly associated with failure to lay eggs and that it was not very virulent in chickens. However, the pathogenicity of IBV is very complex, as it is influenced by the chicken breed, environmental conditions and mixed infection with bacteria or mycoplasma [1]. Further investigation of the pathogenicity of this novel genotype is needed, and to control IBV infection, it is important to continue the surveillance of IBV in Japan as well as to determine its worldwide prevalence.

ACKNOWLEDGMENT. We would like to thank Dr. Y. Shimazaki (National Veterinary Assay Laboratory, Tokyo, Japan) for kindly supplying the anti IBV-4/91 strain chicken serum.

REFERENCES


<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>JP/KH/64 JP-I</td>
<td>4.0*</td>
<td>&lt; 0.5</td>
<td>67.0</td>
</tr>
<tr>
<td>C78 (vaccine) JP-I</td>
<td>4.0</td>
<td>&lt; 0.5</td>
<td>67.4</td>
</tr>
<tr>
<td>JP/Toyama/2000 JP-I</td>
<td>4.5</td>
<td>&lt; 0.5</td>
<td>65.9</td>
</tr>
<tr>
<td>Miyazaki (vaccine) JP-II</td>
<td>4.5</td>
<td>&lt; 0.5</td>
<td>64.8</td>
</tr>
<tr>
<td>JP/Yamanashi/93 JP-II</td>
<td>3.3</td>
<td>&lt; 0.5</td>
<td>64.3</td>
</tr>
<tr>
<td>JP/Fukui/2000 JP-III</td>
<td>3.0</td>
<td>&lt; 0.5</td>
<td>68.5</td>
</tr>
<tr>
<td>H120 (vaccine) Mass</td>
<td>2.5</td>
<td>&lt; 0.5</td>
<td>63.9</td>
</tr>
<tr>
<td>ON (vaccine) Gray</td>
<td>4.0</td>
<td>&lt; 0.5</td>
<td>63.7</td>
</tr>
<tr>
<td>4/91 (vaccine) 4/91</td>
<td>3.0</td>
<td>&lt; 0.5</td>
<td>68.9</td>
</tr>
</tbody>
</table>

* Figure indicates the neutralization index.


