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NOTE

Avian Pathology

Detection of avian encephalomyelitis virus in chickens in Japan using RT-PCR

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ABSTRACT. A reverse transcription-polymerase chain reaction (RT-PCR) method was developed for broadly detecting the avian encephalomyelitis virus (AEV). The new primers were based on conserved sequences of the 5′-untranslated region of AEV, because the virus was not detected using previous reported RT-PCR. By applying this method to the chicken samples with suspected AEV infection in Japan, we successfully obtained PCR products of the predicted size from all samples, and we confirmed the presence of AEV via sequence analysis.

KEY WORDS: avian encephalomyelitis virus, identification, RT-PCR

Avian encephalomyelitis (AE) is an infectious viral disease affecting young chickens, pheasants, quails, and turkeys [9, 11]. The disease is characterized by ataxia and rapid tremors. The transmission of AE virus (AEV) infection generally occurs by vertical transmission, namely through infected eggs (egg transmission) or by horizontal transmission, namely through the fecal–oral route. Mature hens may experience a temporary decline in egg production; however, they do not develop neurological signs. Meanwhile, the morbidity rate in young stock is generally 40–60% if all chicks come from the infected flock. The mortality rate averages 25%; however, it may exceed 50% [9].

AEV, a member of the family *Picornaviridae*, features a small positive-sense, single-stranded RNA genome [7, 8]. This genome, which is 7 kb nucleotides in length, comprises a 5′-untranslated region (5′-UTR) followed by a long open reading frame encoding a large polyprotein. AEV includes four structural proteins (VP-4, VP-2, VP-3, VP-1) from the P1 region and seven nonstructural proteins from regions P2 and P3 [5, 7].

Clinically, AEV infection reveals similar symptoms as several other diseases, such as Newcastle disease, Marek’s disease, rickettsial diseases, vitamin B1 or B2 deficiency, aspergillosis, salmonellosis, coccidiosis, omphalitis, and mycoplasmosis [1, 9]. For the differential diagnosis, isolation and identification of the causal agent are extremely important. In general, intracerebral inoculation of 1-day-old chicks and yolk-sac inoculation of specific pathogen free embryonated chicken eggs are performed to diagnose the AEV infection [11]; however, these methods are extremely laborious and time-consuming. To conquer this problem, molecular methods such as reverse transcription-polymerase chain reaction (RT-PCR) are useful for rapid diagnoses.

Previously, Xie *et al.* reported a RT-PCR method using primers targeting to the VP2 gene [12], which has been applied to certain field cases of AE in Japan. Recently, we also applied this method to the suspected cases of AE in Iwate Prefecture in Japan; however, we did not obtain successful results in some cases, which were diagnosed based on the pathological findings. Therefore, improvement of this method is necessary for the accurate diagnosis of recent AE cases in Japan. In this study, we designed new primers for RT-PCR to detect the AEV strains including the recent cases in Iwate Prefecture and applied this method to certain field samples obtained from chickens in other prefectures in Japan.

In Iwate Prefecture, 120 chicks among 3,000 birds hatched on September 30, 2015 exhibited clinical symptoms suspicious of AE. Neurological symptoms such as tremors, loose feathers, ataxia, and leg paralysis starting approximately at 5 days of age, were observed. Eight samples including brains were collected from 13 affected chicks for diagnosis on October 5 (5 day-old) and October 8 (8 day-old), 2015 (Table 1).

