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<Original Article>

Development of inactivated Akabane and bovine ephemeral fever vaccine for cattle

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Abstract : Akabane and bovine ephemeral fever (BEF) viruses cause vector-borne diseases. In this study, inactivated *Akabane virus* (AKAV)+*Bovine ephemeral fever virus* (BEFV) vaccines with or without recombinant vibrio flagellin (revibFlaB) protein were expressed in a baculovirus expression system to measure their safety and immunogenicity. Blood was collected from mice, guinea pigs, sows, and cattle that had been inoculated with the vaccine twice. Inactivated AKAV+BEFV vaccine induced high virus neutralizing antibody (VNA) titer against AKAV and BEFV in mice and guinea pigs. VNA titers against AKAV were higher in mice and guinea pigs immunized with the inactivated AKAV+BEFV vaccine than in animals inoculated with vaccine containing revibFlaB protein. Inactivated AKAV+BEFV vaccine elicited slightly higher VNA titers against AKAV and BEFV than the live AKAV and live BEFV vaccines in mice and guinea pigs. In addition, the inactivated AKAV+BEFV vaccine was safe, and induced high VNA titers, ranging from 1 : 64 to 1 : 512, against both AKAV and BEFV in sows and cattle. Moreover, there were no side effects observed in any treated animals. These results indicate that the inactivated AKAV+BEFV vaccine could be used in cattle with high immunogenicity and good safety.

Keywords : *Akabane virus*, bovine ephemeral fever, cattle, vaccine

Introduction

Akabane and bovine ephemeral fever (BEF) diseases are designated as second-class infectious diseases in the Infectious Disease Control and Prevention Act in Korea; both are important arthropod-borne viral diseases in ruminants. Arthropod-borne viruses (arboviruses) are widely distributed, from the tropical to temperate zones of the world, and are associated with hematophagous arthropod vectors such as *Culicoides* (*C.*) biting midges and mosquitoes. Particular vector species linked to *Akabane virus* (AKAV) and *Bovine ephemeral fever virus* (BEFV) include *C. brebitarsis*, *C. oxystoma*, *C. nebuchlsus*, and *C. nipponensis* [5, 8, 17].

AKAV is a member of the genus *Orthobunyavirus* in the family *Bunyaviridae,* and contains three segments of single-stranded negative RNA, designated large, medium, and small according to their size [11]. AKAV causes congenital abnormalities of the central nerve system in ruminants, such as cattle, sheep, and goats. AKAV infection has been reported in Australia, Israel, Japan, and Korea. Asymptomatic AKAV infection has been demonstrated serologically in horses and swine in endemic areas [10, 20].

BEFV, an arthropod-borne rhabdovirus, belongs to the genus *Ephemerovirus* in the family *Rhabdoviridae*, and consists of a minus-sense single-stranded RNA genome [18]. BEF disease, also known as 3-day sickness, is outbreak in cattle. BEFV causes serious economic losses in a short period, as well as decreased body condition and decreased weight gain in cattle because of high fever. BEF infection has been reported in Japan, Taiwan, and Australia, and a large-scale outbreak of BEF was reported in Turkey in 2010 [16]. The symptoms in infected cattle include a short fever, shivering, lameness, and muscular stiffness [4].

Flagellin causes signal transduction in mammalian cells via Toll-like receptor 5 (TLR5), and has been considered a potent adjuvant for vaccines [2, 9]. A bacterial flagellin, *Vibrio vulnificus* FalB, has strong mucosal adjuvant activity, inducing protective immunity [9]. Many outbreaks of AKAV and BEFV occurred in the southern regions of Korea in 2010 (Korea Animal Health Integrated System, Animal and Plant Quarantine Agency [QIA], Korea). The isolation of various AKAVs has been reported, and the genetic and pathogenic characteristics of AKAV isolates have been described [13, 14, 19]. Live attenuated vaccines for AKAV and BEFV have been used for the prevention of the disease since the 1980s. In addition, an inactivated vaccine for AKAV, Aino virus

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(AINV), and Chuzan virus (CHUV) was commercialized in 2011 [5, 6]. However, the inoculation rates of live AKAV or BEFV vaccine and inactivated AKAV, AINV, and CHUV vaccines seem to be low based on a serological survey [7, 12]. Among the arboviruses, AKAV and BEFV are the most significant vector-borne viral agents in Korea.

