Journal Club

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Construction of recombinant baculovirus vaccines for Newcastle disease virus and an assessment of their immunogenicity

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Introduction

Newcastle disease virus (NDV)

Baculovirus expression system

Newcastle disease (ND)

- Newcastle disease is a highly contagious bird disease affecting many domestic and wild avian species, it is transmissible to humans
- ND is characterized by acute morbidity and high mortality especially in chickens and OIE List A disease
- It is the global issue leading to huge economic losses in poultry industry and trade restrictions
- Nowadays, ND is endemic in several developing countries with severe outbreaks have occurred in year by year
- Therefore, controlling the spread of NDV is an urgent unmet need in the global poultry industry

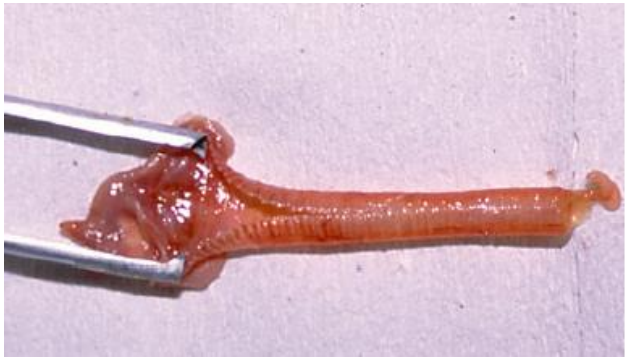
Clinical signs of ND

- Signs of infection with NDV vary greatly depending on factors such as the strain of virus, age and species of the host
- The incubation period for the disease ranges from 2-15 days
- An infected bird may exhibit several signs including:
  - Respiratory signs: gasping, coughing
  - Nervous signs: muscular tremors, drooping wings, twisting of head and neck, circling, complete paralysis
  - Swelling of the tissues around the eyes and neck, greenish, watery diarrhea
  - Misshapen, rough- or thin-shelled eggs and reduced egg production

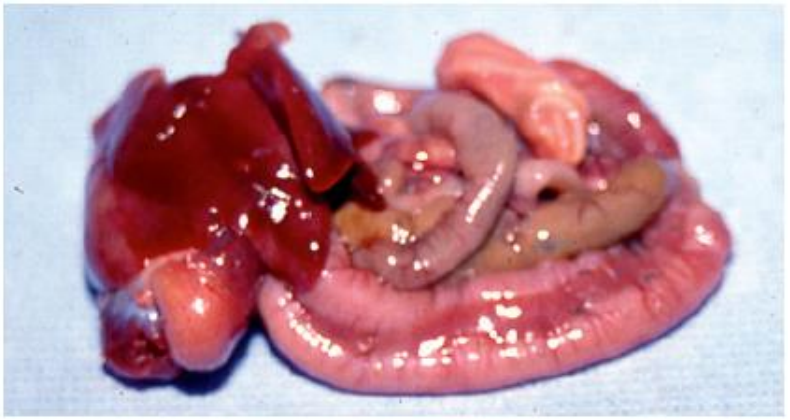
Clinical signs of ND

Soft-shelled, irregular-shaped and off-coloured eggs produced by hens affected by ND

Layer hen, naturally infected with ND virus, velogenic neurotropic pathotype, exhibiting serious nervous signs with torticollis and paresis



Haemorrhages in the larynx



Pancreatitis and duodenitis

Source: Avian Influenza and Newcastle Disease, A Field and Laboratory Manual

Newcastle disease virus (NDV)

TAXONOMY

Group V: Negative sense ssRNA viruses

Order: *Mononegavirales*

Family: ***Paramyxoviridae***

Subfamily: *Paramyxovirinae*

Genus: *Aquaparamyxovirus*
Avulavirus
Ferlavirus
Henipavirus
Morbilivirus
Respirovirus
Rubulavirus

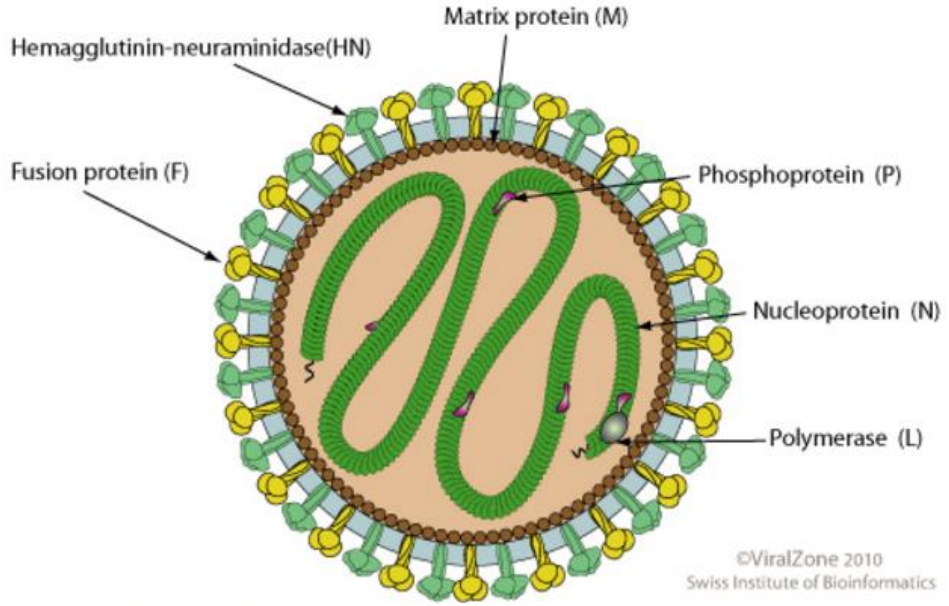
Subfamily: *Pneumovirinae*

Genus: *Metapneumovirus*
Pneumovirus

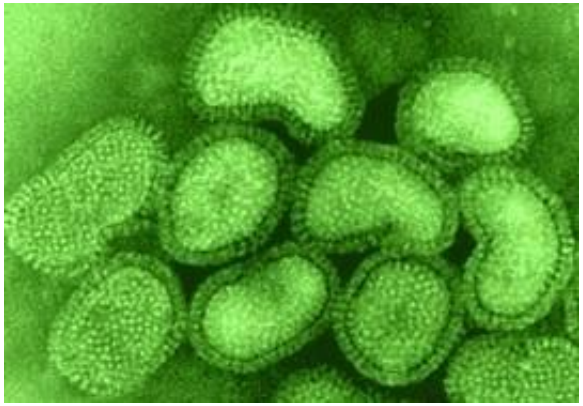
Genus	Species	Virus (Abbreviation)
Avulavirus	*Avian paramyxovirus 2*	avian paramyxovirus 2 (APMV-2)
	Avian paramyxovirus 3	avian paramyxovirus 3 (APMV-3)
	Avian paramyxovirus 4	avian paramyxovirus 4 (APMV-4)
	Avian paramyxovirus 5	avian paramyxovirus 5 (APMV-5)
	Avian paramyxovirus 6	avian paramyxovirus 6 (APMV-6)
	Avian paramyxovirus 7	avian paramyxovirus 7 (APMV-7)
	Avian paramyxovirus 8	avian paramyxovirus 8 (APMV-8)
	Avian paramyxovirus 9	avian paramyxovirus 9 (APMV-9)
	Avian paramyxovirus 10	avian paramyxovirus 10 (APMV-10)
	Avian paramyxovirus 11	avian paramyxovirus 11 (APMV-11)
	Avian paramyxovirus 12	avian paramyxovirus 12 (APMV-12)
	*Newcastle disease virus**	avian paramyxovirus 1 (APMV-1)

Avian Paramyxoviruses (APMVs) almost are classified in the genus *Avulavirus*
All strains of Newcastle disease virus (NDV) belong to serotype APMV-1

Newcastle disease virus (NDV)

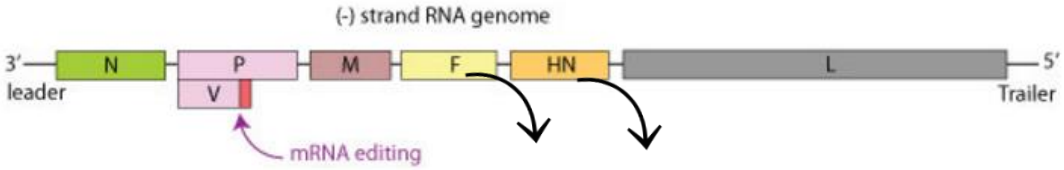


Newcastle disease virus virion



Pleomorphic enveloped particles

GENOME



F and HN proteins are the major protective antigens in NDV

HN and F glycoproteins are important for virus infectivity and pathogenicity

Negative-stranded RNA linear genome, about 15 kb in size. Encodes for seven proteins

Source: http://viralzone.expasy.org/viralzone/all_by_species/84.html

Newcastle disease virus (NDV)

- NDV causes a highly contagious disease in chickens
- NDV isolates vary widely in virulence and are divided into three broad pathotypes based on the severity of the disease produced in chickens;

  Lentogenic → avirulent
  Mesogenic → moderately virulent
  Velogenic → highly virulent

- Lentogenic NDV strains are widely used as live vaccines around the world
- Furthermore, there are reports that has been a major antigenic drift in the types of NDV strains

Newcastle disease virus vaccine

- Current strategies for preventing ND utilize inactivated and attenuated vaccines, but it is always possible that immune failure
- Conventional live vaccines against NDV are available, but they can revert to virulent strains and do not protect against mutant strains of the virus
- Therefore, there is a critical unmet need for a novel vaccine that is safe, efficacious, and cost effective
- Previous studies have achieved a 100% rate of protection by immunizing chickens using a recombinant NDV vaccine containing the F and HN gene using genetically engineered vaccines

Baculovirus expression system (BES)

