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Short communication

Comparison between dot-immunoblotting assay and clinical sign determination method for quantifying avian infectious bronchitis virus vaccine by titration in embryonated eggs

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A B S T R A C T

A sensitive and specific method for measuring the vaccine titer of infectious bronchitis virus (IBV) is important to commercial manufacturers for improving vaccine quality. Typically, IBV is titrated in embryonated chicken eggs, and the infectivity of the virus dilutions is determined by assessing clinical signs in the embryos as evidence of viral propagation. In this study, we used a dot-immunoblotting assay (DIA) to measure the titers of IBV vaccines that originated from different pathogenic strains or attenuation methods in embryonated eggs, and we compared this assay to the currently used method, clinical sign evaluation. To compare the two methods, we used real-time reverse transcription-PCR, which had the lowest limit of detection for propagated IBV. As a clinical sign of infection, dwarfism of the embryo was quantified using the embryo: egg (EE) index. The DIA showed 9.41% higher sensitivity and 15.5% higher specificity than the clinical sign determination method. The DIA was particularly useful for measuring the titer of IBV vaccine that did not cause apparent stunting but propagated in embryonated chicken eggs such as a heat-adapted vaccine strain. The results of this study indicate that the DIA is a rapid, sensitive, reliable method for determining IBV vaccine titer in embryonated eggs at a relatively low cost.

Infectious bronchitis virus (IBV) is a gamma coronavirus that causes a highly contagious disease in chickens. The virus causes severe economic losses to the poultry industry worldwide because it can affect the upper respiratory and reproductive tracts, and some strains can cause nephritis in chickens (Jackwood, 2012). Despite intensive vaccination using both live attenuated and killed vaccines to prevent the disease, the emergence of new variant strains that do not serologically cross-react has complicated disease control and demonstrates the importance of vaccinating chickens with the disease-causing IBV types (Cavanagh, 2005, 2007).

Vaccination is considered the most cost-effective approach for controlling IBV infection (Meeusen et al., 2007). To prevent the economic losses caused by IBV, live attenuated vaccines and inactivated oil-emulsion vaccines containing both the KM91 and Massachusetts 41 (M41) strains are widely used in Korea. More recently, vaccine strains providing broad cross-protection have been developed, including the K2 and K40/09 strains (Kim et al., 2013; Lim et al., 2012, 2015). In Korea, the viral content of a vaccine preparation is quantified by IBV titration according to standard procedure approved by the Animal and Plant Quarantine Agency. For this procedure, the chicken embryos are inoculated with serial dilutions of the virus preparation, and then the embryos are examined for the presence of specific lesions caused by the virus, i.e., dwarfing, curling, and stunting (Doherty, 1967). However, as a variety of live-attenuated vaccines have been developed for variant field strains, it is unclear whether the clinical sign determination method reflects viral propagation in IBV-infected embryos. Furthermore, as there are no specific standards for measuring clinical signs, the measurements of specific lesions can differ among observers.

Our previous study revealed that a novel dot-immunoblotting assay (DIA) using monoclonal antibodies against several IBVs could detect viruses propagated in embryonated chicken eggs (Song et al., 1998). To accurately, reproducibly, and efficiently measure vaccine titers, we used the DIA to detect IBV propagated in inoculated

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embryonated eggs. The aim of this study was to evaluate and compare the sensitivity and specificity of the DIA to the clinical sign determination method for detecting IBV in inoculated embryonated eggs during titration of IBV vaccines.

A respiratory strain belonging to the Mass group (M41), a nephropathogenic strain belonging to the KM91-like subgroup (KM91), and a recombinant nephropathogenic strain belonging to Korean new cluster 1 (K40/09) were used to evaluate the titer of IBVs used in killed vaccines as described by Kim et al. (2013). Two nephropathogenic strains that were attenuated by 170 serial passages (K2p170) or heat-adapted passages (K40/09HP40) in chicken embryos were used to evaluate the live-attenuated vaccine strains (Lee et al., 2010). All viruses were propagated in 10-day-old specific-pathogen-free embryonated chicken eggs (ECE; Hy-Vac, Adel, IA) at 37 °C for 72 h. Allantoic fluid from each egg was harvested, aliquoted, and frozen at −70 °C until use.

All animal protocols used in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee of Konkuk University. To confirm the detection limit of the DIA, each vaccine strain was serially diluted 2-fold in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) and analyzed by DIA and real-time reverse transcription (RT)-PCR. Concurrently, triplicate 10-fold serially diluted samples were used to determine the detection limit of real-time RT-PCR. To measure vaccine titer based on the infectivity of chicken embryos, 10-fold serial dilutions of a 10^0 virus solution (10^{-1}–10^{-9}) were generated by mixing 1 mL of the virus with 9 mL of PBS containing 50 μg/mL gentamicin sulfate (Sigma–Aldrich, St. Louis, MO); all dilutions were kept on ice. Next, 0.1 mL of the 10^{-4} through 10^{-9} dilutions were inoculated into five 10-day-old SPF ECE (Hy-Vac), and the eggs were incubated at 37 °C. No eggs had to be discarded due to non-specific death of embryos within one day of inoculation. After three days of incubation, 500 μL of allantoic fluid was extracted from the inoculated eggs using a 1 mL syringe and was used to detect propagated IBV by the DIA and real-time RT-PCR. Eggs were then resealed with paraffin for further incubation. Seven days after inoculation, the embryo:egg (EE) index was calculated for all eggs. Propagation of the inoculated virus was determined using real-time RT-PCR, DIA, and the EE index method, and the 50% egg infectious dose (EID_{50}) of the five vaccines was calculated based on the method of Reed and Muench (1938).

