Efficacy of a novel avian metapneumovirus live vaccine candidate based on vaccination route and age

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ABSTRACT This article describes a series of animal studies for the development of an avian metapneumovirus (**aMPV**) live vaccine. Although aMPV causes continual economic loss in the poultry industry, there are no live aMPV vaccines available in Korea. Furthermore, information is limited with respect to standard field practices for vaccinations at an early age. Here, the development of an aMPV live vaccine was attempted, and its efficacy was investigated with respect to the vaccination route and age to develop a method for controlling aMPV. Before vaccine development, an animal challenge model was established using the aMPV field isolate to identify the most effective time and site for collecting samples for evaluation. After attenuation of the virulent aMPV in Vero cells, a safety and efficacy test was conducted for the vaccine candidate. As a novel aMPV live vaccine candidate, aMPV K655/07HP displayed sufficient safety in day-old chicks with 10 vaccine doses. The efficacy test using 1-week-old chicks showed weaker humoral immune response than that in 4-week-old chicks. However, the candidate vaccine provided complete protection against infection caused by the challenge virus for all ages of vaccinated chicks. In conclusion, an effective aMPV challenge model was established for studying aMPV in chickens, which offered important, insightful information. The safety and efficacy study suggested that the new aMPV candidate vaccine could be used to effectively reduce the economic losses incurred because of aMPV infection.

Key words: avian metapneumovirus, pathogenicity, vaccine, animal model, chicken

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INTRODUCTION

Avian metapneumovirus (**aMPV**) is a member of the subfamily *Pneumovirinae* in the family, *Paramyxoviridae.* This virus, previously known as avian pneumovirus, is the causative agent of rhinotracheitis in turkeys. In chickens, the virus causes the swollen head syndrome (**SHS**) and leads to a drop in egg production along with discolored eggs. The clinical signs can be more severe because of complications caused by infections with other respiratory pathogens (Ganapathy and Jones, 2007). The economic losses by aMPV in broiler chickens are more considerable than those of layers, because aMPV causes delays in feeding time by severe nasal discharge, followed by decreased uniformity and productivity. Among the 4 subtypes (A, B, C, and D) of aMPV, subtype A and B are the major subtype of virus in the chicken industry causing those clinical signs (Patnayak et al., 2005; Clubbe and Naylor, 2011). Vaccination against aMPV is the most effective way to control the disease. Several live attenuated and inactivated vaccines have been developed to prevent aMPV infection, which are used as commercial products in many countries (Ganapathy and Jones, 2007).

Since the first report of SHS in Korea in 1992 (Kim et al., 1992), the presence of aMPV has been demonstrated by the specific antibodies in infected birds (Lee 1995; Choi et al., 2010) and isolation of the virus (Lee et al., 2007; Kwon et al., 2010). Studies have proven that aMPV causes significant economic losses in the Korean poultry industry (Paudel et al., 2016). Several inactivated aMPV vaccines have been available in Korea since 2011 for the prevention of aMPV infection. However, live aMPV vaccines have not yet been registered. Although many experts agree that the combined use of live and killed aMPV vaccines is much more effective than using the killed vaccine alone (Cook et al., 1996), there are some concerns regarding the use of a foreign strain of live aMPV vaccines. Introducing foreign live vaccine strain derived from other countries can result

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in new types of virulent viruses if the viruses revert to a virulent state (Lupini et al., 2011; Franzo et al., 2015). In addition, if there are antigenic differences between the endemic field virus and foreign vaccine virus, the foreign vaccine may not be effective in controlling the domestic field stain (Catelli et al., 2010; Cecchinato et al., 2010). For these reasons, developing live vaccines using endemic strains is preferred.