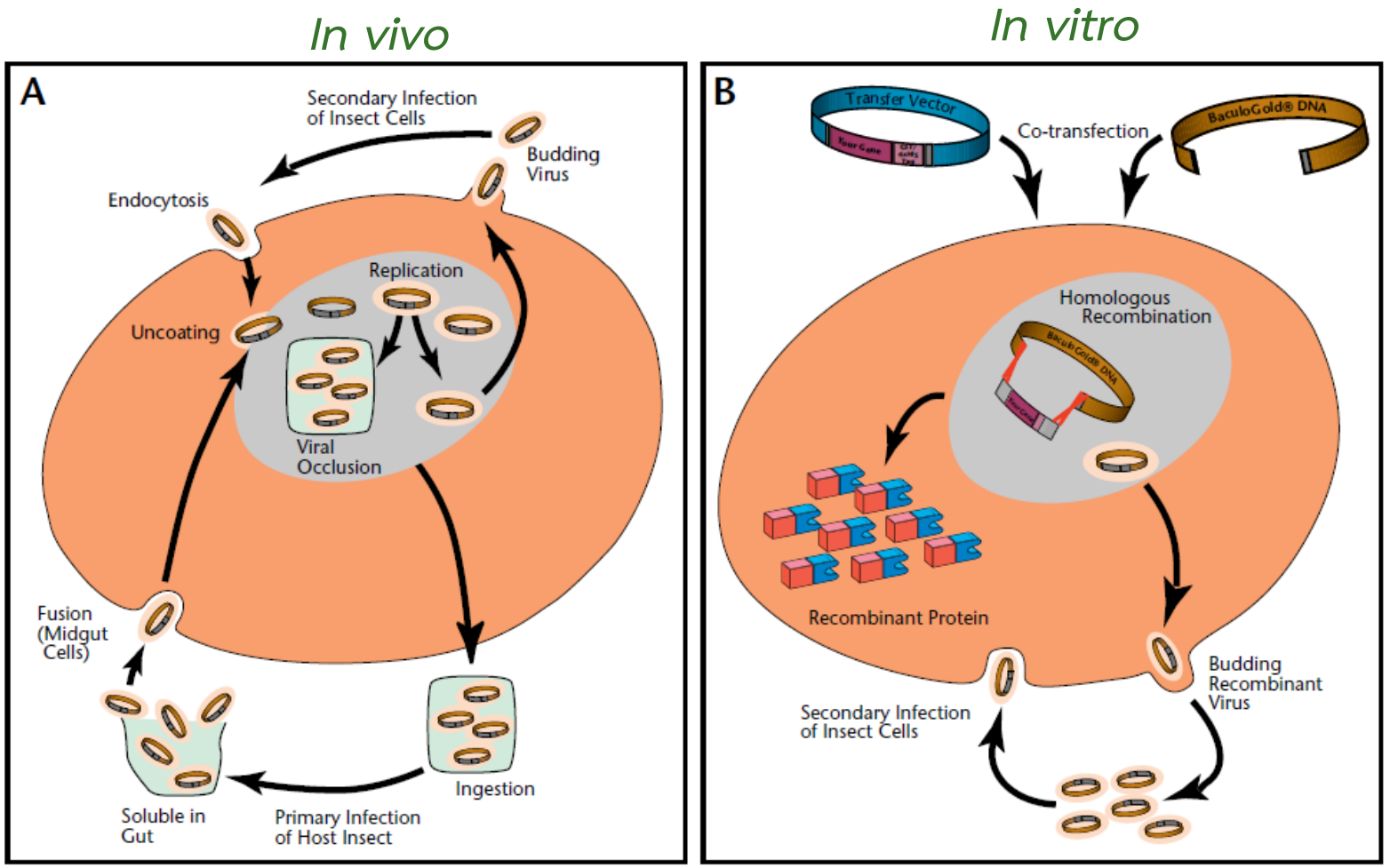
- **BES has distinct advantages with other recombinant expression systems**

  - ✓ It can accommodate large fragments of exogenous gene
  - ✓ It has post-translationally modify products without causing cytotoxic effects
  - ✓ It can express multiple genes simultaneously at high levels
  - ✓ The expressed products also retain their biological activity

- **Most notably, the BES is generally considered a very safe way to express exogenous genes**

Baculovirus expression system (BES)

The Baculovirus life cycle *in vivo* and *in vitro*



Advantages of using the BES

Comparison of BEVS and bacterial expression systems

Features	BEVS	Bacterial
Simple to use	✓	✓
Protein size	unlimited	<100 kD
Multiple gene expression	✓	
Signal peptide cleavage	✓	
Intron splicing	✓	
Nuclear transport	✓	
Functional protein	✓	sometimes
Phosphorylation	✓	sometimes
Glycosylation	✓	
Acylation	✓	

Performing many of the processing events that are required for forming biologically active, foreign proteins

Recombinant proteins expressed in bacterial systems are insoluble, aggregated and incorrectly folded

Proteins expressed in BEVS are, in most cases, soluble and functionally active

Baculovirus expression system (BES)

- The baculovirus system has been modified in many different ways to optimize the expression of exogenous genes; For example
  - ✓ The mammalian cell promoters such as cytomegalovirus **(CMV)**, strong promoter that controls expression from recombinant baculovirus expression platforms in mammalian and poultry cells
  - ✓ The woodchuck hepatitis virus post-transcriptional regulatory element **(WPRE)** added to the 3'untranslated region of the expressed gene can also improve the expression efficiency of target gene expression
  - ✓ The transduction efficiency of the baculovirus system can be increased by displaying a truncated vesicular stomatitis virus G protein **(VSV-GED)** on the surface of the baculovirus
  - ✓ The inverted terminal repeats **(ITRs)** from AAV extend the length of time that target genes are expressed *in vivo*
- Thus modified baculovirus systems could be an ideal platform for NDV vaccine development

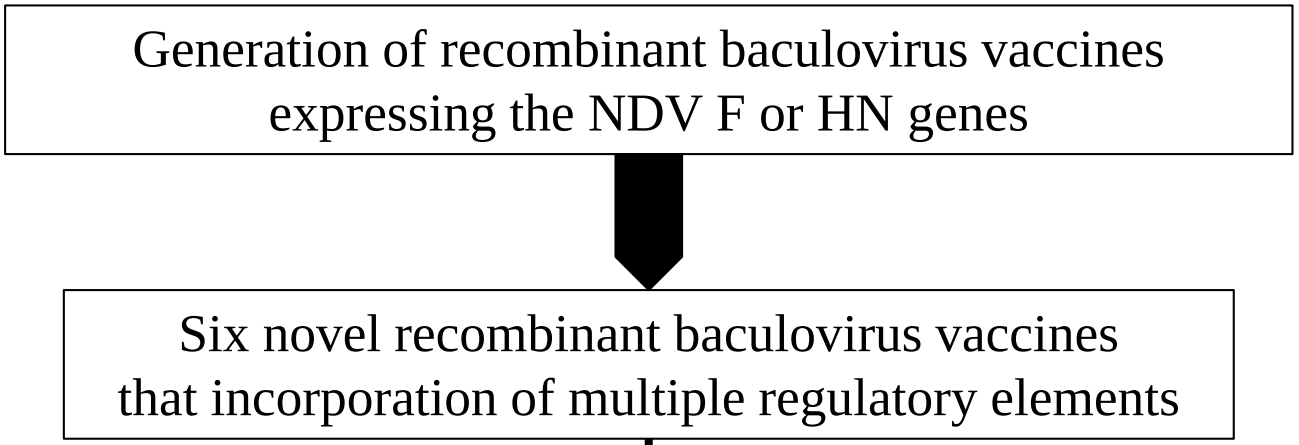
Aim of Study

1. To investigate the effects of the VSV-GED, WPRE, and ITRs regulatory elements on expression of the NDV F and HN genes controlled by the CMV promoter in a recombinant baculovirus vaccine for NDV
2. To assess the efficacy of the vaccine, assessed the humoral and cellular immune response *in vitro* to the F and HN proteins in the presence and absence of each regulatory element
3. To assess the efficacy of the vaccine *in vivo,* the humoral and cellular immune response to vaccination was characterized

Methods and Results

- Construction of NDV recombinant baculovirus vaccines
- Testing the *in vitro* efficacy of recombinant baculovirus vaccines
- Testing the *in vivo* efficacy of recombinant baculovirus vaccines

Overview of research



Testing the *in vitro* efficacy of vaccines

- Expression of the NDV F and HN recombinant proteins in CEFs with different regulatory elements

Testing the *in vivo* efficacy of vaccines

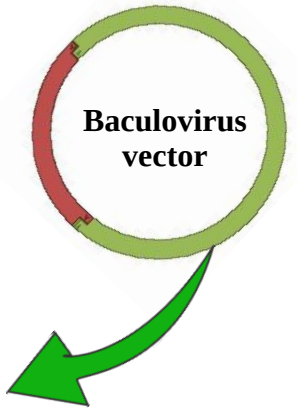
- Chicken immunization: Survival & Protection rates
- Humoral: neutralizing antibodies & IgG titers
- Assessing lymphoproliferative responses
- Cytokine levels in immune serum

Methods and Results

Construction of NDV recombinant baculovirus vaccines

Generation of NDV recombinant baculovirus vaccines

Construction of baculovirus vectors



Six (6) plasmids were constructed:

Baculovirus vectors	Gene cassette					
	CMV promoter	SV40 poly(A)	gp64 signal peptide	Regulatory elements		
				WPRE	VSV-GED	ITRs
pLM(–)-F	√	√				
pLM-F	√	√	√	√	√	
pLM-ITRs-F	√	√	√	√	√	√
pLM(–)-HN	√	√				
pLM-HN	√	√	√	√	√	
pLM-ITRs-HN	√	√	√	√	√	√