RNA was extracted from each inoculated allantoic fluid sample using the ExiPrep viral RNA/DNA extraction kit (BIONEER Co., Daejeon, Korea) according to the manufacturer's instructions, and eluted in a 50 μL volume. Real-time RT-PCR analysis of the extracted RNA samples was conducted as previously described (Callison et al., 2006). The primers and TaqMan dual-labeled probe were synthesized by Macrogen (Seoul, Korea). The primers and probe were utilized in a 25 μL reaction containing 12.5 μL of Quantitect Probe RT-PCR 2× mix (Qiagen, Hilden, Germany), 0.25 μL of RT enzyme (Qiagen), 0.5 mol of primers, 0.1 mol of probe, and 5 μL of the RNA sample from 50 μL of extracted RNA. Amplification was performed in an ABI Prism 7500 real-time PCR System using the following program: 50 °C for 30 min, 95 °C for 15 min, and 45 cycles of 94 °C for 1 s and 60 °C for 60 s, with emitted fluorescence measurement. Cycle threshold (Ct) values below the detection limit were considered positive for IBV.

The DIA was conducted as described previously with slight modifications (Song et al., 1998). Allantoic fluids from ECEs inoculated with the serially diluted vaccines were centrifuged at 1000 × *g* for 1 min. Next, 150 μL of the supernatant was dotted onto a nitrocellulose membrane (0.45 μm pore size) using a Hybri-Dot 96-well filtration manifold (Bio-Rad Laboratories, Hercules, CA). Uninfected normal allantoic fluid was used as a negative control. The membrane was then blocked with 3% bovine serum albumin in Tris-buffered saline (TBS; 100 mM Tris, 0.9% sodium chloride, pH 7.5) at 37 °C overnight. The primary monoclonal antibodies (3F5) were diluted 1:1000 with Tris Tween-buffered saline (TTBS; 100 mM Tris, 0.2% Tween 20, 0.9% sodium chloride, pH 7.5) and incubated with the membrane for 30 min at 37 °C. After washing the membrane in TTBS three times for 15 min each with gentle agitation, the membrane was incubated for 30 min at 37 °C with biotinylated anti-mouse immunoglobulin G (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA) diluted in TTBS. Following washing, the membrane was incubated for 30 min at 37 °C with biotin and avidin-conjugated peroxidase complex (Vectastain ABC kit) diluted in TTBS. After the final washing, the membrane was developed using 6 mg of diaminobenzidine (Pierce, Rockford, IL) in 10 mL of 50 mM Tris and 10 μL H_2O_2 for 1 min. The reaction was then stopped by rinsing with distilled water (three times) and the membrane was allowed to air-dry. Dark brown-colored dots were considered positive for IBV.

Dwarfing of the infected chicken embryos was detected by determining the EE ratio. The weights of the eggs were measured 7 days after inoculation of the serially diluted viruses. The respective eggs and embryos were weighed using an electronic balance (Ohaus, Florham Park, NJ). The EE ratio was defined as the weight of embryo divided by the weight of respective egg. A total of 120 SPF eggs (Hy-Vac) were used to determine the EE ratio of control ECE (mock-inoculated). Briefly, we inoculated 10-day-old eggs with 100 μL of sterile PBS as a mock-inoculated control. The EE ratio of 20 eggs was determined at 7 days post-inoculation. The average EE ratio and the standard deviation of the mock control were calculated. The EE index was determined by dividing the EE ratio of inoculated eggs by the mean EE ratio of mock-inoculated eggs (Dhinakar Raj et al., 2004).

To assess the receiver operating characteristics (ROC) using MedCalc® version 15.8 statistical software (Mariakerke, Belgium), positive or negative results confirmed by real-time RT-PCR were assigned as "true-positive" or "true-negative," respectively. The area under the ROC-curve (AUC index) was then calculated for both the DIA and clinical sign detection methods. High values (close to 1) indicate a highly accurate test (Greiner et al., 2000). The concordance of both assays to the properly classified samples as positive or negative was estimated by calculating the weighted kappa statistic (κ test). κ test values of 0.41–0.60 indicate moderate agreement, values of 0.61–0.80 indicate substantial agreement, and values of 0.81–0.99 indicate nearly perfect agreement (Viera and Garrett, 2005).

The 2-fold diluted virus was detected concurrently by DIA and real-time RT-PCR (Supplementary material Fig. 1). The highest dilution that showed dark-brown dots in the DIA was 2^{-7} for K2p170 and K40/09 and 2^{-6} for M41, KM91, and K40/09HP40. The 10-fold diluted viruses were also examined by real-time RT-PCR (Supplementary material Fig. 2). For the tested pathogenic or attenuated IBVs, there were no detectable Ct values at the 10^{-7} dilution. Therefore, the detection limit of the real-time RT-PCR assay was set to the Ct value of the 10^{-6} dilution. The real-time RT-PCR method was found to be approximately 2^{15}-fold more sensitive than the DIA method. Thus, real-time RT-PCR is suitable for detecting propagated viruses and can be used to compare the DIA and EE ratio detection methods for propagated IBV during titration. Because the DIA does not distinguish between infectious and noninfectious virus, we had to confirm that the assay does not detect inoculated virus in the eggs. As shown by the detection limit of the DIA, we observed that the input virus was not detected at dilutions higher than 2^{-6}–2^{-7}. Thus, none of the dilutions used in the titering (10^{-4}–10^{-9}) would give a positive result without viral replication, especially after further dilution in the allantoic fluid. After 7 days of incubation, the mean EE ratio and standard deviation of mock-inoculated eggs was 0.375 ± 0.036. To detect dwarfism, the EE index was defined as the EE ratio of IBV-inoculated ECE divided by the mean EE ratio of mock-inoculated ECE. Embryos with EE