A follow-up study based on a serological survey after the mass outbreaks of AKAV and BEFV in Korea indicated that mass vaccination would be required to prevent disease caused by AKAV and BEFV. Thus, we prepared an inactivated AKAV and BEFV vaccine for cattle, and evaluated its safety and immunogenicity in several animal species.

Materials and Methods

Viruses and cells

Outbreak of Akabane viral infection in a Korean native cattle farm located on Jeju-do was reported to QIA in 2005. Twelve cattle bloods of the farm were collected and one virus, KV0505 strain, was isolated, was isolated from the blood samples [20]. The AKAV (KV0505 strain) was propagated in baby hamster kidney cells (BHK-21), and BEFV (DS11 strain) was also grown in BHK-21 cells. The BHK-21 cells were regularly maintained in α-minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (10 μg/mL), and amphotericin B (0.25 μg/mL). To propagate the AKAV and BEFV, the BHK-21 cells grown in α-MEM were washed three times with PBS, and the two viruses were inoculated. After adsorption, α-MEM was added, and the cells were incubated until a 90% cytopathic effect (CPE) was observed in the infected BHK-21 cells. After harvesting, the antigens were frozen and thawed three times, and centrifuged (3,000 × g, 30 min) to remove the cellular debris.

Construction and expression of recombinant vibrio flagellin (revibFlaB) baculovirus

A flagellin (FlaB) protein of *Vibrio alginolyticus* was synthesized based on GenBank accession no. FJ617267, and cloned into the pGEM-T easy vector. For the construction of a revibFlaB baculovirus, the Bac-N-Blue DNA (Invitrogen, USA) and 10 μg/μL of purified pFastBacFlaB plasmid DNA was mixed with Cellfectin, a commercial lipid-based transfection reagent (Invitrogen), in Grace's insect medium without supplements. After incubation for 15 min at room temperature, the transfection mixture was added to the 60 mm dish, in which *Spodoptera frugiperda* (Sf9) cells had been cultivated at 27°C. After 3 days, the supernatant was harvested and the cells were incubated continuously in fresh medium containing FBS.

A plaque assay to purify recombinant baculovirus was performed in 1% agarose medium containing 150 μg/mL X-gal. A PCR assay against revibFlaB baculovirus was performed to confirm the isolation of a pure plaque using specific baculovirus primers (Table 1). Passage of revibFlaB baculovirus was conducted three times using Sf9 cells infected with 0.1 multiplicity of infection. The third passage was used as a viral stock for expression.

For the vaccine adjuvant, the Sf9 cells infected with the revibFlaB baculovirus were frozen and thawed three times, and centrifuged (5,000 × g, 30 min) to remove the cellular debris. The revibFlaB baculovirus was titrated in 96-well microplates using 10-fold dilutions. The viral titer determined by the CPE was calculated according to the Reed and Muench method. The titer of the revibFlaB baculovirus was $10^{7.0}$ $TCID_{50}$/mL, and the antigen was added to the test vaccine formula at 10% volume (v/v).

Western blotting

For identification of the expressed revibFlaB protein, the cell lysate, cell culture supernatant, and normal cell lysate were dissolved in SDS-PAGE sample buffer with β-mercaptoethanol, and boiled for 5 min. The three protein samples were separated on a 12.5% Tris-glycine gel, and electrophoretically transferred onto a nitrocellulose (NC) membrane. The NC membrane was blocked in 5% skim milk solution for 2 h. After washing three times with Tris-buffered saline Tween-20 (TBST), the NC membrane was incubated with a 1/1,000 dilution of an anti-His_6 monoclonal antibody (Sigma, USA) for 1 h. The NC membrane was washed three times, and incubated for 1 h with anti-mouse IgG horseradish peroxidase conjugate, diluted 1 : 1,000, at room temperature. After thorough washing with TBST, the membrane was developed in TMB solution.

Virus inactivation

AKAV and BEFV were inactivated with binary ethylene imine (BEI) using the method by Bahnemann [1]. Briefly, 0.1 M BEI was prepared from 2% 2-bromo-ethylamine hydrobromide (2-BEA) in a solution of 0.2 N NaOH that was incu-

Table 1. Oligonucleotide primers to clone and identify flagellin gene of *Vibrio alginolyticus*

Primer	Oligo nucleotide sequences (5'- 3')	Genomic region
FlaBF	**CC<u>GGATCC</u> <u>ATG</u>** GCA GTG AAT GTA AAC A	flagellin gene of *Vibrio alginolyticus*
FlaBR	CC **<u>CTCGAG</u>** ACC TAG AAG ACT TAG CGC T	
Bac F	TTT ACT GTT TTC GTA ACA ACA GTT TTG	Baculovirus multi cloning site
Bac R	CAA CAA CGC ACA GAA TCT AGC	