Baculovirus vector for expression Fusion (F) protein

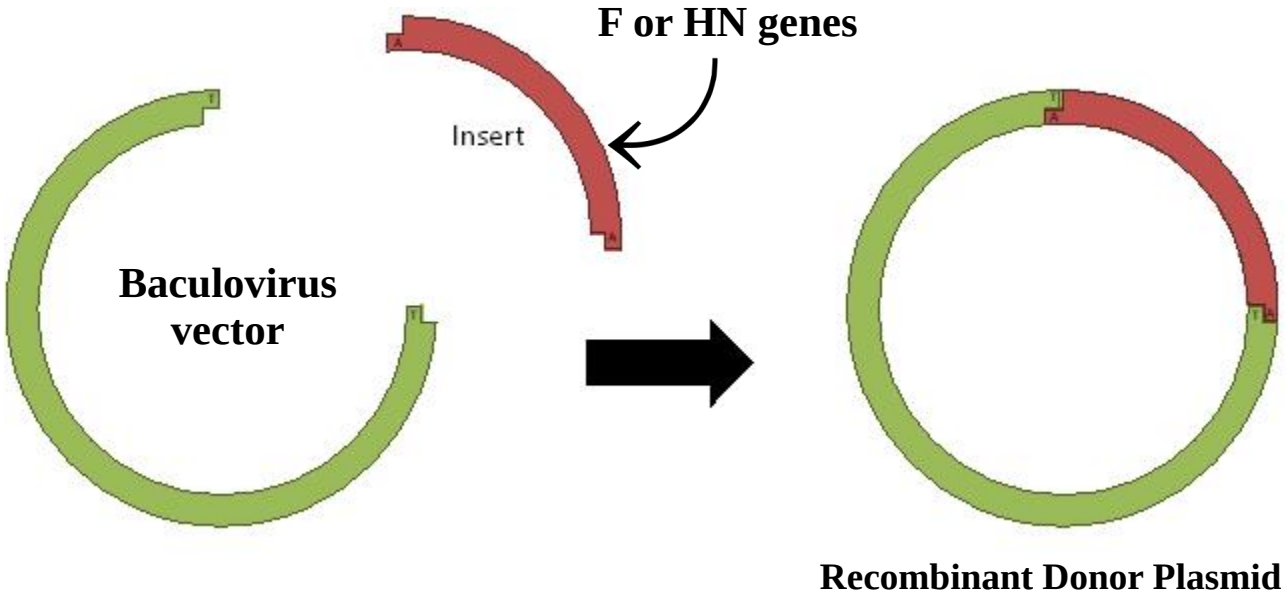
Baculovirus vector for expression hemagglutinin-neuraminidase (HN) protein

truncated vesicular stomatitis virus G protein (VSV-GED)
woodchuck hepatitis virus post-transcriptional regulatory element (WPRE
inverted terminal repeats (ITRs) of adeno-associated virus (AAV Serotype II)

Generation of NDV recombinant baculovirus vaccines

Generation Recombinant Donor plasmid vectors

- The F or HN genes were individually amplified with primers that also inserted a the 6xHis-tag



pLM(–)-F
pLM-F
pLM-ITRs-F
pLM(–)-HN
pLM-HN
pLM-ITRs-HN

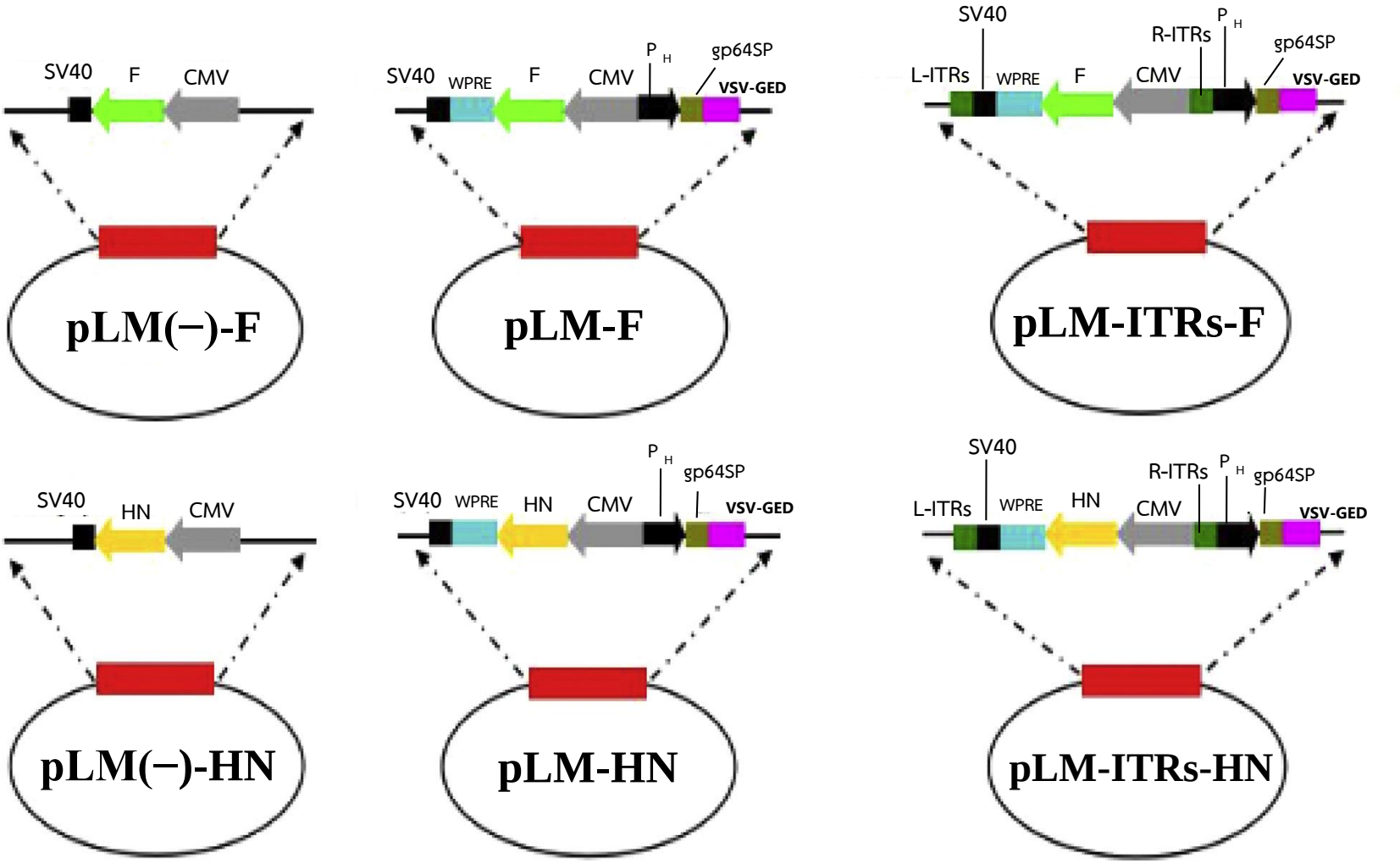
Plasmid controls :

pLM(–)

pLM

pLM-ITRs

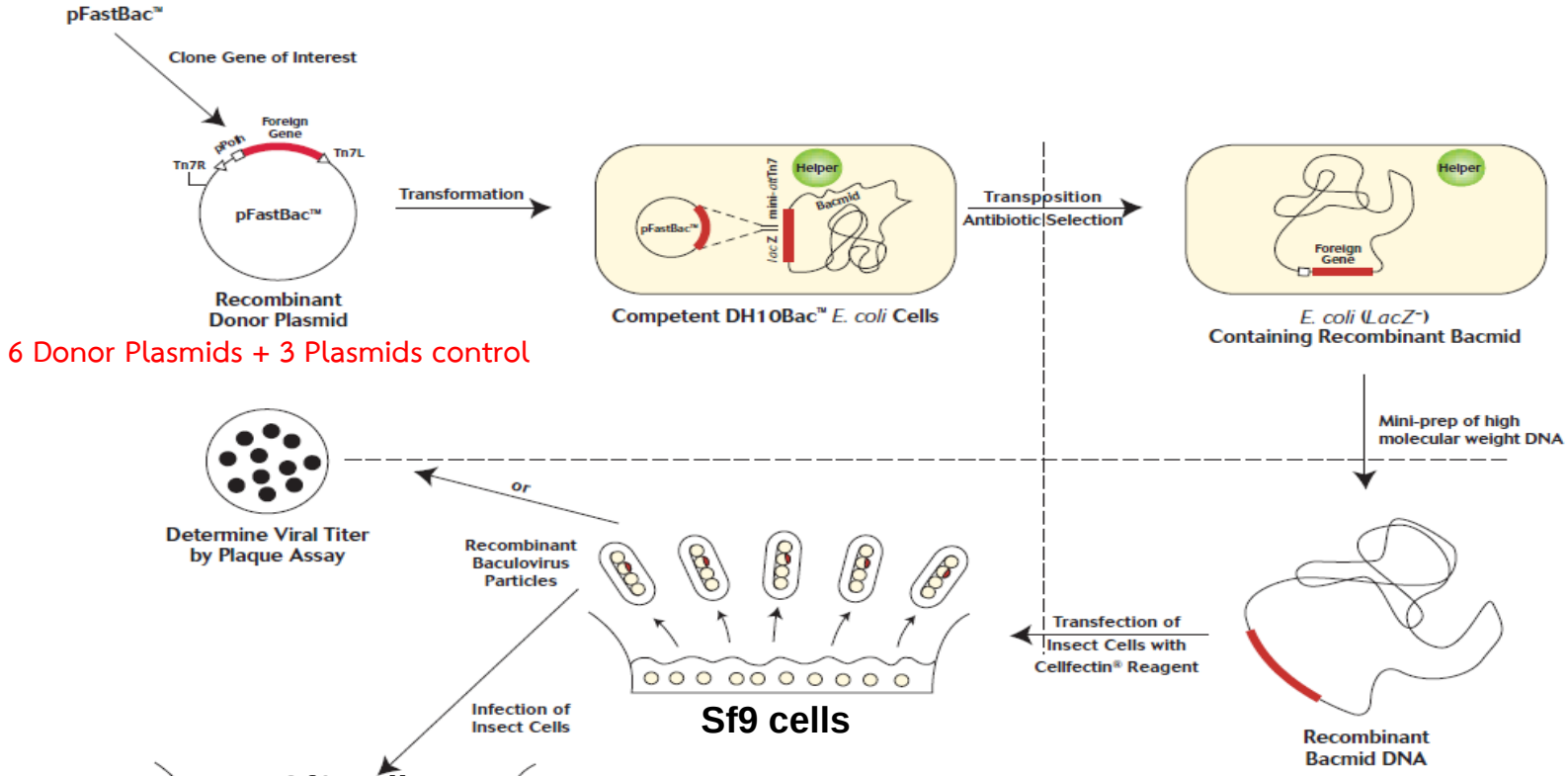
Plasmid constructions : Expression cassettes



- The gp64SP and VSV-GED expression cassettes were inserted under the polyhedron promoter
- WPRE expression cassettes including the F or HN genes were controlled by the CMV promoter

Generation of NDV recombinant baculovirus vaccines

Generation recombinant baculovirus vaccines



6 Donor Plasmids + 3 Plasmids control

To obtain high titer viral stocks

rBac-LM(−)-F | rBac-LM-F | rBac-LM-ITRs-F

rBac-LM(−)-HN | rBac-LM-HN | rBac-LM-ITRs-HN } Recombinants contain F or HN proteins

rBac-LM(−) | rBac-LM | rBac-LM-ITRs → Recombinants control group

Generation of NDV recombinant baculovirus vaccines

The titer of the baculovirus stocks were measured by plaque assay

Table 1
Titers of P_3 recombinant baculovirus vaccine strains in Sf9 cells.