The vaccination of 1-week-old chicks via the drinking water route is a common and preferred method for mass vaccination of live vaccines against respiratory diseases in Korea (Kim et al., 2016). Because aMPV is widely spread in Korea (Lee et al., 2010), it is essential to secure early protection against the disease. However, there are concerns that the immune response induced by vaccination on early age of chickens with immature immune system is not adequate compared with the response on mature-aged chickens (Smialek et al., 2015). However, there is limited information comparing the efficacies of aMPV vaccines with respect to the age of the vaccinated chickens, even though this type of information is vital for establishing an optimal program for disease prevention. In addition, young birds with an immature immune system can show stronger vaccine reactions (Mazija et al., 2010). Therefore, identifying the minimum age for safe vaccination is essential for establishing a recommended vaccination age.

This study aimed to develop a live aMPV vaccine for the practical control of aMPV. For this purpose, an aMPV challenge model was established using Korean field isolates. After confirmation of the adequate safety of attenuated viruses in day-old chicks, the protective efficacy of the attenuated live vaccine candidate using different vaccination routes and the age of chickens was compared using the challenge model.

MATERIALS AND METHODS

Chickens

Specific pathogen-free (**SPF**) White Leghorn chickens (Namdeog Sanitek, Korea) were randomly allocated into groups for each study and placed in separate isolators (3 Shine, Daejeon, Korea). Food and water were provided ad libitum in a biosafety level 2 laboratory.

Virus

The aMPV K655/07 strain (Ck/A/Kr/655/07), which was used for vaccine development, was isolated from a broiler breeder flock with SHS. For isolation of the virus, oropharyngeal swabs and nasal turbinate from the flock were re-suspended in minimum essential medium with gentamicin (Kukjae Pharmoco, Gyeonggi-do, Korea). After centrifugation at 1,000 $\times$ *g* for 10 min, supernatant was passaged 5 times in Vero cell. Avian metapneumovirus was identified by an immunofluorescence assay with the monoclonal antibody against aMPV. The aMPV subtype A was confirmed by reverse transcriptase polymerase chain reaction, followed by nucleotide sequencing analysis of the G glycoprotein gene of aMPV using a previously described method (Kwon et al., 2010). This virulent field strain K655/07 (K655/07parent) was later used as the challenge virus.

Animal Study 1: Pathogenicity Test of aMPV K655/07parent

Before developing an aMPV vaccine candidate from the K655/07parent virus, the pathogenicity of the K655/07 parent was evaluated for the following challenge studies. First, 4-wk-old SPF chickens (n = 135) were divided into 2 groups. The first group (n = 110) was ocularly (50 μL) and nasally (100 μL) inoculated with $10^{5.0}$ 50% tissue culture infectious doses (**$TCID_{50}$**) of K655/07parent per chicken. This method was used to ensure that accurate virus doses were delivered to each chicken. The 25 chickens in the second group were not inoculated with aMPV. Twenty chickens from the first group and 5 chickens from the second group were euthanized daily between 2 and 6 D postinoculation (**DPI**), and the nasal turbinate and trachea were collected from each chicken. The viral amount of nasal turbinate, trachea, oropharyngeal swabs, and nasal discharge from 10 birds was each measured daily by quantitative real-time reverse transcriptase polymerase chain reaction (**qRRT-PCR**). Other remaining nasal turbinate and trachea from 10 birds of each group were fixed with formalin and processed for histopathological examination. The histopathological lesions were scored as follows: 0 = normal, 1 = extensively focal lesions, 2 = multifocal lesions, and 3 = diffuse lesions. A separate set of oropharyngeal swabs and nasal discharge were taken daily from the remaining 10 chickens of the first group during the same period.

Animal Study 2: Safety Test of Attenuated aMPV K655/07HP

For attenuation of the K655/07 strain, the virus was passaged in Vero cells. Attenuation was confirmed by comparing the histopathological scores between the parent (K655/07parent) and passaged virus (K655/07HP; HP, high passage). The safety of the vaccine candidate was tested in day-old chicks. For the test, 61-day-old SPF chicks were randomly allocated into 3 groups. In 2 groups, the chicks were inoculated with $10^{4.5}$ $TCID_{50}$ of K655/07parent or K655/07HP. The control group was sham-inoculated with phosphate-buffered saline (**PBS**). Each bird received the virus ocularly (50 μL) and nasally (100 μL). At 5 DPI, all birds were euthanized, and turbinate were collected for histopathological studies. The samples were processed for histopathological examinations using the same methods as those specified for study 1.