Underlined sequences show restriction enzyme sites (*Bam* H1 and *Xho* 1) and start codon.

bated in a 37°C water bath for 1 h. The final concentration of BEI was adjusted to 0.001 M of antigen, and the pH of the antigen was also adjusted to 8.0 with 1 M NaOH. Inactivation was done at 37°C for 10 h. A recombinant FlaB baculovirus was inactivated with 0.2% formaldehyde at 37°C for 12 h. BHK-21 and Sf9 cells were used to check whether the viruses had been inactivated. After confirming inactivation of the viruses, the antigens were used for preparation of the vaccine.

Preparation of vaccine

The KV0505 strain of AKAV and the DS11 strain of BEFV, which had undergone five serial passages in BHK-21 cell culture, were used for preparation of the vaccine. Montanide IMS1313VG (Seppic, France) and the revibFlaB baculovirus protein were used for the adjuvant for the inactivated AKAV and BEFV vaccine. For the inactivated AKAV+BEFV vaccine, the AKAV, BEFV, and IMS1313VG adjuvant were blended at 35% : 35% : 30% under agitation. For the inactivated AKAV+BEFV vaccine containing revibFlaB baculovirus protein, the AKAV, BEFV, revibFlaB baculovirus protein, and IMS1313VG were blended at 40% : 40% : 10% : 10% under agitation. Commercial live attenuated AKAV and BEFV vaccines produced in Korea were used to compare immunogenicity in experimental animals. They were all licensed for use in cattle in Korea.

Safety test

Four vaccines (live AKAV vaccine, live BEFV vaccine, inactivated AKAV+BEFV vaccine, and inactivated AKAV+BEFV vaccine with revibFlaB protein) were inoculated into mice, guinea pigs, sows, and cattle to check the safety of the vaccines. Ten mice were inoculated with 0.5 mL vaccines intraperitoneally, four guinea pigs with 1.0 mL, and three sows and two cattle with 3 mL vaccines intramuscularly (IM). Mice, guinea pigs, sows, and cattle inoculated with the vaccines were observed for 14 days post-inoculation.

Immunogenicity of vaccines

Four groups of six mice were inoculated with 0.2 mL of the four vaccines IM to measure immunogenicity, and four guinea pigs were inoculated twice with 0.5 mL of the vaccines IM, with a 2-week interval. Blood samples were collected 2 weeks after the second immunization. Control mice and guinea pigs received no treatment, except that blood samples were taken. One dose (3 mL) of the inactivated AKAV+BEFV vaccine was inoculated into three sows and two cattle twice with a 2-week interval, and blood was collected 2 weeks after the second immunization.

Virus neutralization test

The virus neutralization (VN) test was performed in 96-well plates in duplicate using sera inactivated at 56°C for 30 min. Then, 50 µL aliquots of two-fold serially diluted serum were mixed with equal volumes of AKAV and BEFV containing 200 $TCID_{50}$/0.1 mL. After incubation of the mixtures at 37°C for 1 h, 100 µL of Vero cell suspension containing 20,000 cells was added to each well. The plates were incubated for 3 days in a humidified incubator with 5% CO_2. Each well was examined under a microscope to detect viral-specific CPE. The virus neutralizing antibody (VNA) titers were expressed as the reciprocal of the highest serum dilution that inhibited CPE completely.

Statistical analysis

All data are expressed as means ± SD. A one-way ANOVA test was performed by GraphPad Prism (ver. 6.05; GraphPad Software, USA) for statistical analysis. Statistical significance was set at $p < 0.05$.

Results

Expression of revibFlaB protein in insect Sf9 cells

The FlaB gene (1134 bp) of *Vibrio alginolyticus* was cloned into the pGEM-T and pFastBac vector, which contains a six-histidine tag in the C-terminal region. After transfection into insect cells, plaque-purified revibFlaB baculovirus was identified by CPE and RT-PCR (Figs. 1 and 2). The revibFlaB protein was identified with Western blotting using specific monoclonal antibodies against His_6, and the molecular weight was ~43 kDa (Fig. 3). The revibFlaB baculovirus revealing titer of $10^{7.0}$ $TCID_{50}$/mL in Sf9 cells was inactivated with 0.2% formaldehyde, and was added to the inactivated AKAV +BEFV vaccine.

