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Short Communication

Molecular identification of the vaccine strain from the inactivated oil emulsion H9N2 low pathogenic avian influenza vaccine

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In order to control the H9N2 subtype low pathogenic avian influenza (LPAI), an inactivated vaccine has been used in Korea since 2007. The Korean veterinary authority permitted the use of a single H9N2 LPAI vaccine strain to simplify the evolution of the circulating virus due to the immune pressure caused by the vaccine use. It is therefore important to determine the suitability of the vaccine strain in the final inactivated oil emulsion LPAI vaccine. In this study, we applied molecular rather than biological methods to verify the suitability of the vaccine strain used in commercial vaccines and successfully identified the strain by comparing the nucleotide sequences of the hemagglutinin and neuraminidase genes with that of the permitted Korean LPAI vaccine strain. It is thought that the method used in this study might be successfully applied to other viral genes of the LPAI vaccine strain and perhaps to other veterinary oil emulsion vaccines.

Keywords: H9N2 avian influenza, identification of vaccine strain, oil emulsion vaccine

The H9N2 low pathogenic avian influenza (LPAI) has become a serious problem in the poultry industry worldwide [2,6] and concerns about a possible zoonotic disease have been raised [8].

Since 2007, the Korean veterinary authority has permitted the use of inactivated H9N2 LPAI vaccine to control the disease and has decided to use a single strain to simplify the antigenic variation of the H9N2 LPAI virus which is circulating in the country [3]. It is therefore necessary to eliminate any possibility that the vaccine has been unintentionally contaminated during production or that it has, without permission, been deliberately altered by other vaccine strains (e.g. autogenous vaccines) to achieve better efficacy.

However, it is difficult to identify the strain through biological (serological) methods, because the strain most antigenically similar to other circulating pathogens has been chosen as the vaccine strain. In addition, antigen extraction is difficult, particularly in inactivated oil emulsion vaccines due to the stability of the emulsion. The same situation exists with the Korean H9N2 LPAI vaccine [3].

To overcome these limitations, we used isopropyl myristate (IPM) to separate the antigen from the oil adjuvant according to the method employed by Maas *et al*. [7]. We then applied molecular methods to detect genetic characteristics of the Korean H9N2 LPAI vaccine strain through conventional reverse transcription-polymerase chain reaction (RT-PCR) and direct nucleotide sequencing.

The H9N2 subtype LPAI viruses used in this study for comparing the nucleotide sequences of viral genes:were A/chicken/Korea/01310/2001 (CE3; 01310/CE3) (CE number represents the passage number through the chicken embryo), a parent virus of the Korean H9N2 LPAI vaccine strain; A/chicken/Korea/01310/2001 (CE20; 01310/ CE20), a vaccine strain; A/chicken/Korea/Q30/2004 (CE2; Q30/04), A/chicken/Korea/Q99/2006 (CE1; Q99/ 06), A/Duck/Korea/GJ84/2007 (CE1; GJ84/07), and A/chicken/Korea/ GJ180/2008 (CE1; GJ180/08), recent field isolates [3,5]. All viruses were propagated in 9-11 day old specific pathogen free embryonated chicken eggs (ECE) via the allantoic cavity. Commercial H9N2 LPAI vaccines produced by 5 Korean vaccine companies were used. All vaccines used formalin as an inactivating agent and were emulsified with mineral oil adjuvant.

Viral RNA from infectious allantoic fluids and IPM treated antigens were extracted with the Viral Gene-spin Viral DNA/RNA Extraction Kit (iNtRON Biotechnology, Korea). RT-PCR was done with the AccuPower RT/PCR Premix Kit (Bioneer, Korea) according to the manufacturer's directions. Amplified fragments were visualized using a 1.5% agarose gel electrophoresis with ethidium bromide staining.

Hemagglutinin (HA) and neuraminidase (NA) proteins of

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influenza viruses are surface glycoproteins and act as major immunogenic antigens. Therefore, we focused on these two genes in identifying the vaccine strain through RT-PCR and direct nucleotide sequencing.