Virus name-P_3	Virus titer (pfu/mL)	Dilution	Number of plaques
BV-LM(−)-F	$1.90 \pm 0.26 \times 10^8$	10^{-7}	19
BV-LM-F	$1.11 \pm 0.82 \times 10^9$	10^{-7}	111
BV-LM-ITRs-F	$4.90 \pm 0.41 \times 10^8$	10^{-7}	49
BV-LM(−)-HN	$8.60 \pm 0.97 \times 10^8$	10^{-7}	86
BV-LM-HN	$1.00 \pm 0.34 \times 10^9$	10^{-7}	100
BV-LM-ITRs-HN	$6.10 \pm 0.23 \times 10^8$	10^{-7}	61

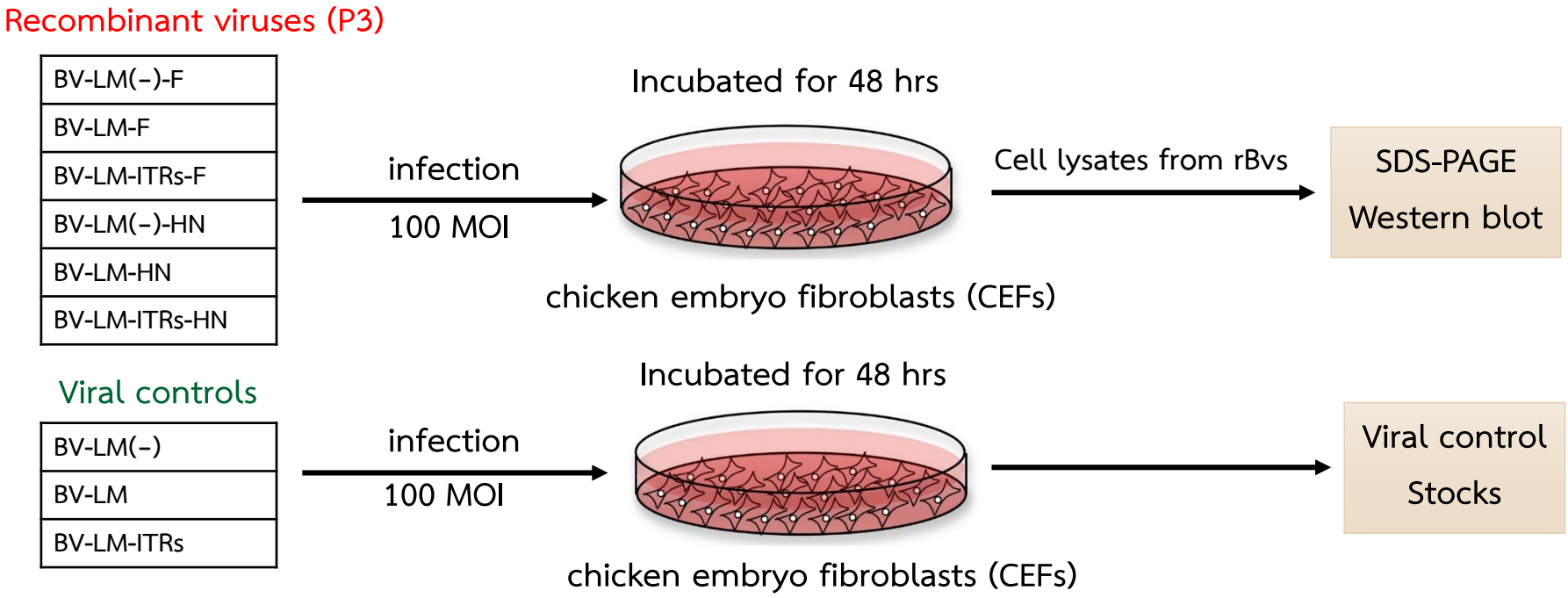
Baculovirus vectors	Gene cassette					
	CMV promoter	SV40 poly(A)	gp64SP	Regulatory elements		
				WPRE	VSV-GED	ITRs
BV-LM(−)-F	√	√				
BV-LM-F	√	√	√	√	√	
BV-LM-ITRs-F	√	√	√	√	√	√
BV-LM(−)-HN	√	√				
BV-LM-HN	√	√	√	√	√	
BV-LM-ITRs-HN	√	√	√	√	√	√

Methods and Results

Testing the *in vitro* efficacy of recombinant baculovirus vaccines

Expression of the F and HN proteins from chicken embryo fibroblasts

- To assessed whether the F and HN proteins were successfully expressed from the baculovirus expression systems
- To compared the effect of different regulatory elements (WBRE, ITRs, VSV-GED) on expression levels of the NDV proteins

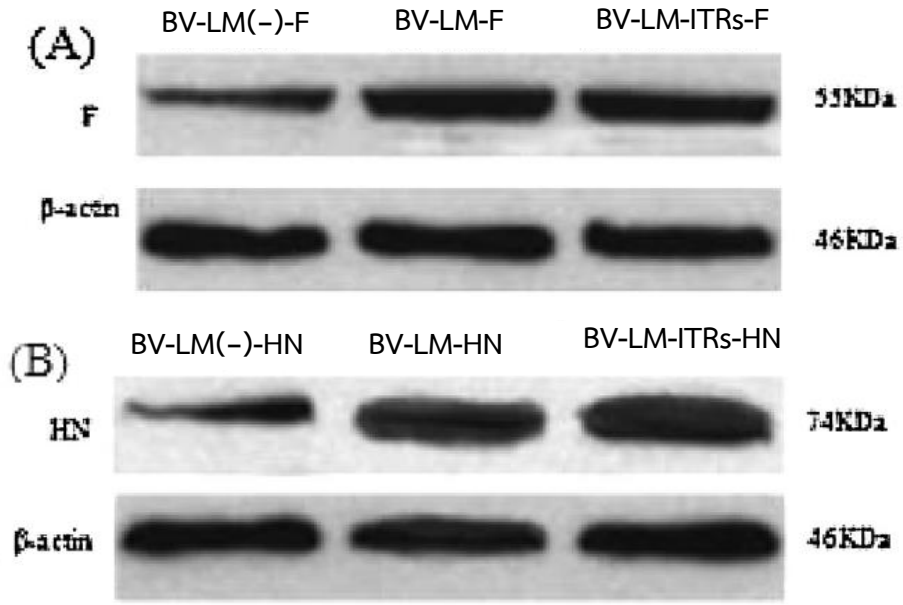


Results

Detection of F and HN proteins by Western blot from Cells Transduced with BV-F and BV-HN

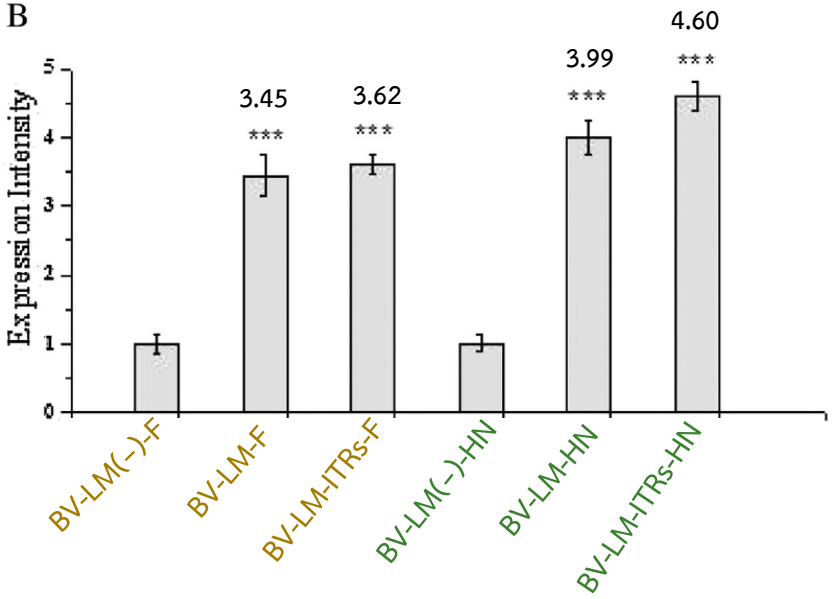
A

Western blot



Indicating successful expression from the baculovirus constructs

Level of protein expression



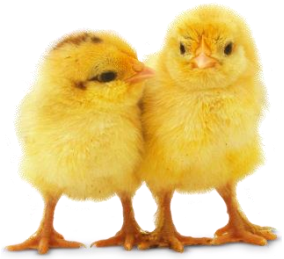
Indicated that the VSV-GED and WPRE regulatory elements markedly improved the expression of the target protein

The level of protein expression was quantified using the Biology Software Gel-Pro analyzer 4.5

Methods and Results

Testing the *in vivo* efficacy of recombinant baculovirus vaccines

Chicken immunization

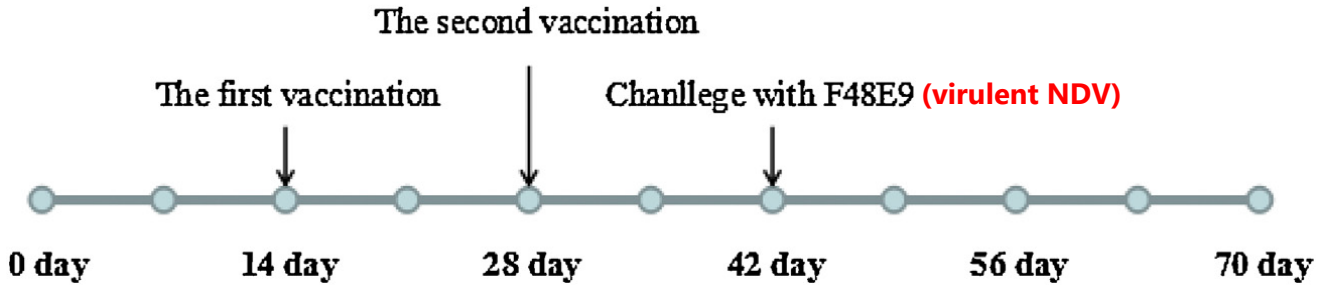


14-day old SPF chickens (80 total chickens)

randomly divided into ten groups

Chickens groups	Vaccines immunization 
A	BV-LM(–)-F
B	BV-LM-F
C	BV-LM-ITRs-F
D	BV-LM(–)-HN
E	BV-LM-HN
F	BV-LM-ITRs- HN
G	BV-LM-ITRs-F + BV-LM-ITRs- HN
H	BV-LM-ITRs (empty vector)
I	PBS (unvaccinated control)
J	Lasota commercial vaccine