Safety and immune response in animals

Mice and guinea pigs inoculated with the vaccines showed no clinical symptoms 14 days after inoculation. The sows and cattle immunized with one dose of the inactivated AKAV+BEFV vaccine IM showed no symptoms related to non-suppurative encephalitis of a viral infection in the period of observation. Figure 4 shows that the geometric mean VNA titers against AKAV were higher in mice and guinea pigs immunized with the inactivated AKAV+BEFV vaccine

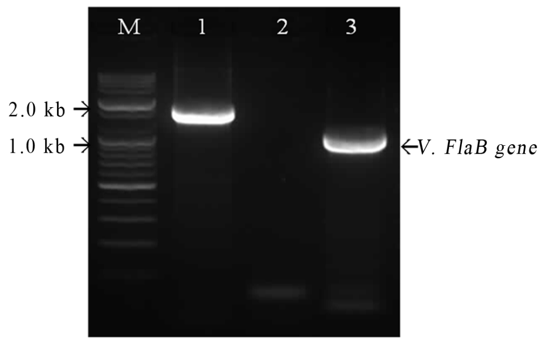

Fig. 1. Identification of flagellin (*FlaB*) gene in recombinant vibrio flagellin (revibFlaB) baculocvirus. M, 1 kb DNA ladder; lane 1, *revibFlaB* gene detected with baculovirus primer sets; lane 2, normal Sf9 cells; lane 3, *revibFlaB* gene detected with primer sets for *FlaB* gene of *Virio* (*V.*) *alginolyticus*.

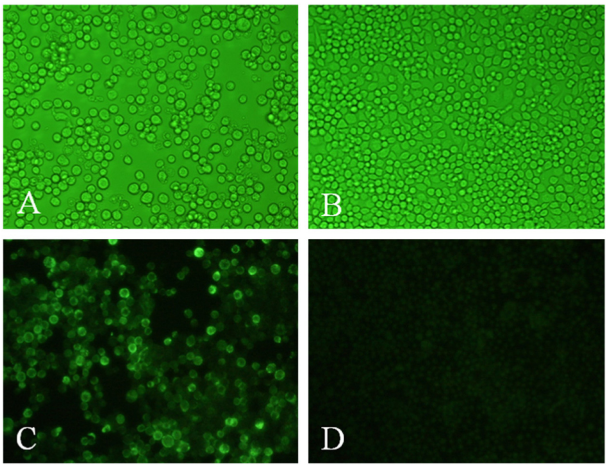

Fig. 2. Cytopathic effect in insect cells inoculated with revibFlaB baculovirus (A) and normal Sf-9 cells (B). Identification of revibFlaB baculocvirus using specific monoclonal antibodies against six-his_6 (C) and normal insect cells as a control (D). Magnification: 200× (A-D).

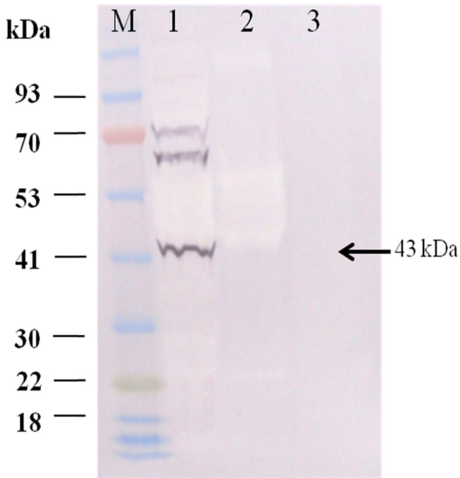

Fig. 3. Identification of the revibFlaB protein with Western blotting using specific monoclonal antibodies against six-his_6 and the molecular weight was found to be approximately 43 kDa. M, protein ladder; lane1, revibFlaB-infected cell lysate; lane 2, revibFlaB-infected cell culture supernatant; lane 3, mock-infected insect cell culture supernatant.

than in those animals inoculated with the vaccine containing revibFlaB protein. Based on the results of immunogenicity tests in mice and guinea pigs, the inactivated AKAV and BEFV vaccine without revibFlaB protein was selected for inoculation in sows and cattle. As shown in Figure 5, the sows and cattle inoculated with the inactivated AKAV+BEFV vaccine induced high VNA titers against AKAV and BEFV, ranging from 1 : 64 and 1 : 512, and the geometric mean VNA titer was higher in cattle inoculated with the vaccine than in sows. Moreover, after the second vaccination at day 14, the VNA titers of AKAV+BEFV were significantly increased in the vaccinated animals. These findings indicated that immunization with inactivated AKAV and BEFV vaccine could induce high level of VNA titers in cattle.