For virus isolation, eight brains were ground using sterile sand and diluted using phosphate buffered saline to create 10% (w/v) homogenates. These homogenates were then centrifuged at low speed (3,000 × *g* for 10 min). The supernatants were inoculated into the yolk sacs of 5–7-day-old specific antibody-negative embryonated chicken eggs, and chicks were observed for any disease symptoms during the first 10 days after hatching, as described previously [2]. When clinical signs were observed, the brains were collected from six affected chicks. These six brain homogenates were centrifuged at 12,000 rpm for 10 min, and the supernatants

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Table 1. Sample contents of Iwate 2015

No	Name	Contents
1	Iwate/1/2015	Brain homogenate from experimentally infected chicken (inoculate was Iwate/brain/1/2015)
2	Iwate/2/2015	Brain homogenate from experimentally infected chicken (inoculate was Iwate/brain/2/2015)
3	Iwate/3/2015	Brain homogenate from experimentally infected chicken (inoculate was Iwate/brain/3/2015)
4	Iwate/9/2015	Brain homogenate from experimentally infected chicken (inoculate was Iwate/brain/9/2015)
5	Iwate/12/2015	Brain homogenate from experimentally infected chicken (inoculate was Iwate/brain/12/2015)
6	Iwate/13/2015	Brain homogenate from experimentally infected chicken (inoculate was Iwate/brain/13/2015)
7	Iwate/brain/1/2015	Brain homogenate from affected field chicken (5-day-old)
8	Iwate/brain/2/2015	Brain homogenate from affected field chicken (5-day-old)
9	Iwate/brain/3/2015	Brain homogenate from affected field chicken (5-day-old)
10	Iwate/brain/4/2015	Brain homogenate from affected field chicken (5-day-old)
11	Iwate/brain/5/2015	Brain homogenate from affected field chicken (5-day-old)
12	Iwate/brain/9/2015	Brain homogenate from affected field chicken (8-day-old)
13	Iwate/brain/12/2015	Brain homogenate from affected field chicken (8-day-old)
14	Iwate/brain/13/2015	Brain homogenate from affected field chicken (8-day-old)