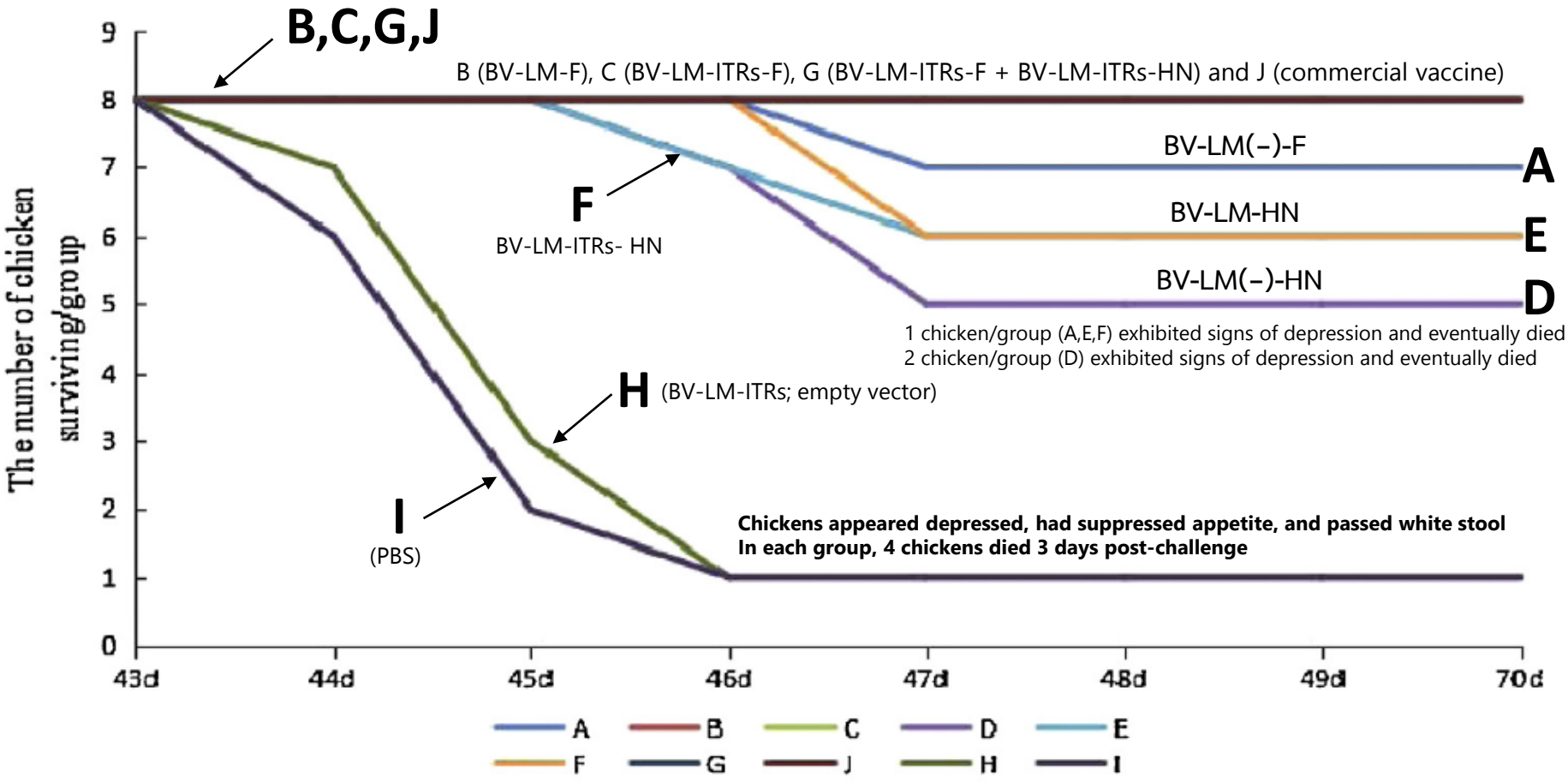
The Experimental Timeline (since the chicken birth)



- Blood was collected at days 14, 28, 42, 56 and 70 to test of serological assays (HI)
- PBMCs from vaccinated chickens were measured at 21, 36, 49 and 56 days (CMI)
- The chickens were monitored for clinical symptoms and the rate of protection was determined

Results

Survival plot of the immunized chickens post challenge with F48E9



There were no obvious clinical symptoms following challenge in groups; B,C,G and J

Results

Five (5) days after the challenge (47 days after chicken birth), the protection rate in each experimental group was calculated

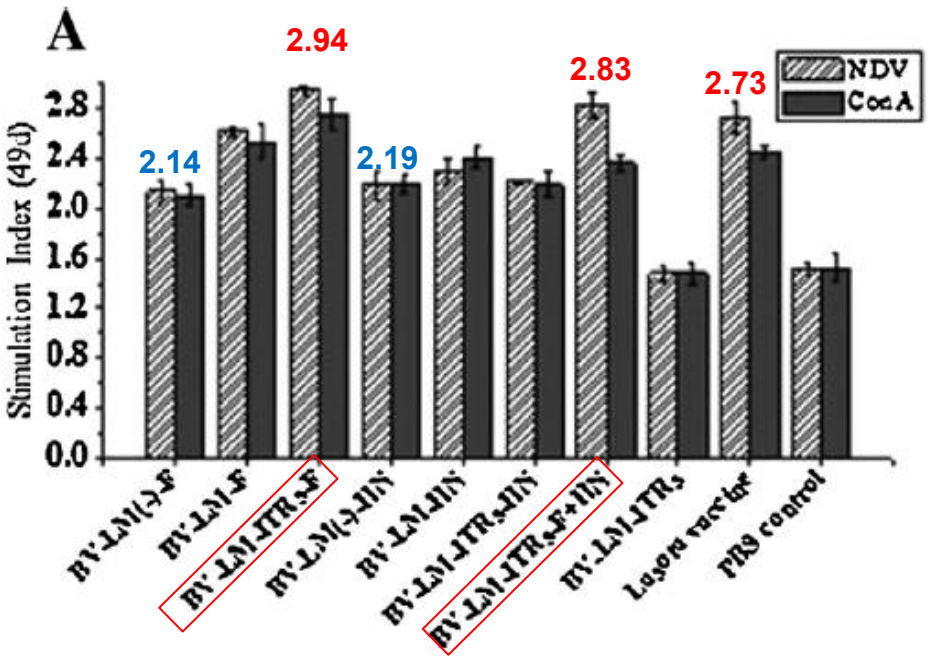
Table 2
Rate of protection following baculovirus immunization and challenge with F48E9.

Cohorts	Vaccines	No. deaths after challenge with F48E9					Protection rate
		2d	3d	4d	5d	No. deaths/total number	
A	BV-LM(−)-F	0	0	0	1	1/8	87.5%
B	BV-LM-F	0	0	0	0	0/8	100%
C	BV-LM-ITRs-F	0	0	0	0	0/8	100%
D	BV-LM(−)-HN	0	0	1	2	3/8	62.5%
E	BV-LM-HN	0	0	1	1	2/8	75.0%
F	BV-LM-ITRs-HN	0	0	0	1	1/8	87.5%
G	BV-LM-ITRs-F+BV-LM-ITRs-HN	0	0	0	0	0/8	100%
J	Lasota attenuated	0	0	0	0	0/8	100%
H	BV-LM-ITRs	1	4	2	0	7/8	12.5%
I	PBS	2	4	1	0	7/8	12.5%

- The results indicated that the recombinant baculovirus vaccines could protect against challenge with a virulent NDV strain
- The protection rate of the F-series immunized groups (95.83%) were higher than the HN-series (75%)
- Suggesting that the F protein elicited stronger protective immunity than the HN protein

Lymphoproliferation assay

Cellular immunity was measured by assessing the lymphoproliferative responses to F48E9 stimulation in PBMCs collected from vaccinated chickens at 21, 36, 49 and 56 days



After challenge with F48E9, the SI values were highest in

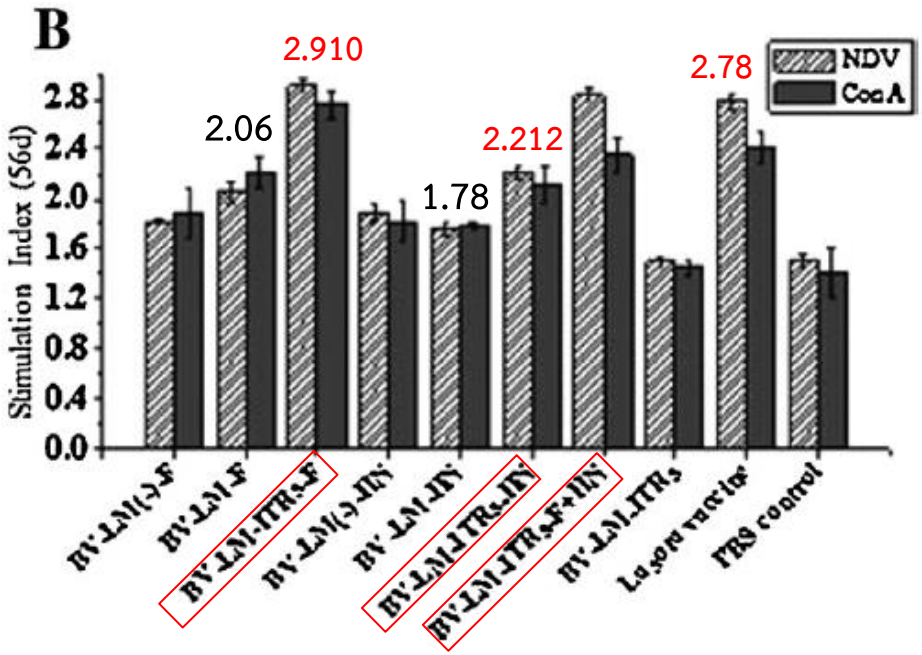
- BV-LM-ITRs-F (2.94)
- BV-LM-ITRs-F + BV-LM-ITRs-HN (2.830)
- commercial vaccine (2.73) groups