Animal Study 3: Serological Responses to Different Doses of aMPV K655/07HP

Serological responses were evaluated with respect to the viral dose to determine the minimum dose of the

Table 1. Viral amount of aMPV inoculated in 4-week-old SPF chicks detected by qRRT-PCR.

Tissue	Number of samples	Mean CT[1] value ± SE				
		2 DPI[2]	3 DPI	4 DPI	5 DPI	6 DPI
Nasal discharge	10	23.7 ± 1.5^{A,B}	21.0 ± 0.7^{A}	21.7 ± 0.4^{A}	25.5 ± 0.5^{A,B}	26.8 ± 1.1^{A}
Turbinate tissue	10	27.0 ± 1.3^{A,B,C}	22.5 ± 0.5^{A}	20.0 ± 0.4^{A}	23.1 ± 0.5^{A}	25.3 ± 1.2^{A}
Oropharyngeal swab	10	28.0 ± 1.1^{B,C}	26.2 ± 0.5^{B}	21.9 ± 0.3^{A}	27.3 ± 1.2^{B}	28.2 ± 1.0^{A}
Trachea tissue	10	31.8 ± 1.0^{D}	27.3 ± 1.0^{B}	28.2 ± 1.1^{B}	23.7 ± 0.8^{A}	32.0 ± 1.0^{B}

Different letters represent a significant difference ($P < 0.05$) between different samples within the same DPI.
Abbreviations: aMPV, avian metapneumovirus; SPF, specific pathogen-free; qRRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction.
[1]Cycle threshold.
[2]Day postinoculation.

vaccine. Seventy-six 4-week-old SPF chickens were assigned to 7 groups of 10 to 13 chickens each. Next, 5 groups were vaccinated ocularly or orally with K655/07HP ($10^{3.5}$ $TCID_{50}$, $10^{4.0}$ $TCID_{50}$, and $10^{4.5}$ $TCID_{50}$), whereas the remaining groups remained nonimmunized and were later sham-inoculated with PBS. For 4 wk after vaccination, the chickens were examined for clinical symptoms, and the severity of these symptoms was scored as described by Jones et al. (Jones et al., 1992). Briefly, a score of 0 = no sign, 1 = clear nasal exudate, 2 = turbid nasal exudate, and 3 = frothy eyes or swollen infraorbital sinuses in conjunction with nasal exudates. Blood samples were obtained 4 wk postvaccination (**WPV**), and antibodies against aMPV were measured via the serum neutralization (**SN**) test (Paudel et al., 2016).

Animal Study 4: the Protective Efficacy of aMPV K655/07HP

To evaluate the efficacy of aMPV K655/07HP, Twenty-five 4-week-old SPF chickens were randomly allocated into 3 groups of 7 to 9 birds each. Two groups of chickens were immunized with $10^{3.5}$ $TCID_{50}$ of K655/07HP orally or intraocularly. The control (unvaccinated) group received PBS. At 4 WPV, blood samples were obtained from the birds, and aMPV specific antibody levels were measured through SN test. On the same day, all chickens were challenged with $10^{5.0}$ $TCID_{50}$ of K655/07parent. Each chicken was inoculated with the challenge virus ocularly (50 μL) and nasally (100 μL). The chickens were euthanized 5 D postchallenge. Sections of the turbinate and trachea from the chickens were removed for the detection of aMPV RNA by qRRT-PCR, as described by Kwon et al. (2010).

The same study was conducted with different groupings consisting of 2 groups of ten 1-week-old SPF chickens to determine the efficacy of the vaccines. Chickens in the vaccine group were vaccinated with $10^{3.5}$ $TCID_{50}$ of K655/07HP orally. The control group was not vaccinated. The experimental schedule and evaluation methods were the same as that described above.