The high growth rate of the Korean H9N2 LPAI vaccine strain in chicken eggs is achieved by performing over 20 serial blind passages with the parent virus through the ECE [3]. During each passage the virus acquires nucleotide changes in the HA and NA genes which lead to an additional potential N-linked glycosylation site near the receptor binding site of HA and to an eighteen amino acid deletion in the NA stalk region [3]. Therefore, we amplified the 204 bp region of the HA gene, including the additional potential glycosylation site in the vaccine strain. For the NA gene, primers were designed to check the 54 bp deletion in the NA stalk region of the vaccine strain. The amplified product sizes are 127 bp for the vaccine strain and 181 bp for the parent and field viruses.

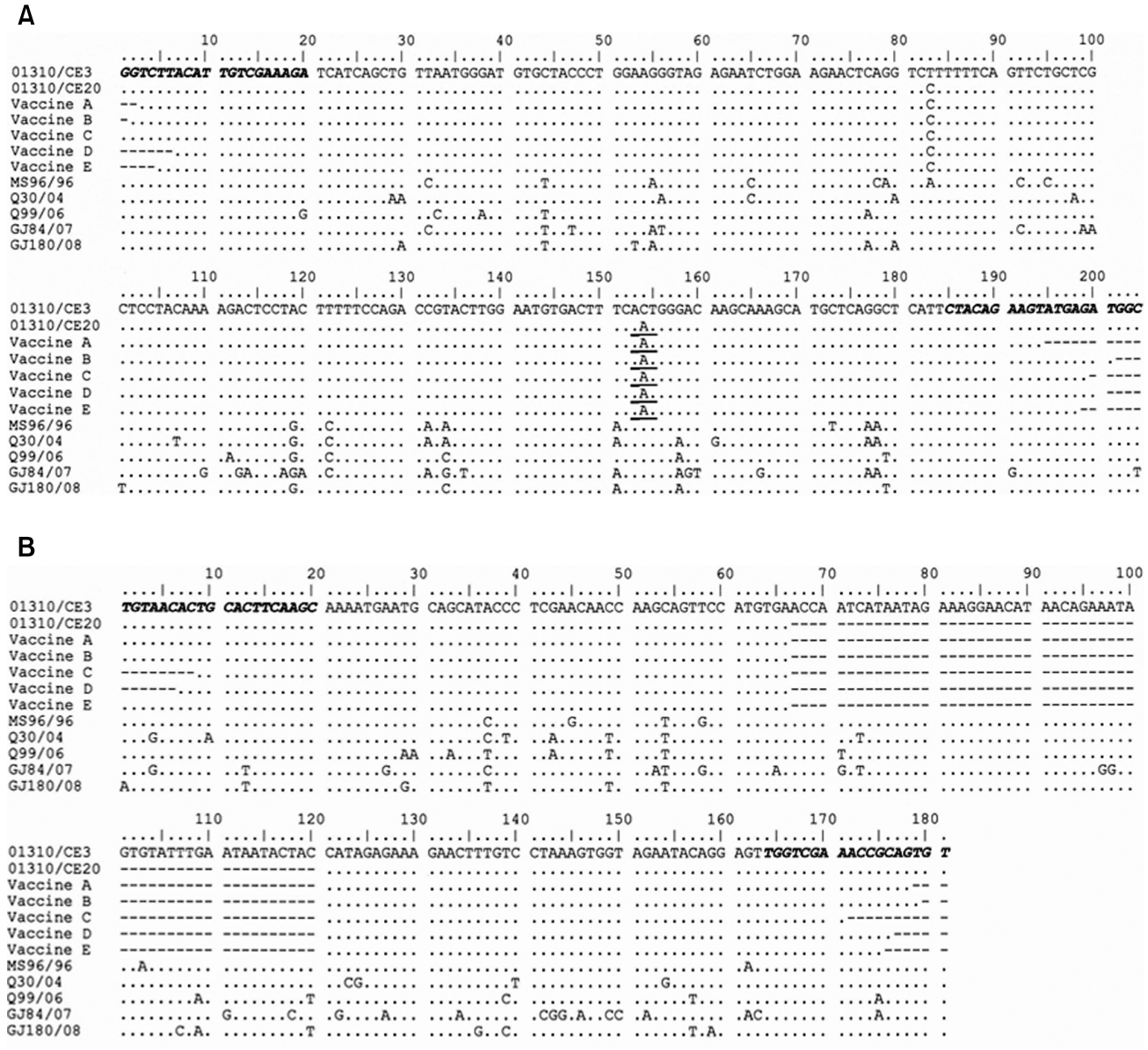

Fig. 1. Comparison of the nucleotide sequences of the hemagglutinin (A) and neuraminidase (B) genes of the vaccine parental strain (01310/CE3), vaccine strain (01310/CE20), five commercial LPAI vaccines (vaccine A to E), and representative Korean H9N2 LPAI viruses (field isolates). The GenBank accession number for the hemagglutinin and neuraminidase genes of A/chicken/Korea/MS96/1996 (MS96/96) are AF203008 and AF203786, respectively. Q99/06, GJ84/07 and GJ180/08 were sequenced in this study. Dots indicate residues identical to 01310/CE3 and dashes stand for missing nucleotides. The primer sequences are indicated with italic bold fonts. Nucleotide sequences for the additional potential N-linked glycosylation site are indicated with underlines.

To determine the specificity of the primer sets to the Korean H9N2 LPAI vaccine virus, we used 3 type A influenza viruses (01310/CE20, A/Puerto Rico/8/1934 (H1N1, PR/8) and A/Chicken/Korea/IS/2006 (H5N1, IS/06), Newcastle disease virus (NDV, LaSota) and infectious bronchitis virus (IBV, M41). The primer sets for the HA and NA genes reacted only to the 01310/CE20 but not to the other influenza viruses, NDV or IBV. The amplified products for the HA and NA gene specific primer sets were 204 bp and 127 bp, respectively (data not shown). For the sensitivity of the primer sets, we performed RT-PCR with 10-fold serially diluted 01310/CE20 ($10^{8.8}EID_{50}$/0.1 mL). The HA and NA gene primer sets could detect the virus to dilution factors of 10^{-7} ($10^{1.8}EID_{50}$/0.1 mL) and 10^{-6} ($10^{2.8}$ EID_{50}/0.1 mL), respectively (data not shown).