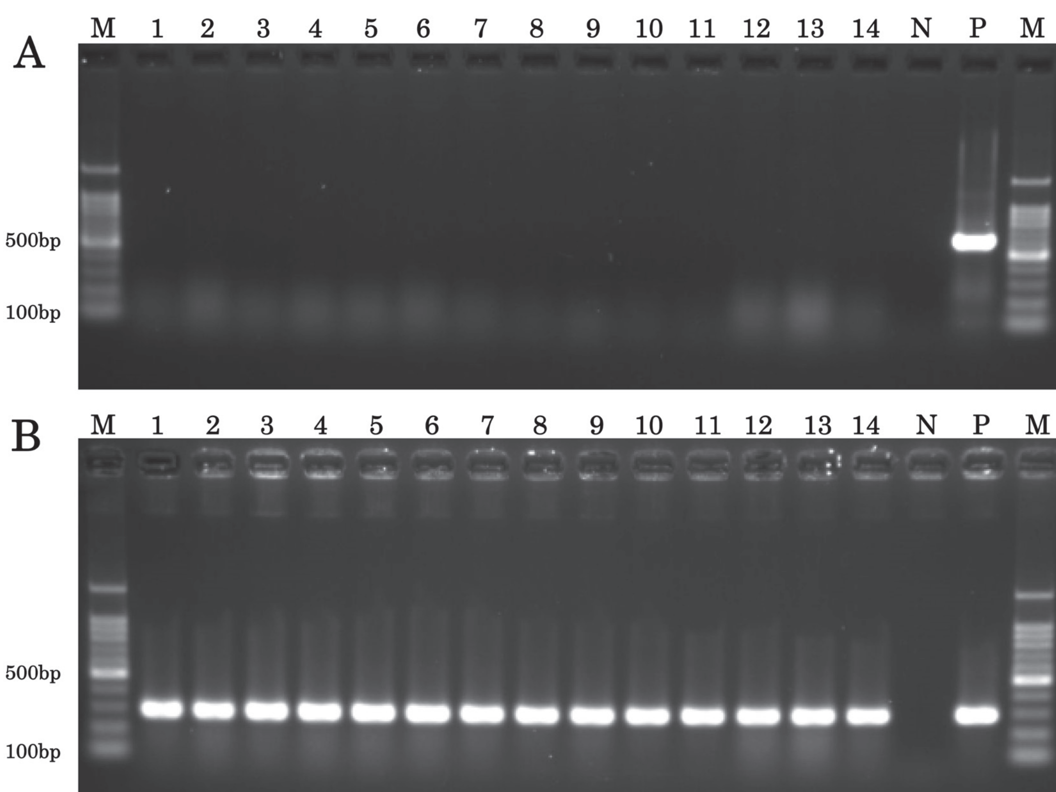

Fig. 1. Agarose gel electrophoresis of AEV products by RT-PCR. Xie's primers were used [12] (A). Our primers designed in this study were used (B). Lane M, 100-bp DNA ladder marker. The lane numbers (1–14) indicate the numbers listed in Table 1. Lane N, negative control. Lane P, AEV vaccine strain #1010 was used as a positive control.

were considered as AEV isolates (Table 1). Together with the results of this virus isolation assay and histopathological findings, we diagnosed this case as AE infection. No bacteria related with the observed symptoms were isolated from the major organs of the affected chicks.

To detect the AEV genes by RT-PCR, viral RNA was extracted from the clinical samples and AEV isolates using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. As aforementioned, we did not obtain any PCR products from the clinical samples and AEV isolates using Xie *et al.*'s method (Fig. 1A).

As a mismatch with the nucleotide sequence used to generate the PCR primers was suspected, we designed new PCR primers for AEV detection using conserved sequences identical to the 5′-UTR as follows:
AEV205f, 5′-CTTTGCGTTTCACAGAACCATCC-3′ (from the Calnek strain 5′-UTR, nucleotides 205–227, sense) and AEV489r,

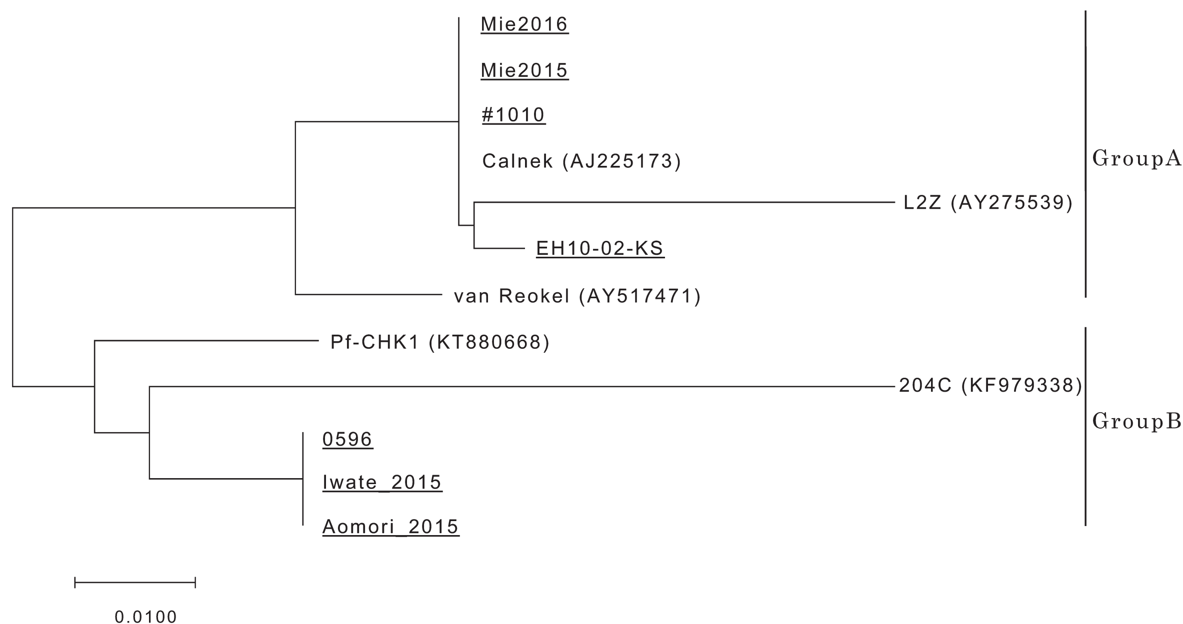

Fig. 2. Phylogenetic tree based on the 5′-untranslated region of AEV. Nucleotides 228–468 (241 bases) of the complete genome of AEV Calnek (GenBank Accession No. AJ225173) were subjected to phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join the nodes and sequences. The samples employed in this study are underlined.