Ethics Statement

All animal procedures carried out in this study (permit number: KU19110) were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee of Konkuk University.

RESULTS

Animal Study 1: Pathogenicity Test of aMPV K655/07parent

The results of aMPV-specific qRRT-PCR on turbinate, trachea tissue, oropharyngeal swabs, and nasal discharge are shown in Table 1. During the study, aMPV was detected in all collected samples. Generally, turbinate tissue and nasal discharge showed higher viral amounts than the trachea tissue and oropharyngeal swabs. In turbinate tissues, the cycle threshold values were lowest at 4DPI and increased from then on. Cycle threshold values of nasal discharge showed similar patterns but were lowest at 3DPI.

When histopathological scores were compared, score at 5 DPI in the K655/07parent inoculated group was significantly higher ($P = 0.003$) than that of the control group (Figure 1). Tissue samples at 3 DPI were excluded from the result because they were not appropriately processed for histopathological scoring. No substantial score

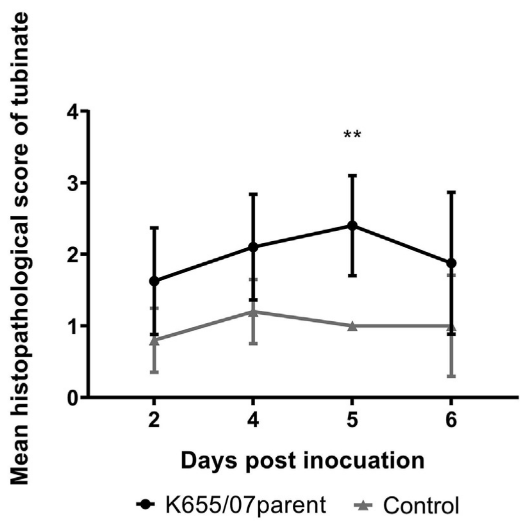

Figure 1. Histopathological scores after inoculation with K655/07parent in 4-week-old SPF chicks. Abbreviations: SPF, specific pathogen-free. $^{**}P < 0.01$ by two way ANOVA test compared with control group.

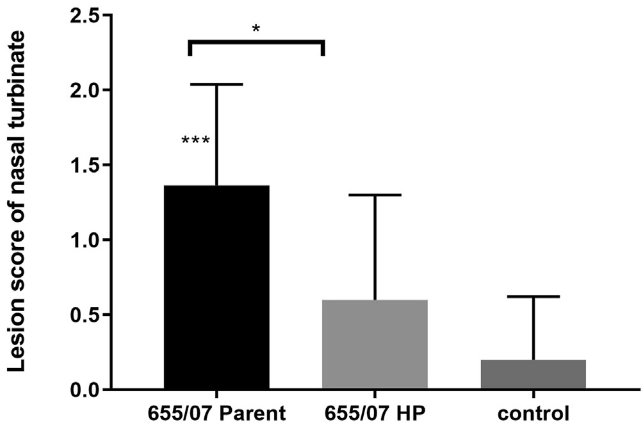

Figure 2. Histopathological lesion scores of turbinate in the safety test of K655/07HP compared with its parental virus in day-old SPF chicks. Abbreviations: SPF, specific pathogen-free. $*P < 0.05$ by one way ANOVA test compared with control group. $***P < 0.001$ by one way ANOVA test compared with control group.

was obtained for the tracheal sample up on histological analysis.

Animal Study 2: Safety Test of Attenuated aMPV K655/07HP

The K655/07 parental virus was pathogenic to day-old chicks and induced significantly higher ($P < 0.001$) inflammatory responses than that in the control group with K655/07HP (Figure 2). In contrast, there was no significant difference between the aMPV K655/07HP inoculated group and the negative control group. This showed that aMPV K655/07 was adequately attenuated by passaging in Vero cells.