Discussion

When vector-borne viral disease in Korean ruminants occurred, local veterinary authorities have reported the outbreak to QIA in accordance with the regulations. Among the arboviral diseases, AKAV and BEFV infections have been reported in Korean cattle continuously (Table 2) and in many other countries, including Japan, China, and Taiwan [5, 21]. However, there has been no outbreak of AINV, CHUV, or IBAV infection in Korea since 2007.

In the present study, an inactivated AKAV and BEFV vaccine was developed and evaluated for its safety and immunogenicity in mice, guinea pigs, sows, and cattle inoculated IM. Generally, as important factors inducing VNA responses in

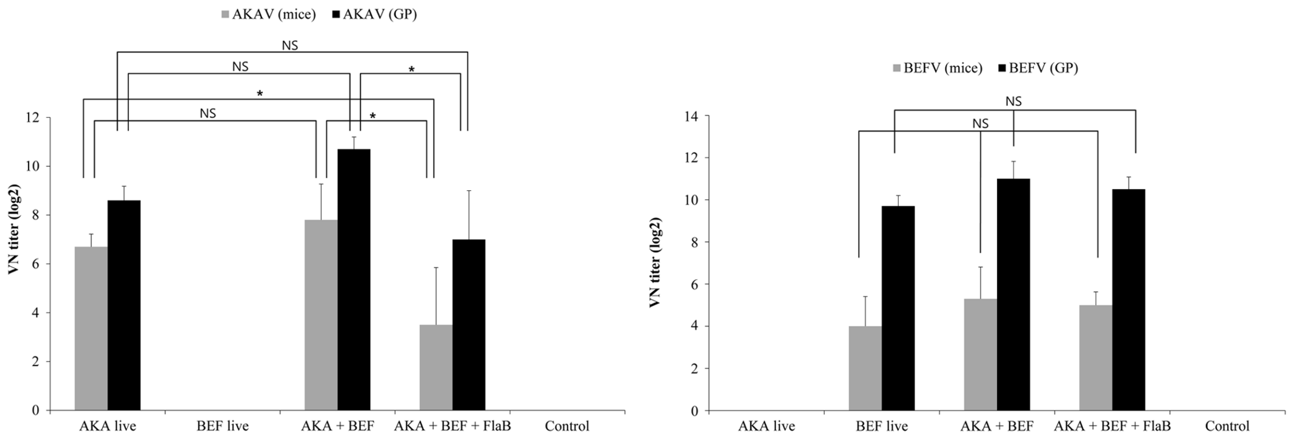

Fig. 4. Virus neutralizing antibody (VNA) titers of live *Akabane virus* (AKAV) and *Bovine ephemeral fever virus* (BEFV) vaccine, inactivated AKAV+BEFV vaccine with or without revibFlaB protein in mice and guinea pigs (GP). Data shown as expressed mean ± SD of six mice and four guinea pigs in each group. *$p < 0.05$. NS, not significant (one-way analysis of variance: ANOVA).

