The rise of fowl adenovirus-associated diseases in Asia

Fowl adenovirus type 1-associated disease such as inclusion body hepatitis (IBH) and hepatitis-hydropericardium syndrome (HHS) were initially described in the 1960s in the United States and since then FAdV has been found widely distributed throughout the world.

FAdVs are grouped into five species (A-E) based on restriction fragment length polymorphisms of the full FAdV genome, and they are further divided into 12 serotypes by cross-neutralisation tests.

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At present, IBH is ubiquitous in Indonesia and other Asian countries. FAdVs have been known to cause economic losses to the poultry industry since 2017.

At present, three prevalent serotypes: 8b, 11 and 8a have been isolated from broilers and broiler breeders in Indonesia.

Horizontal transmission and subsequent mortalities have been observed in 3-4 week old broilers which could be infected mainly via the oral-faecal route. In addition, with the spread of type A avian influenza viruses of H5N1 and H9N2 subtypes in Indonesia, major breeding operations in Indonesia have elevated their biosecurity procedures and sanitary status, generating immunologically naive breeding stocks.

Consequently, these breeding stocks become susceptible to FAdV type 1 infection during high stress periods, in particular at the onset of egg production without compromising laying performance.

These infected breeder flocks will spread the virus vertically during lay, resulting in low hatchability, poor chick quality, and high mortality in young broilers.

Hence an effective control measure should be initiated at broiler breeder farms to provide maternal antibody against FAdV that can be transferred and prevent FAdV-associated diseases in their progenies. Several studies have reported the control of FAdV infection and revealed successful protection by immunisation with either attenuated vaccines or inactivated vaccines. At present, commercial FAdV vaccines are not available in Indonesia.

However, sero-monitoring of broiler breeders over the last decade and sporadic outbreaks of IBH indicate the prevalence of FAdV in Indonesia. In this study, three FAdV serotypes, namely 8a, 8b and 11, are found circulating in Indonesia.

The growing number of field reports suggests an increase in pathogenicity of FAdV, in particular strain 8b over 8a and 11 in highly selected modern yield-type broilers. Unlike broilers, commercial layers do not seem to be affected in this wave of outbreaks.

Current commercially available FAdV vaccines in the world contain mostly serotypes 4 with variation in antigen content and are reported to be limited in their efficacy against specific FAdV serotypes, such as 8b and 11, circulating in Asia, while autogenous vaccines which are often used in primary breeder companies still lack potency and efficacy data.

In this study, we standardise the optimum antigen content by formulating antigen matching oil emulsion vaccines with antigen contents ranging from $10^{5.5}$-$10^{7.0}$ $TCID_{50}$ per dose and they are assessed based on the vaccination immune response using commercial ELISA kit and serum neutralisation test (SNT).

Materials and methods

● Virus isolation and characterisation:
From September 2017 to July 2018, liver samples were collected from broiler and broiler breeder chickens infected by FAdV throughout Indonesia.

Liver suspension of field sample were homogenised and 8-day-old specific pathogen-free (SPF) chicken embryos were inoculated with 0.2ml supernatant via the embryo yolk sac. Liver from dead embryos showing clinical liver pathology were taken, and FAdV purified in primary embryo cell culture. FAdV nucleic acid were analysed by polymerase chain reaction (PCR) and DNA-sequencing.

Three serotypes: 8a, 8b and 11 were isolated and identified to be circulating in Indonesia.

● Vaccine production:
Serotype 8b and 11 FAdV were grown on primary chicken embryo liver cells (CEL) until rounding and detachment of cells which appeared refractile under a light microscope were observed.

In order to identify the minimum antigen content in FAdV vaccines, a monovalent FAdV serotype 8b vaccine was produced in view of its significant prevalence in Indonesia.

This vaccine had four different formulations based on different antigenic concentrations as $10^{5.5}$, $10^{6.0}$, $10^{6.5}$ and $10^{7.0}$ $TCID_{50}$ per dose respectively.

These inactivated vaccines were then blended with mineral-oil adjuvants and were administered by intramuscular injection into pectoral muscles of six week-old SPF chickens at the dose of 0.5ml per bird.

Sera were taken every week until eight weeks post-vaccination and tested by ELISA and SNT.

In the second part of the study, the respective SPF chicken groups were boosted with a second dose of FAdV vaccines as per aforementioned four formulated antigen concentration in order to mimic field practice (data not shown).