5′-AAATGCTACCCTTAATCTCTC-3′ (from the Calnek strain 5′-UTR, nucleotides 469–489, antisense). PCR was performed using a SuperScript™ II One-Step RT-PCR kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and the following amplification program: 50°C for 30 min, 94°C 2 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec, and a final extension of 72°C for 10 min. The RNA of commercially available AE live vaccine strains, namely AEV of #1010 (Vaxxinova, Tokyo, Japan), EH10-02-KS (Kyouritsu Seiyaku, Tokyo, Japan), and 0596 (Nisseiken, Tokyo, Japan), were used as positive controls. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and were detected under UV light. The predicted size of the PCR products was approximately 285 bp.

We obtained PCR products of the predicted size from eight clinical samples, six AEV isolates (Table 1), and AEV vaccine strains using the newly designed primers (Fig. 1B). For further confirmation, the PCR products were purified by a QIAquick PCR Purification kit (Qiagen) for direct sequencing. The purified PCR products were used as templates for sequencing on an Applied Biosystems 377 automated DNA sequencer (Thermo Fisher Scientific) using a Big Dye™ Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The obtained nucleotide sequences of the PCR products of samples derived from the affected chicks in the Iwate Prefecture (abbreviated as Iwate 2015) were all identical. By GenBank BLAST database analysis, the obtained sequences of the 5′-UTR were closest to the 204C strain (accession No.KF979338), and Iwate 2015 was identified as AEV.

Moreover, we applied the developed RT-PCR to AEV samples obtained from other prefectures. We obtained brain homogenate samples from cases in Mie Prefecture in 2015 (Mie 2015) and 2016 (Mie 2016) and in Aomori Prefecture in 2015 (Aomori 2015). All samples were brain homogenates from the affected chicks. RNA extraction and RT-PCR were conducted, as described previously. Using the new method, PCR products of the expected size were obtained from all samples.

For further genetic analysis, we conducted phylogenetic analysis using the nucleotide sequences of the obtained PCR products. The phylogenetic tree was constructed via the maximum likelihood method [10] using MEGA 7 [4]. The nucleotide sequences of strains from other countries were obtained from GenBank using accession numbers AJ225173 (Calnek strain), AY275539 (L2Z strain), AY517471 (van Reokel strain), KF979338 (204C strain), and KT880668 (Pf-CHK1 strain) and were used for phylogenetic analysis.

The nucleotide sequences of all samples (Iwate 2015, Aomori 2015) obtained from Iwate Prefecture and Aomori Prefecture were identical, although the epidemiological relationship remains unclear. The sequences were also identical to that of the AEV vaccine 0596 strain. The nucleotide sequences of Mie 2015 and Mie 2016 were identical, although the epidemiological relationship remains unclear. Interestingly, the nucleotide sequences of Mie 2015 and Mie 2016 differed from those obtained from the Iwate 2015 and Aomori 2015, suggesting that prevalent AEV strains in Japan are genetically diverse.