With this specificity and sensitivity data, we attempted to apply the primer sets to the commercial H9N2 AI vaccines (monovalent) from 5 Korean vaccine companies. As a result, we successfully obtained 204 bp fragments from the viruses and vaccine samples for the HA gene. In the NA gene, we could differentiate the vaccine strain from the parent virus and the recent field isolate by size through agarose gel electrophoresis. The 181 bp region of the NA gene was amplified from 01310/CE3 and Q30/04, and the smaller 127 bp fragment from 01310/CE20, and the extracted antigens from all of the five commercial inactivated H9N2 LPAI vaccines (data not shown).

The amplified fragments were sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, USA) with ABI PRISM 3730XL Analyzer (Applied Biosystems, USA). The nucleotide sequences were aligned with the Clustal W algorithm packaged in the BioEdit program (version 7.0.9.0.; North Carolina State University, USA).

The HA and NA gene sequences of the amplified fragments of the extracted antigens from the vaccines were identical to the vaccine strain (01310/CE20) and were distinguishable from parent and other Korean isolates (Figs. 1A and B). We also found the additional potential N-linked glycosylation site in HA genes and the 54 bp deletion in NA genes of the extracted antigens; these findings are characteristic of the Korean H9N2 LPAI vaccine strain [3].

We next tested the practical applicability of this assay with a total of 19 commercial LPAI (H9N2) inactivated oil emulsion vaccines, which were produced by 5 Korean vaccine companies. Six monovalent, 5 bivalent (for LPAI and ND), 6 tetravalent (for LPAI, ND, American and Korean type IB), and 2 pentavalent (for LPAI, ND, American and Korean type IB, and egg drop syndrome) vaccines were tested. 17 of the vaccines had been stored for less than 9 months, 1 for over 15 months and 1 for over 18 months from the date of manufacture. We were able to obtain appropriately sized amplicons from all 19 vaccines with the primer sets (data not shown) and this strongly supported the practical usefulness of the method.

We successfully amplified genes of the antigens extracted from oil emulsified vaccines treated with formalin. However, other inactivating agents, such as beta propiolactone and binary ethylenimine, have been used for inactivated vaccines [1,4] and their mode of action is different than that of formalin [1,9]. Therefore, further study is needed to elucidate the applicability of this method to vaccines inactivated with other agents.

Although the amplified regions targeted in this study are relatively short and limited to only the HA and NA genes, this method may be an efficient tool for identifying the properly licensed H9N2 LPAI vaccine in Korea and might be applied to other genes of the vaccine strain or to other vaccines in other countries.

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