- The proliferative response to F48E9 and ConA was increased in all of the vaccinated chickens following challenge indicating that the recombinant baculovirus vaccine can elicit a cellular immune response
- The SI index in these groups was significantly higher than the BV-LM(–)-F (2.14) and BV-LM(–)-HN (2.19) groups ($P < 0.05$)
- **Thus, recombinant baculovirus vaccines containing the WPRE and VSV-GED regulatory elements significantly improved the level of cellular immunity**

Concanavalin A (ConA) was used as the positive control

Lymphoproliferation assay

To determine whether the increased cellular immunity was persistent, we repeated the lymphoproliferative response assay 56 days after the chicken birth



Baculovirus constructs containing ITRs

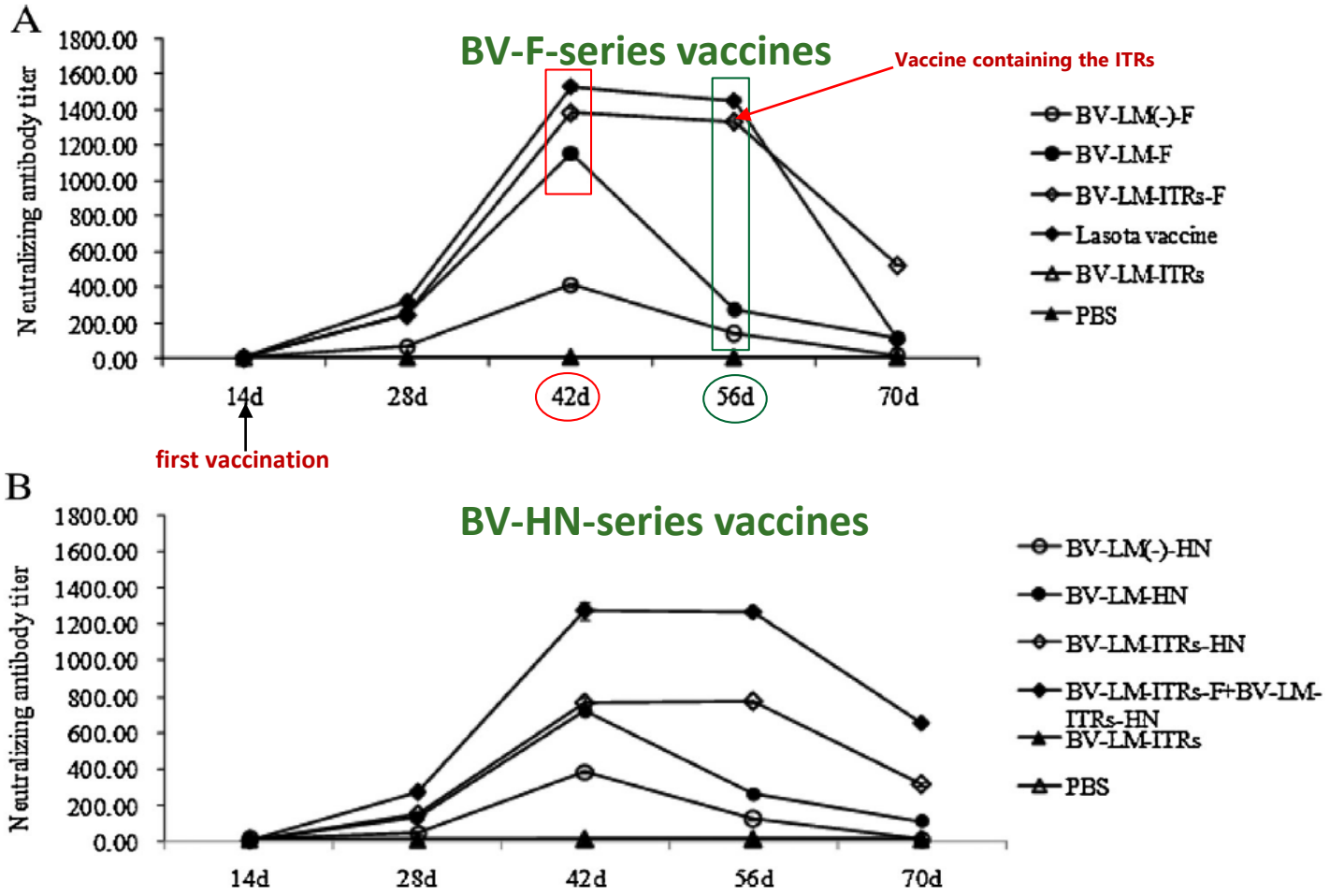
- BV-LM-ITRs-F = 2.910
- BV-LM-ITRs-HN = 2.212
- commercial vaccine = 2.78

- The SI values for groups vaccinated with baculovirus constructs containing ITRs and commercial vaccine remained significantly higher than SI values without ITRs (BV-LM-F = 2.06; BV-LM-HN = 1.78;$P < 0.05$)
- The SI values in the BV-LM-ITRs-F + BV-LM-ITRs-HN group, was slightly lower than the BV-LM-ITRs-F group, but significantly higher than the BV-LM-ITRs-HN group
- **These results indicated that ITRs effectively extended the duration of the cellular immune response to the vaccine**

Concanavalin A (ConA) was used as the positive control

Serological assays: Serum neutralization test

A serum neutralization test was conducted to determine whether the serum was able to neutralize NDV infection

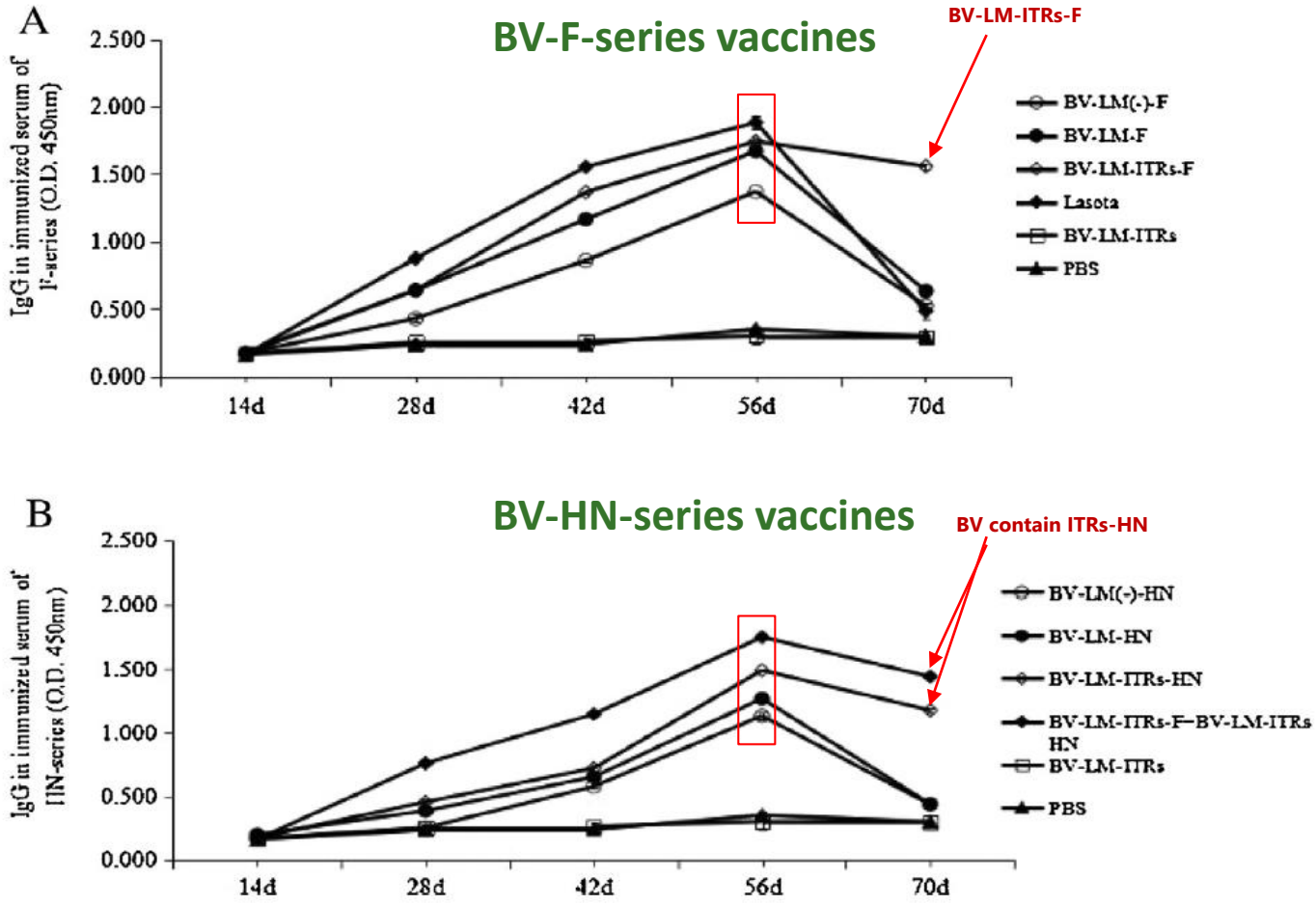


F antigen vaccine series tended to induce a greater humoral immune response in immunized chickens than the HN antigen series