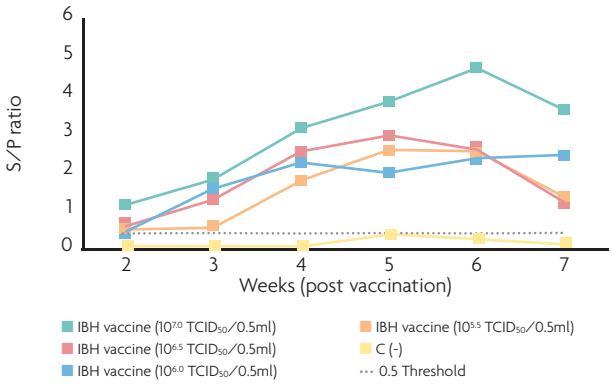

Fig. 1. Mean group of sample-to-positive (S/P) ratios in serum from vaccinated groups and negative control on different weeks (at 2-7 weeks post vaccination).

● Serology:
Host response to FAdV vaccination was evaluated based on the change of pre-vaccination and post-vaccination antibody titers.

● Enzyme-linked immunosorbent assay (ELISA):
Enzyme-linked immunosorbent assay (ELISA) techniques for the detection of group-specific or type-specific antibodies have been widely used throughout the world for the diagnosis of FAdV. ELISA typically represents the method of choice to screen breeder flocks for the presence of adenovirus antibodies.

Cross-reactivity between various FAdV serotypes is a basis for sensitive detection of exposure to heterologous FAdV serotypes by ELISA.

However, ELISAs from different manufacturers and even different batches from the same manufacturer cannot be compared, and there is no clear determination on exposure and convalescence antibodies level derived from infections and vaccinations.

Continued on page 15

Continued from page 13

In order to compare the results between batches and manufacturers we use S/P (sample-to-positive) ratio to measure immune response to vaccination. SPF chickens were bled every week and sera tested using a commercial ELISA kit (BioChek, Reeuwijk, The Netherlands).

Sera were diluted by 1:100 and OD measured at 405nm. According to the manufacturer's manual positive antibody response cut off of BioChek ELISA is at S/P ratio >0.5 and at positive titer >1070.

● **Serum neutralisation test (SNT):** The serum neutralisation test (SNT) is the gold standard to measure and quantify neutralising antibodies against specific FAdV serotypes. In our study neutralising antibodies to FAdV serotype 8b were detected in a SNT with the constant virus, varying serum method. SNTs were carried out in 96-well cell culture plates as described previously by Van Oirschot (2000).

Sera were diluted in twofold dilutions (1:2) and the serum-virus mixtures (equal volumes of 50µl) were incubated at 37°C for one hour before the addition of CEL cells (5 x 10^5 cells/0.1ml/well of 50µL).

Tests were performed against the FAdv 8b strain, with 100 tissue culture infectious doses ($TCID_{50}$).

Plates were incubated at 37°C in a moist atmosphere with 5% CO_2 for 5-7 days until the final reading, which was performed by microscopic control every 24 hours with basis on the presence or absence of a cytopathic effect (CPE).

Results were calculated by the method of Reed and Muench and expressed as positive neutralising antibody titer ≥2 or negative neutralising antibody titer <2.

Results

● **Virus isolation and characterisation**

The PCR products of the hexon loop-1 gene were sequenced. Nucleotide sequences of the Hexon genes were aligned using the Geneious (Biomatters Ltd, Auckland, New Zealand), and were compared to the sequences of other reference FAdVs in the Genebank.

A phylogenetic tree was created using the neighbour-joining method in Geneious R10.

Serotype 8b and serotype 11 are widely distributed in Asia. FAdVs of serotype 8a and serotype 1 were isolated from broilers in 2016.

In vivo pathogenicity in young SPF chicks serotype 8b was observed, indicating higher pathogenicity than serotype 11 and 8a (data not shown).

● **Vaccine production:** A highly virulent serotype 8b FAdV strain, ID/Malang/035/2017, was selected as a candidate vaccine strain and prepared as an oil-adjuvant inactivated virus vaccine.

Four types of vaccine with virus contents of $10^{5.5}$, $10^{6.0}$, $10^{6.5}$ and $10^{7.0}$ $TCID_{50}$ per dose, respectively, were produced. Formaldehyde (0.1% in final product) was added to the cultures containing ID/Malang/035/2017 strain over night at 37°C during the process of vaccine inactivation.

● **Detection of antibodies by ELISA:** Serum samples were examined by indirect ELISA (BioChek). As the manufacturer's ELISA kit is coated with FAdV serotype 1 antigen, only a positive antibody response threshold to vaccination or field infection is determined as >1070, we are not able to utilise the ELISA to produce a quantitative protective antibody baseline for our breeding farm.

Hence this study was assessed based on three criteria as follows:

● Time of vaccine take (seroconversion).
● Uniformity of vaccine take.
● Duration of immunity.