Animal Study 3: Serological Responses by Different Doses of aMPV K655/07HP

Clinical signs and serological responses were monitored after the inoculation of K655/07HP with different doses to evaluate safety and immunogenicity. Over 4 wk of observation, no aMPV infection–associated clinical signs were observed despite administration of viral dose of K655/07HP (data not shown). All inoculated chickens exhibited increasing levels of SN in the SN test at 4 WPV (Table 2). In these groups, regardless of the administrated viral amount and routes, the values rose and were significantly higher than those in control group, which showed no serological response. Among the inoculated groups, there were no significant differences between the different doses and vaccination routes of K655/07HP. Based on the results of this study, the minimum dose of the candidate vaccine was designated as $10^{3.5}$ $TCID_{50}$ through both ocular and oral routes.

Animal Study 4: the Protective Efficacy of aMPV K655/07HP

The aMPV live vaccine candidate K655/07HP was administered orally or intraocularly to 1- or 4-week-old SPF chickens to evaluate its efficacy. In blood samples collected from unvaccinated chickens, no antibodies against aMPV were detected (Table 3). Chickens vaccinated at 4 wk showed significantly higher ($P < 0.015$) values in the SN test than the control group. Chickens vaccinated when they were 1-week-old did not show any significant difference with respect to the control group in the SN test. Regardless of serological results, all turbinate tissues and trachea samples from the vaccinated groups were negative for aMPV after the parent strain challenge, as determined by reverse transcriptase polymerase chain reaction. For the 2 control groups, the number of positive samples was 7/7 or 10/10 for turbinate and 5/7 or 5/10 for trachea tissues.

DISCUSSION

Establishing an appropriate animal challenge model is essential when developing a vaccine and evaluating its safety and efficacy. Although there have been several studies about aMPV live vaccine on chicken, many of them evaluated the safety and efficacy based on the clinical sign alone (Ganapathy et al., 2006; Cook et al., 2010; Ganapathy et al., 2010; Awad et al., 2015). Although some studies conducted isolation and detection of the virus for evaluation of the aMPV vaccine, the studies did not show a statistical difference between groups (Ganapathy and Jones, 2007). Experimentally, aMPV infected chickens often show only mild respiratory signs unlike those seen in the field (Kwon et al., 2010). To prove the efficacy and safety of the attenuated live vaccine, there should be an adequate evaluation way providing enough differences for vaccinated group from

Table 2. Serological responses to different doses of aMPV K655/07HP in 4-week-old SPF chickens.

Administrated viral dose	Administration route	Number of birds	Mean value of serum neutralization test ± SE
$10^{3.5}$ $TCID_{50}$[1]	Ocular	10	808 ± 140
$10^{4.0}$ $TCID_{50}$	Ocular	10	960 ± 107
$10^{4.5}$ $TCID_{50}$	Ocular	10	873 ± 245
$10^{3.5}$ $TCID_{50}$	Oral	13	763 ± 123
$10^{4.5}$ $TCID_{50}$	Oral	13	800 ± 117
PBS	Ocular	10	0 ± 0
PBS	Oral	10	0 ± 0

Abbreviations: aMPV, avian metapneumovirus; PBS, phosphate-buffered saline; SPF, specific pathogen-free.
[1]50% tissue culture infectious doses.

Table 3. Results of the protective tests of the aMPV K655/07HP vaccine through different administration routes in 1- or 4-week-old SPF chickens.

Group	Mean value of serum neutralization test at 4WPV ± SE	Positive sample number/tested sample number 5 DPC	
		Turbinate tissue	Trachea
4-week-old control	0 ± 0^{B}	7/7	5/7
4-week-old ocular vaccination	653 ± 171^{A}	0/9[3]	0/9[2]
4-week-old oral vaccination	573 ± 147^{A}	0/9[3]	0/9[2]
1-week-old control	0 ± 0^{B}	10/10	5/10
1-week-old oral vaccination	$272 \pm 52^{A,B}$	0/10[3]	0/10[1]

Different letters represent a significant difference ($P < 0.05$) between different groups.