Serological assays: Detection of NDV-specific IgG

The IgG titer is as an important index of humoral immunity in immunized chickens

for a response specific to NDV



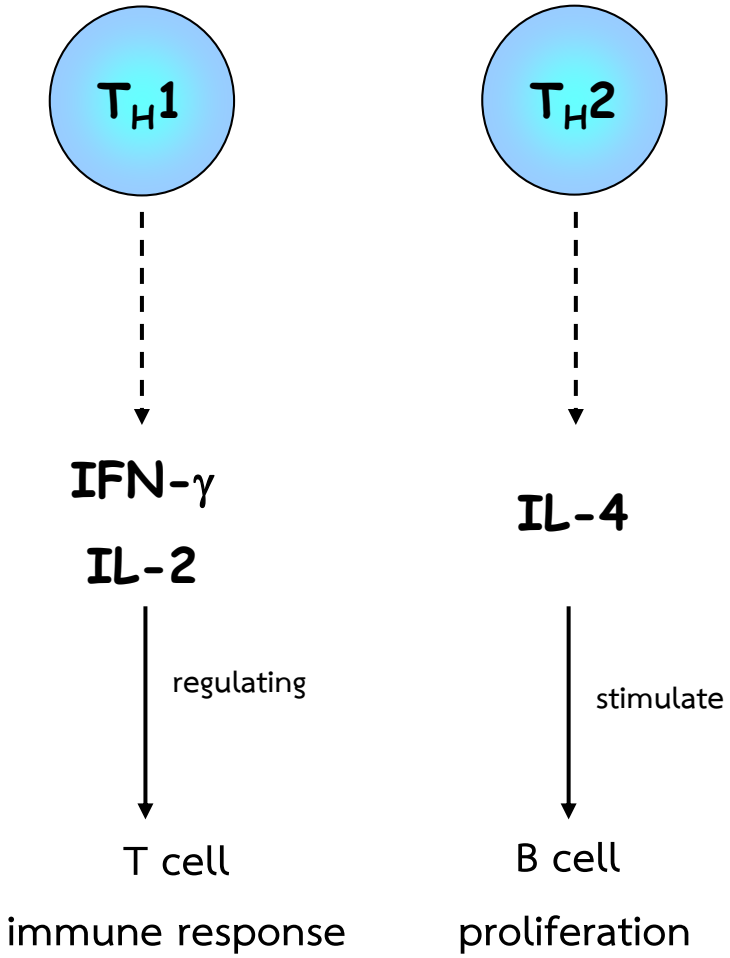
- **The BV-LM-F constructs containing the WPRE and VSV-GED regulatory elements had significantly higher NDV-specific IgG titers (1.673) than constructs without the regulatory elements (1.372; $P < 0.05$) and similarly in BV-HN-series vaccines**
- **Vaccines containing the F antigen elicited more NDV-specific IgG antibodies than the HN gene**

Serological assays: Cytokine levels in serum

The cytokine levels in immune sera can be used to measure the immune state of the host

- The levels of IL-2, IL-4, and IFN- γ were determined by ELISA (Abcam, UK)
- IFN- γ and IL-2 are secreted by Th1 cells and play important roles in regulating the cellular (T cell) immune response
- IL-4 is secreted by Th2 cells and can stimulate B cell proliferation, and antibody production involved in the humoral immune response

To determine whether the baculovirus constructs are eliciting a predominately Th1 or Th2 response

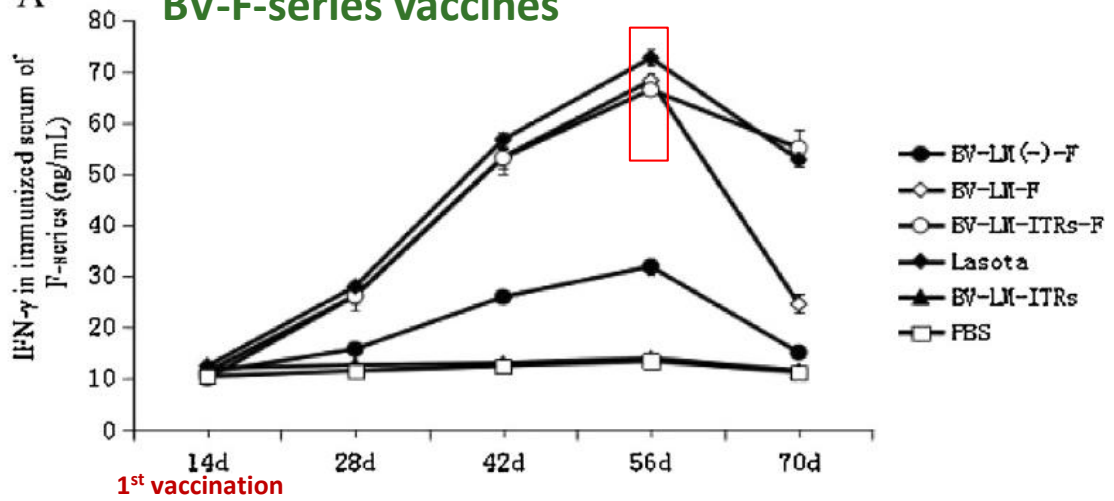


Result

Serum IFN- γ concentration following vaccination with baculovirus vaccines

A

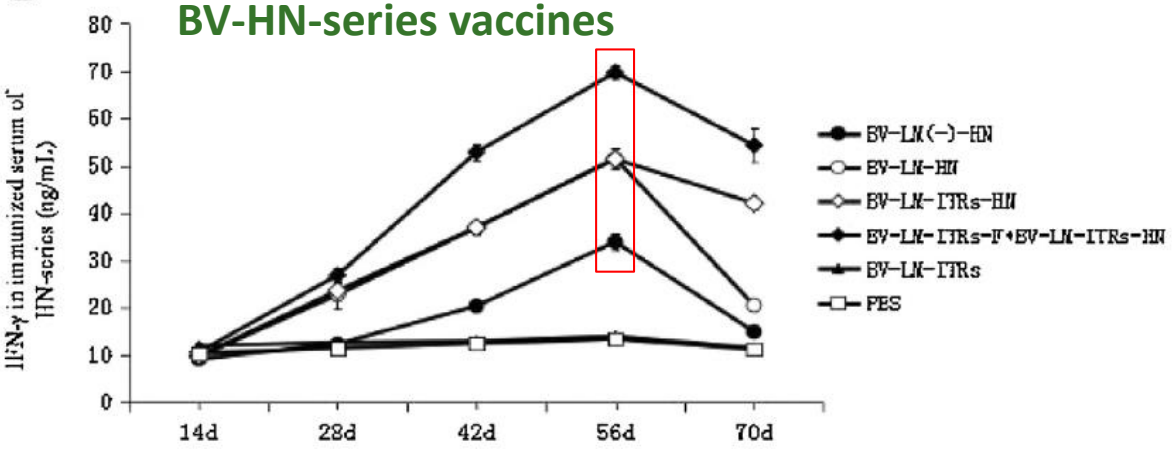
BV-F-series vaccines



- ✓ The concentration of IFN-γ peaked at 56 days in all of the immunized groups
- ✓ The highest concentrations in commercial vaccine

B

BV-HN-series vaccines

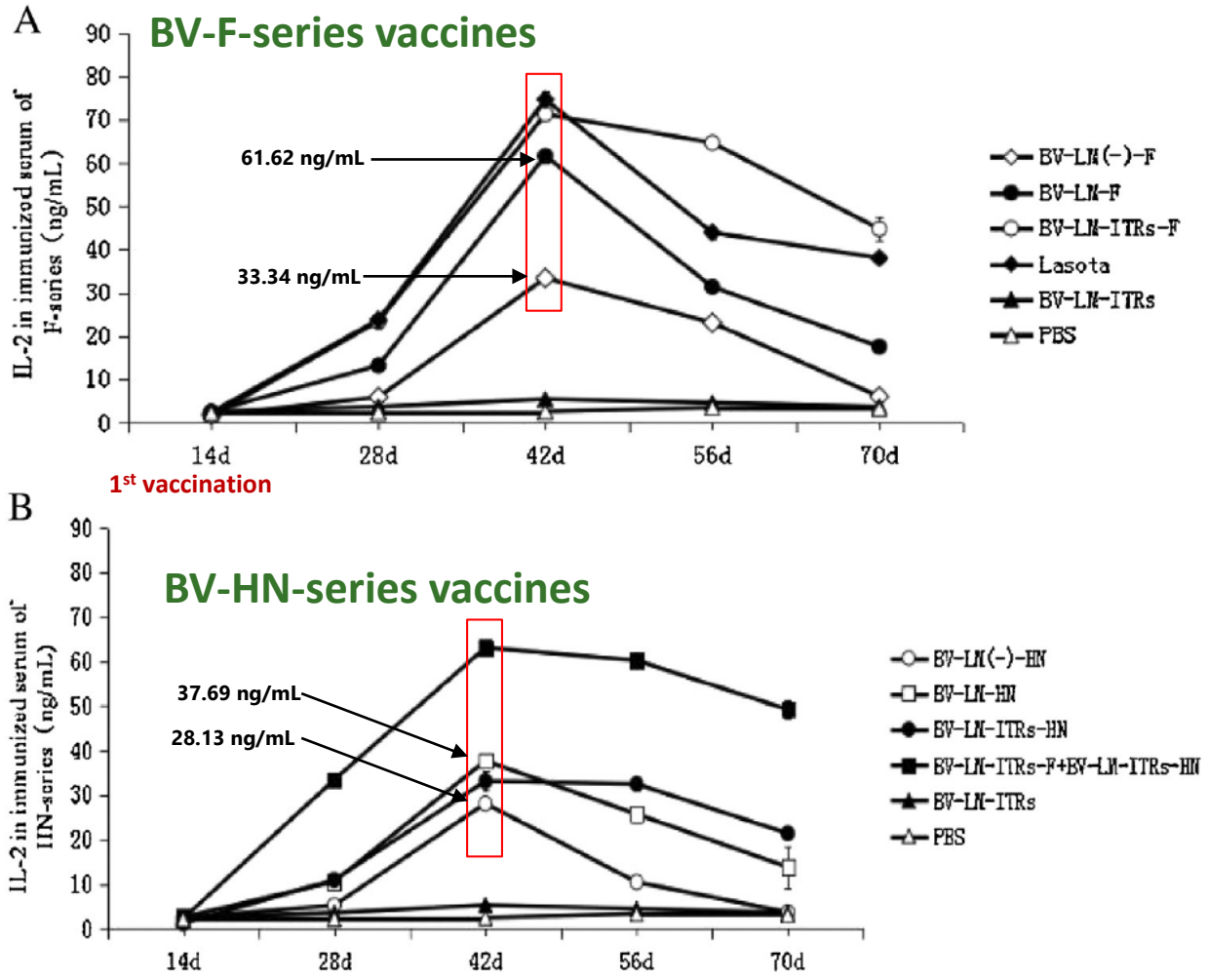


- ✓ The highest concentrations in the BV-LM-ITRs-F + BV-LM-ITRs-HN (69.65 ng/mL) groups

The IFN-γ concentration present in the blood of chickens was measured by IFN-γ ELISA assay at each time point