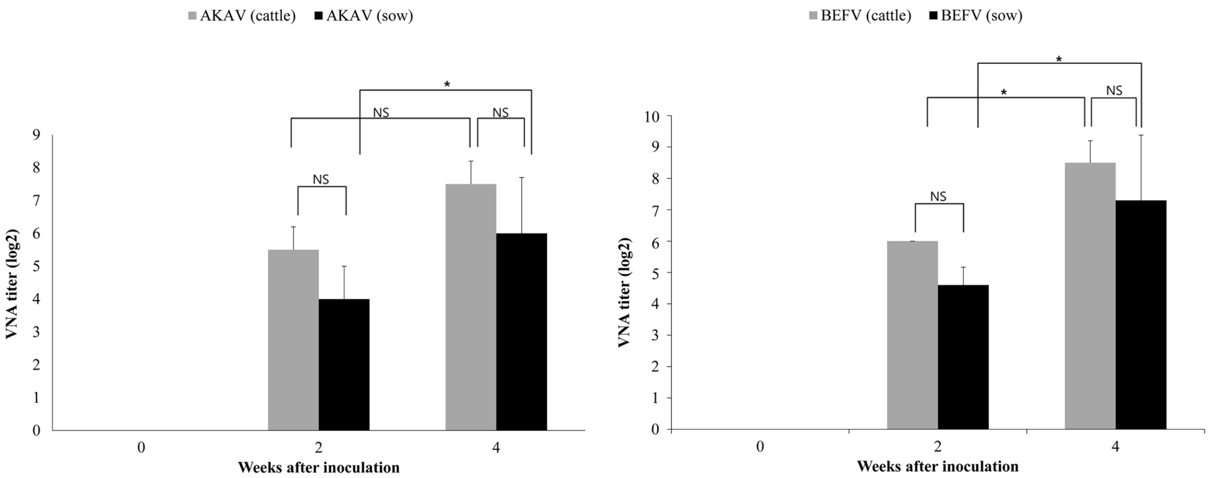

Fig. 5. VNA titers of inactivated AKAV+BEFV vaccine containing 30% IMS1313VG in cattle and pigs. Data shown as expressed mean ± SD of two cattle and three sows in each group. *$p < 0.05$.

Table 2. Outbreak case of five vector-borne diseases in Korean cattle, 2002-2014

Year	AKAV	AINV	CHUV	IBAV	BEFV
2002	8	–*	–	–	0
2003	2	2	13	0	1
2004	0	2	0	0	0
2005	2	3	1	0	0
2006	2	6	1	0	0
2007	4	0	0	0	1
2008	0	0	0	0	0
2009	1	0	0	0	0
2010	107	0	0	0	92
2011	2	0	0	0	1
2012	1	0	0	0	4
2013	0	0	0	0	8
2014	0	0	0	0	1

*not reported. AKAV, *Akabane virus*; AINV, Aino virus; CHUV, Chuzan virus; IBAV, Ibaraki virus; BEFV, *Bovine ephemeral fever virus*.

animals to vaccines, several factors such as viral strain, viral titer, kind of inactivated agent, and type of adjuvant should be considered [15]. The AKAV and BEFV strains isolated from cattle were propagated in BHK-21 cells, and viruses showing over titer of $10^{7.0}$ $TCID_{50}$/mL were used to prepare the inactivated vaccine. Generally, a high concentration of antigen in an inactivated vaccine makes the vaccine elicit higher VNA titers in animals.

Recently, it was reported that FlaB proteins in pathogenic bacteria cause signal transduction in mammalian cells via TLR5 and are considered a potent adjuvant for vaccines [9]. Lee *et al*. [9] reported that FlaB could also elicit strong antitumor activity. In addition, Montanide IMS has been used as a ready-to-dilute adjuvant for the production of veterinary vaccines [1, 6]. IMS1313VG does not need any special procedure for manufacturing a vaccine, and can be blended with inactivated antigens.

Our results indicated that the inactivated AKAV+BEFV vaccine could induce high VNA against AKAV and BEFV in mice and guinea pigs, but the inactivated AKAV+BEFV vaccine containing 10% revibFlaB protein and 10% IMS1313VG induced lower VNA titers in the experimental animals compared to the inactivated AKAV+BEFV vaccine containing 30% IMS1313VG. The first reason why the AKAV+BEFV vaccine containing revibFlaB protein induced low VNA titer may be the low percentage of IMS1313VG adjuvant. The adjuvant is recommended for vaccine at a ratio between 30 and 50% according to sensitivity of animal species and reactivity of the antigen. The second reason why the revibFlaB protein did not stimulate TLR5 properly in mice and guinea pigs may be the small amount of revibFlaB protein and the source of the FlaB protein, obtained from *Vibrio alginolyticus*. It was reported that a bacterial flagellin, *Vibrio vulnificus* FlaB, when mixed with antigen and administered intranasally, exerted strong mucosal adjuvant activity, by stimulating TLR5 [2, 3]. The difference in origin of bacterial species may lead to different immune responses in animals. In addition, the inactivated AKAV+BEFV vaccine was found to be safe, and induced high VNA titers, ranging from 1 : 64 to 1 : 512, against both AKAV and BEFV in sows and cattle. The VNA titer in arboviral infection is related to protection against AKAV and BEFV, and a titer of > 1 : 2 in cattle has been considered to provide protective immunity [7].

In conclusion, the results of those trials demonstrated the ability of the inactivated AKAV+BEFV vaccine to be used in cattle with a high immunogenicity and good safety. Further studies of the efficacy and the duration of immunity in cattle and vibrio FlaB activity are necessary in the field trials.

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