Fig. 1 shows mean S/P ratio in sera of chickens that were vaccinated using one dose of IBH serotype 8b vaccine and collected every week from two weeks until seven weeks post vaccination. All vaccine groups revealed seroconversion as early as 2-3 weeks post vaccination, with S/P ratio above the manufacturer's cut off (>0,5), while the negative control group remained negative for antibodies against FAdV.

High and medium antigen content groups ($10^{6.0}$, $10^{6.5}$ and $10^{7.0}$ $TCID_{50}$) showed overall statistically significant ($p<0.05$) antibody increment compared to the negative control on the third week post-vaccination with S/P ratio between 1.0-2.0.

While the low antigen content group ($10^{5.5}$ $TCID_{50}$) seroconverted at four weeks post vaccination, the vaccine group with the highest antigen content ($10^{7.0}$ $TCID_{50}$) showed the fastest onset of seroconversion (two weeks post-vaccination) (Table 1). Meanwhile, there were no differences on antibody response in the mid-range antigen content groups, $10^{6.5}$ and $10^{6.0}$ $TCID_{50}$.

Group	Week post vaccination					
	2	3	4	5	6	7
c (-)	0.1200[a]	0.1005[a]	0.1073[a]	0.4224[a]	0.2997[a]	0.1872[a]
IBH 5.5	0.5716[a]	0.5710[ab]	1.8083[b]	2.5697[b]	2.5400[b]	1.3674[ab]
IBH 6.0	0.4919[a]	1.5826[c]	2.2393[bc]	1.9850[ab]	2.3617[b]	2.4374[bc]
IBH 6.5	0.6161[ab]	1.2949[bc]	2.5325[bc]	2.9359[bc]	2.5753[b]	1.2170[ab]
IBH 7.0	1.1758[b]	1.8249[c]	3.122[c]	3.300[c]	4.6779[c]	3.530[c]

Table 1. Statistical significance comparison of S/P ratio of each group for seven weeks by ANOVA. P>0.05.

Both vaccine groups sero-converted after three weeks post-vaccination. However, uniformity of vaccine response at four weeks post-vaccination for the groups $10^{7.0}$, $10^{6.5}$, $10^{6.0}$ and $10^{5.5}$ $TCID_{50}$ were 100, 90, 90 and 80% respectively (data not shown).

All vaccine groups reached the peak of seroconversion at 5-6 weeks post-vaccination.

This result indicated that a minimum antigen content of $10^{6.0}$ $TCID_{50}$ is required for fast and complete protection of broiler breeders and their progeny.

Detection of neutralising antibodies by SNT

Serum samples were examined for neutralisation antibodies (NAbs) by SNT. Pre-vaccination titer were negative and control SPF chickens remained seronegative throughout the experiment.

The neutralising antibody (NAb) response, evaluated by SNT depends on the immunogenicity of the FAdV serotype 8b and dosage. The mean neutralising antibodies titer against the FAdV-8b in the antigen dose of $10^{5.5}$-$10^{7.0}$ $TCID_{50}$ were measured from 2-7 weeks post-vaccination.

This study revealed a significant antigen dose dependent immune response, meaning the higher the antigen content, the faster the vaccine take and the higher the SNT titer.

The SNT titer of all groups reached the maximum titer at five weeks post-vaccination.

However, the vaccine with the highest antigen content $10^{7.0}$ $TCID_{50}$/dose showed a 2 Log_2 drop in SNT titer, from 11.4 to 10.2 and further to 9.6 Log_2, while the other groups $10^{6.5}$, $10^{6.0}$ and $10^{5.5}$ $TCID_{50}$/dose plateau-out their SN titer at 8 Log_2 (Fig. 2 and Table 2).

All four antigen groups reach their maximum SNT titer at five weeks post-vaccination, but the speed and uniformity for the vaccine take for group 4 ($10^{5.5}$ $TCID_{50}$) is the slowest.

Discussion

Vaccination with homologous autogenous FAdV vaccines in primary breeders in North America and Europe at 10 and 17 weeks of age is a common practice as