Using phylogenetic analysis, AEV strains in Japan were divided into two major groups (Groups A and B; Fig. 2). Group A comprised strains isolated in U.S.A. and China, whereas group B comprised strains isolated in Hong Kong and Hungary. Interestingly, we applied Xie's method [12] to the samples employed in this study; however, no amplification products were obtained from the group B samples. This appeared to be the result of mismatches between the designed primers and group B strains. We also applied another method [6]; however, no amplification product was obtained from many samples employed in this study (data not shown). This failure also appears to be the result of mismatches with their designed primers. The 5′UTR of bovine

enteroviruses was characterized by group-specific nucleotide sequences, having conserved regions that maintain the secondary structures [13]. Therefore, a specific primer targeting the 5′UTR was used for detection of the bovine enteroviruses [3]. Thus, it can be expected that the PCR primers designed in this study could be detect all AEV samples. By using our primers to detect AEV from field samples, an accurate virological diagnosis of AE is possible, and genetic analysis will contribute to the epidemiological analysis.

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REFERENCES

1. Haryanto, A., Ermawati, R., Wati, V., Irianingsih, S. H. and Wijayanti, N. 2016. Analysis of viral protein-2 encoding gene of avian encephalomyelitis virus from field specimens in Central Java region, Indonesia. *Vet. World* **9**: 25–31. [Medline] [CrossRef]
2. Hoekstra, J. 1964. Experiments with avian encephalomyelitis. *Br. Vet. J.* **120**: 322–335. [CrossRef]
3. Jiménez-Clavero, M. A., Escribano-Romero, E., Mansilla, C., Gómez, N., Córdoba, L., Roblas, N., Ponz, F., Ley, V. and Sáiz, J. C. 2005. Survey of bovine enterovirus in biological and environmental samples by a highly sensitive real-time reverse transcription-PCR. *Appl. Environ. Microbiol.* **71**: 3536–3543. [Medline] [CrossRef]
4. Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**: 1870–1874. [Medline] [CrossRef]
5. Lau, S. K. P., Woo, P. C. Y., Yip, C. C. Y., Li, K. S. M., Fan, R. Y. Y., Bai, R., Huang, Y., Chan, K. H. and Yuen, K. Y. 2014. Chickens host diverse picornaviruses originated from potential interspecies transmission with recombination. *J. Gen. Virol.* **95**: 1929–1944. [Medline] [CrossRef]
6. Liu, Q., Yang, Z., Hao, H., Cheng, S., Fan, W., Du, E., Xiao, S., Wang, X. and Zhang, S. 2014. Development of a SYBR Green real-time RT-PCR assay for the detection of avian encephalomyelitis virus. *J. Virol. Methods* **206**: 46–50. [Medline] [CrossRef]
7. Marvil, P., Knowles, N. J., Mockett, A. P. A., Britton, P., Brown, T. D. K. and Cavanagh, D. 1999. Avian encephalomyelitis virus is a picornavirus and is most closely related to hepatitis A virus. *J. Gen. Virol.* **80**: 653–662. [Medline] [CrossRef]
8. Shafren, D. R. and Tannock, G. A. 1992. Further evidence that the nucleic acid of avian encephalomyelitis virus consists of RNA. *Avian Dis.* **36**: 1031–1033. [Medline] [CrossRef]
9. Suarez, D. L. 2013. Avian encephalomyelitis. pp. 486–510. *In*: Diseases of Poultry, 13th ed. (Swayne, D. E., Glisson, J. R., McDougald, L. R., Nolan, L. K., Suarez, D. L. and Nair, V. eds.), Wiley-Blackwell, Ames.
10. Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10**: 512–526. [Medline]
11. Tannock, G. A. and Shafren, D. R. 1994. Avian encephalomyelitis: a review. *Avian Pathol.* **23**: 603–620. [Medline] [CrossRef]
12. Xie, Z., Khan, M. I., Girshick, T. and Xie, Z. 2005. Reverse transcriptase-polymerase chain reaction to detect avian encephalomyelitis virus. *Avian Dis.* **49**: 227–230. [Medline] [CrossRef]
13. Zell, R. and Stelzner, A. 1997. Application of genome sequence information to the classification of bovine enteroviruses: the importance of 5′- and 3′-nontranslated regions. *Virus Res.* **51**: 213–229. [Medline] [CrossRef]