Abbreviations: aMPV, avian metapneumovirus; DPC, days postchallenge; SPF, specific pathogen-free; WPV, wk postvaccination.

[1] $P < 0.05$ by 2-tailed t-test compared with the control group.

[2] $P < 0.01$ by 2-tailed t-test compared with the control group.

[3] $P < 0.001$ by 2-tailed t-test compared with the control group.

challenged control group. The clinical sign is not only a subjective method but also an inappropriate indicator to secure enough difference for statistical analysis. Therefore, we compared histopathological scores and viral quantity by various sampling sites and days after challenge as the first animal study. Based on detailed evaluation methods, we designated the optimum time and the site of sampling for following safety and efficacy study.

Safety is a paramount concern with regards to live vaccines. Based on safety results, the applicable age for vaccination was established, and possible vaccine reactions were predicted. In the safety test on day-old chicks, aMPV K655/07HP—a virus passaged in Vero cells—showed significantly decreased pathogenicity compared with its parent virus, aMPV K655/07parent. In addition, there was no significant difference with respect to the unvaccinated control. The tested viral amount, $10^{4.5}$ $TCID_{50}$ aMPV K655/07HP, corresponds to 10 vaccine doses, which was later determined to be $10^{3.5}$ $TCID_{50}$ per dose. Because the vaccine proved to be safe for day-old chicks even in harsh conditions with high overdoses, this candidate vaccine can be used in chickens of any age without concerns regarding vaccine reactions.

The presence of antibodies in the blood is a convenient indicator of vaccine-take (Awad et al., 2015). The minimum effective dose was designated by SN test. Although many studies have used commercial enzyme-linked immunosorbent assay (**ELISA**), the ELISA values do not reflect the efficacy of live aMPV vaccines (Chary et al., 2005; Ganapathy and Jones, 2007; Ganapathy et al., 2010; Kwon et al., 2010; Awad et al., 2015). Antibody mechanisms, including neutralization of the epitope, show that the SN test is more appropriate to estimate the protective efficacy of the aMPV live vaccine. There was no correspondence between the ELISA value and the decrease in viral amount after the challenge (data not shown). In this study, aMPV-specific antibodies were measured through SN tests, and seroconversion was confirmed after vaccination using different doses and routes.

Animal study 4 was performed to assess the efficacy of aMPV live vaccine candidate against its parent strain with respect to the route and age of the vaccinated chickens. In this article, to our knowledge, for the first time, the difference in vaccine efficacy based on vaccination route and age was investigated. Although there was a difference in the SN test results for chickens vaccinated at 1 wk and 4 wk of age, they were completely protected against the challenge. Smialek et al. also reported similar result (Smialek et al., 2015). The authors explained that differences between birds vaccinated at different age might be influenced by the maturation of the immune and/or respiratory system. For the efficacy test, the proliferation of the challenged virus was assessed through the detection of viral RNA. The perfect protection observed in this study proves the excellent efficacy of the current candidate vaccine. This finding further supports previous reports that contend that humoral antibodies against aMPV play little or no part in the protection of the respiratory tract because of the limited accessibility to the epithelium of the turbinate and trachea (Naylor et al., 1997; Ganapathy et al., 2005; Ganapathy et al., 2007; Ganapathy et al., 2010; Awad et al., 2015). Local and cell-mediated immunity play a significant role in protection against aMPV (Rubbenstroth and Rautenschlein, 2009; Awad et al., 2015; Smialek et al., 2015).

In conclusion, an effective aMPV challenge model was established for studying aMPV in chickens, and several insights have been provided. The safety and efficacy studies indicate that the new aMPV candidate vaccine can effectively assist in reducing economic losses due to aMPV infection.

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DISCLOSURES

The authors did not provide any conflict of interest statement.

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