Result

Serum IL-2 concentration following vaccination with baculovirus vaccines

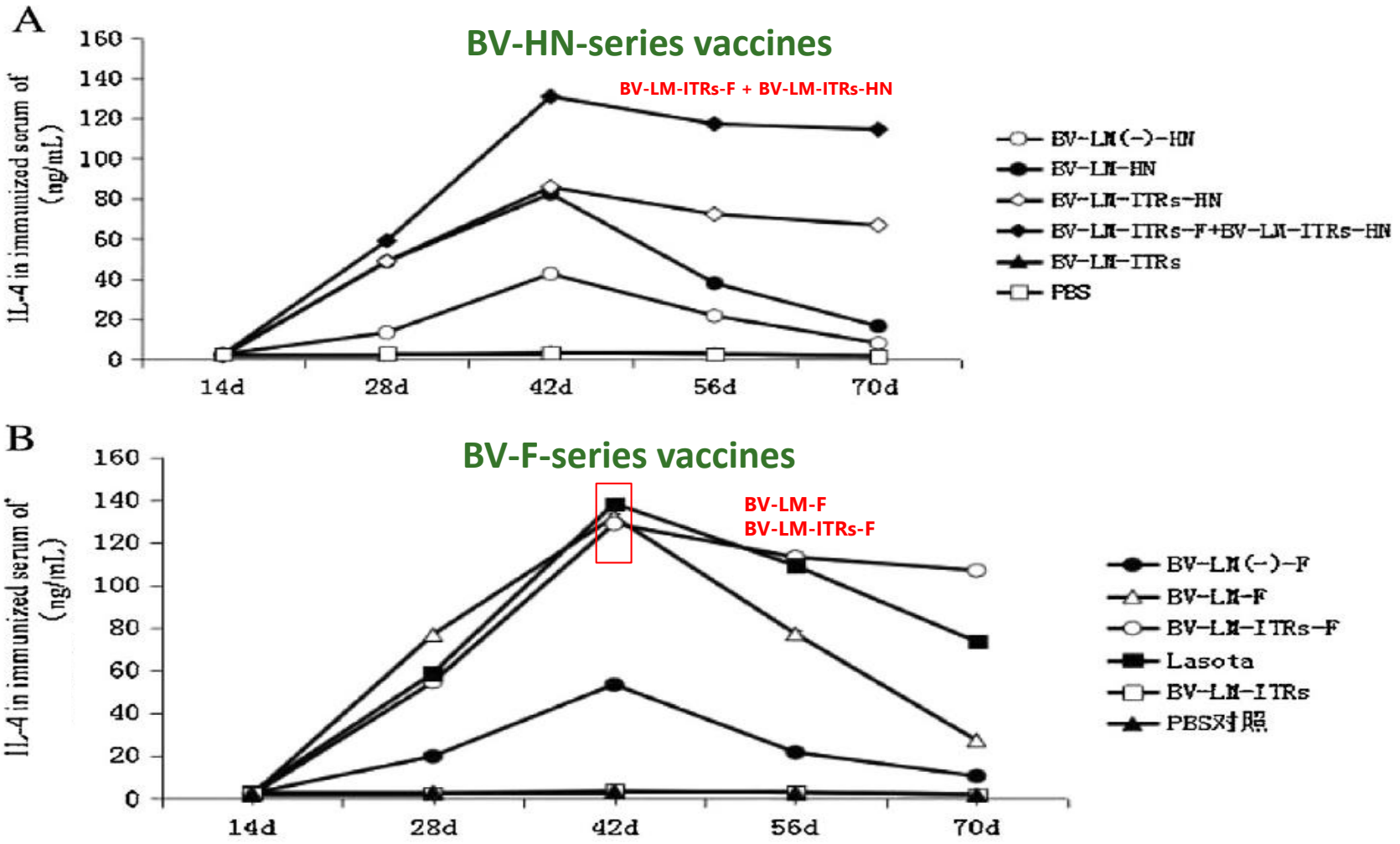


- ✓ Levels of IL-2 were significantly higher than the control groups
- ✓ Different combinations of antigen genes and regulatory elements elicited different levels of IL-2 production
- ✓ Levels of IL-2 production were greater when constructs contained the WPRE and VSV-GED regulatory elements than without

The IL-2 concentration present in the blood of chickens was measured by IFN-γ ELISA assay at each time point

Result

Serum IL-4 concentration following vaccination with baculovirus vaccines



The levels of IL-4 were highest in the groups vaccinated with baculovirus constructs containing the WPRE and VSV-GED elements

Result : Cytokine levels in serum

- The cytokine levels at 70 days to determine whether the vaccines had any lasting effects on cytokine production
- The IFN-γ, IL-2, and IL-4 levels in the BV-LM-ITRs-F and BV-LM-ITRs-HN were significantly ($P < 0.05$) elevated compared to the groups without ITRs (BV-LM-F and BV-LM-HN)
- The ITRs consistently improved the magnitude and duration of the cellular and humoral immune response

Taken together, the cytokine results provided further evidence that the baculovirus constructs elicited a robust cellular and humoral response

inverted terminal repeats (ITRs)

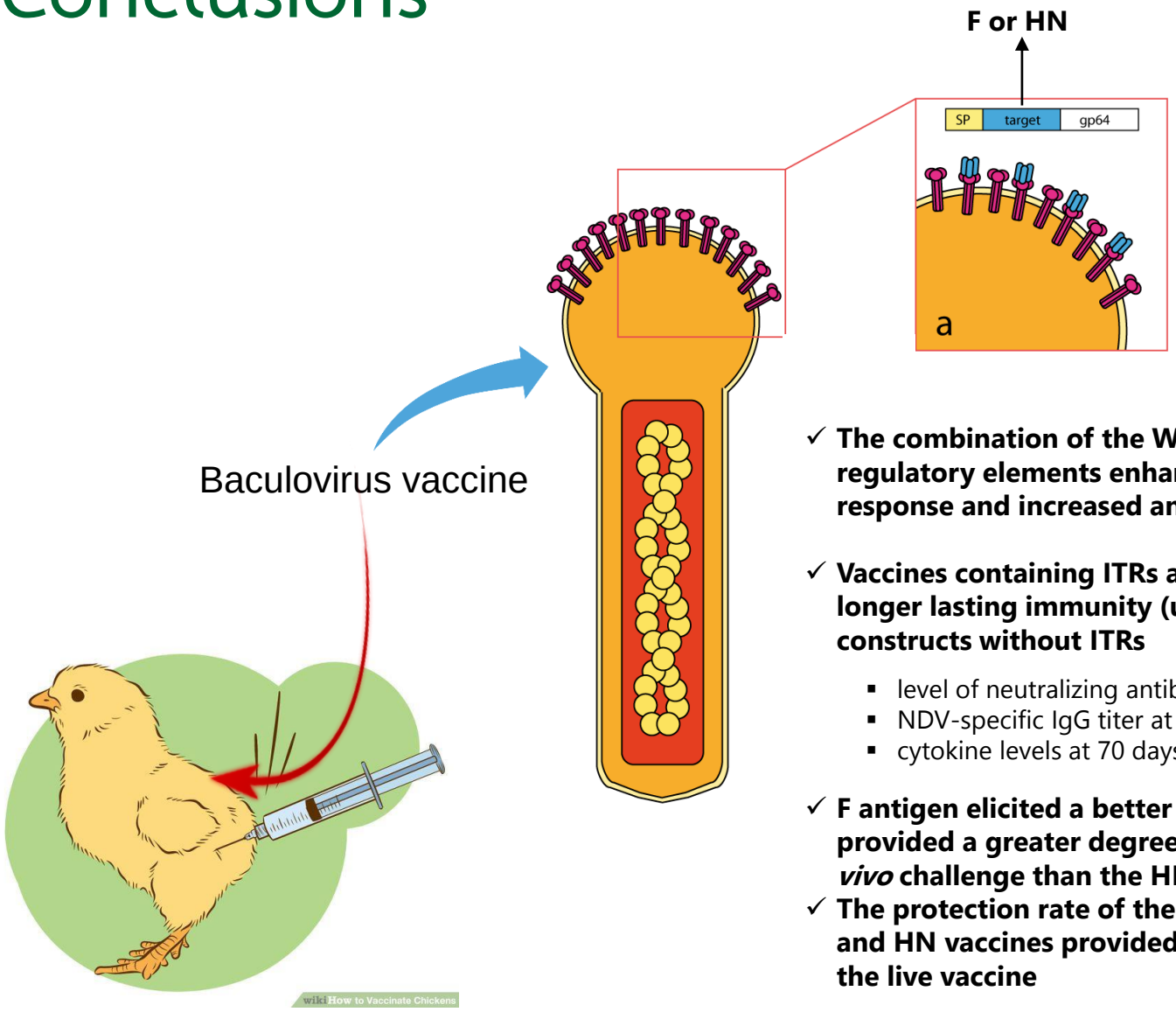
Discussion

Discussion

- The novel Baculovirus vaccine series using the F and HN proteins of NDV that efficiently express the antigen target and elicit a robust immune respons
- Baculovirus vaccines containing the WPRE, VSV-GED, and ITR elements can be directly injected into chickens
- The high neutralizing antibody titer, increased IL-4 levels, and increased IFN-γ, and IL-2 levels
- Indicated that the baculovirus vaccine had the dual advantages of **recombinant viral vector vaccines** and **DNA vaccines**
- The baculovirus vectors have the dual advantages of mimicking natural virus infection by a recombinant viral vector vaccine and the non-replicating characteristic of a DNA

Truncated vesicular stomatitis virus G protein (VSV-GED)
woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)
inverted terminal repeats (ITRs) of adeno-associated virus (AAV Serotype II)

Conclusions



- ✓ **The combination of the WPRE and VSV-GED regulatory elements enhanced the immune response and increased antigen expression**
- ✓ **Vaccines containing ITRs also appeared to elicit a longer lasting immunity (up to 70 days) than constructs without ITRs**
  - level of neutralizing antibodies at 56 days
  - NDV-specific IgG titer at 70 days
  - cytokine levels at 70 days
- ✓ **F antigen elicited a better immune response and provided a greater degree of protection in the *in vivo* challenge than the HN antigen**
- ✓ **The protection rate of the combination of the F and HN vaccines provided 100% which similar to the live vaccine**

The baculovirus system is a promising platform for NDV vaccine development that combines the immunostimulatory benefits of a recombinant virus vector with the non-replicating benefits of a DNA vaccine

Thank you

For paying your attention