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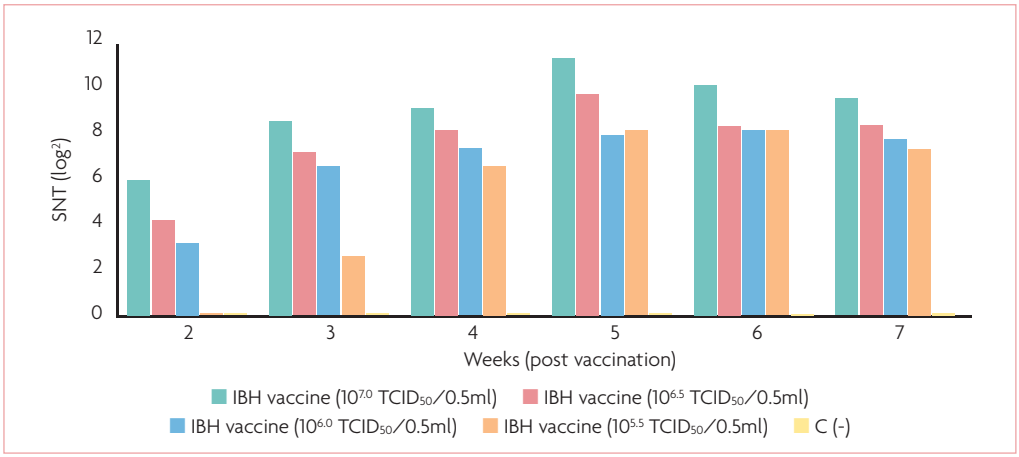
Fig. 2. Serum neutralisation test titers in serum from vaccinated groups and negative control on different weeks (at 2-7 weeks post vaccination).



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infected breeder flocks may spread the virus vertically during the laying period resulting in low hatchability, poor chick quality, and high mortality in young broilers.

With the increased cases of FAdV-associated diseases in Asia, we look at several aspects of FAdV vaccination.

Different groups reported cross protection between serotypes, while others have shown limited cross protection and the ability to cross-protect differs from strain to strain.

Suboptimal protection has been reported from different sources in the industry in India and Malaysia using a FAdV serotype four vaccines (personal communication).

Moreover, we noticed that antigen content of commercial and autogenous FAdV vaccines are not standardised and according to the manufacturer range from $10^{4.0}$ to $10^{7.0}$ $TCID_{50}$ per dose.

This study has demonstrated a minimum antigen content of $10^{6.0}$ $TCID_{50}$, using a homologous strain is important for protection.

Many breeding operations in Asia have no access to either vaccines or ELISA kit for sero-monitoring.

1 x sero-monitoring of FAdV antibody titer prior to egg production using ELISA is common in breeding operations in order to ensure existence of antibody by field infection.

Monitoring of antibody is helpful but meaningless when there is little or no seroconversion during the rearing period due to stringent biosecurity procedures in breeding operations.

Vaccination with homologous monovalent or heterologous vaccines that contain sufficient antigen similar to the circulating strain is crucial for protection.

In addition, commercial ELISAs depend on the virus, dosage and the route of inoculation to provide valid information on protection. Hence monitoring of antibody response to vaccination using ELISA has less benefit in comparison to the neutralising antibody (Ab) response, evaluated by SNT.

In the first part of our study, we show that it is pivotal that antigen content in FAdV vaccines should be $>10^{6.0}$ $TCID_{50}$ per dose per strain. However, considering the low antigen yield during FAdV vaccine production in primary cells or cell lines, FAdV would be very expensive to produce in order to fulfil the recommended minimum antigen content.

In our study we use the SNT to determine the minimum antigen content for FAdV vaccines. All antigen groups reach their maximum SNT titer at five weeks post vaccination, but the speed for the vaccine take for group 4 ($10^{5.5}$ $TCID_{50}$) was slower than other groups.

This group reached an antibody level of Log_2 6 by week four, which is considered as the threshold. Therefore, we suggest minimum $10^{6.0}$ $TCID_{50}$ per dose per strain for FAdV vaccines since it can induce immune response of Log_2 6 by week two.

To further understand the disease control, it is necessary to perform a prime-boost vaccination regime to mimic the common farm practice with booster vaccinations and cross challenged with heterologous FAdV serotypes.

A challenge and protection study in day-old SPF white leghorns is not easy to conduct as leghorns are supposedly less susceptible to FAdV than meat-type broilers.

In order to induce pathological signs in three week old SPF chickens, we may suppress their immunity by chemicals. Alternatively, clinical pathological changes may be detected by histological analysis. ■

Group	Week post vaccination						
	1	2	3	4	5	6	7
IBH 7.0	1.8^{b}	6^{c}	8.6^{d}	9.2^{d}	11.4^{d}	10.2^{d}	9.6^{c}
IBH 6.5	0^{a}	4.2^{b}	7.2^{c}	8.2^{c}	9.8^{c}	8.4^{b}	8.4^{b}
IBH 6.0	0^{a}	3.2^{b}	6.6^{c}	7.4^{c}	8^{b}	8.2^{b}	7.8^{b}
IBH 5.5	0^{a}	0.8^{ab}	2.6^{b}	6^{b}	8.2^{b}	8.4^{b}	7.4^{b}
C (-)	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Table 2. Statistical significance comparison of SNT titer (log2) of each group for seven week by ANOVA. P>0.05.

References are available from the authors on request