Short-term humoral immune response of the pcDNA4-G plasmid expressing the bovine ephemeral fever virus G gene in BALB/c mice

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Antibody response, Bovine ephemeral fever, DNA vaccine.

Summary
Bovine ephemeral fever (BEF) is an arthropod-borne viral disease characterised by a short-term clinical expression that can lead to significant losses in high-yielding cattle and water buffaloes. In this study, we aimed to generate a recombinant plasmid expressing the glycoprotein (G) of the BEF virus (BEFV) and to stimulate a humoral immune response to this protein in BALB / c mice immunised with the recombinant plasmid. Expression of the encoded protein was demonstrated by western blotting and immunoperoxidase tests. The suitable plasmids were intramuscularly administered to BALB/c mice on days 0, 14 and 21. The antibody response in the immunised mice was measured by a plaque reduction neutralization test (PRNT) and enzyme-linked immunosorbent assay (ELISA). According to BEFV ELISA, only two of the seven animals in these groups exceeded the cut-off value. A significant difference was observed in the mean OD values at 450 nm absorbance in the pcDNA4-G-immunised group when compared with those in the plasmid control group at 30 days ($p < 0.05$). According to PRNT50 results, a 1:20 ($p < 0.05$) antibody response was obtained at 30 days in pcDNA4-G (100 µg)-immunised mice, whereas this ratio was 1:80 ($p < 0.001$) in BEFV-immunised mice (1,000 PFU/0.5 ml). We conclude that the humoral immune response was stimulated in experimental mice immunised with the recombinant plasmid. However, disappointingly, the antibody response was markedly low in pcDNA4-G-immunised mice.

Introduction

Bovine ephemeral fever (BEF) can lead to trade restrictions between countries and significant yield losses in high-yielding cattle and buffaloes. BEF is also known as a 3-day sickness because the clinical symptoms persist for 3-4 days. The disease is transmitted among susceptible animals through *Culicoides* species. The causative agent is a bovine ephemeral fever virus (BEFV), which has a negative-sense, single-stranded (ss) RNA genome (St George *et al.* 1995, Hertig *et al.* 1996, Nandi and Negi 1999). The BEFV genome, which is 14,900-nucleotide (nt) long, contains a total of 12 gene regions as well as leader and trailer sequences (Walker 2009, Walker and Klement 2015, Bulut and Azkur 2016). Similar to other rhabdoviruses, the BEFV virion encodes five structural proteins: matrix protein (M), glycoprotein (G), nucleoprotein (N), polymerase protein (L), and phosphoprotein (P) (Walker 2009). Glycoprotein is the only viral protein that stimulates the production of neutralising antibodies that protect cattle from BEF (Uren *et al.* 1994).

BEF is a rare viral disease that benefits from early treatment (e.g. non-steroidal anti-inflammatory administration and calcium supplements) (St George 1988). However, due to the rapid onset of clinical findings, it is likely that late intervention in all animals is possible in breeding sites with high cattle populations. To date, BEFV has caused endemic outbreaks in Africa, the Middle East, Asia and Australia. Unusual serious outbreaks exceeding the 10%-20% mortality rate have recently been reported (Abayli *et al.* 2017). Preventive measures are therefore recommended to limit yeld losses, animal deaths and commercial restrictions (Davis *et al.* 1984, St George *et al.* 1986, St George 1988, Uren 1989, Nandi and Negi 1999, Tonbak *et al.* 2013). Vaccination is the most important measure for protecting animals against BEFV. Inactive, recombinant, attenuated

and subunit vaccines have been developed against BEF, and some of these are commercially available (Walker and Klement 2015). One of the most effective BEFV vaccines is a live attenuated vaccine formulated with the saponin Quil A (Vanselow *et al.* 1995). In this study, we investigated the expression of and short-term humoral immune response to the pcDNA4-G plasmid in BALB/c mice immunised with the attenuated vaccine on days 0, 14 and 21.

Materials and methods

Virus and cell culture

In this study, we use the BEFV/Ad12/TUR strain (Genebank: KY012742), which was isolated from the Turkish BEF outbreak in 2012. The virus was propagated in Vero cells, as previously described (Abayli *et al.* 2017).

RNA isolation and RT-PCR

The viral RNA extraction from the virus culture supernatant was performed using a QIAamp® Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The primers were designed using DNA Baser (Heracle Biosoft S.R.L., Romania) based on the complete genome sequences of the BEFV/Ad12/TUR strain. Reverse transcription polymerase chain reaction (RT-PCR) was performed with the Qiagen OneStep Kit. Briefly, the reaction mixture [5× RT-Buffer (10 µl), 10 mM dNTP mix (2 µl), primers mix (10 pmol) (4 µl), 5'-ATGTTCAAGGTCCTAATAATTACC-3' and 5'-TTAATGATCAAAGAACCTATC-3'), RT-PCR enzyme mix (2 µl) and RNA (50 ng)] was adjusted to a volume of 50 µl with RNase-free water. After the reverse transcriptase step at 50 °C (30 min), amplifications were carried out at 94 °C for 15 min, followed by 35 cycles at 94 °C for 45 sec, 55 °C for 1 min, and 72 °C for 1 min.

Construction of PcDNA4-G

The full-length glycoprotein gene (BEFV/Ad/12TUR, 3060-4931 nucleotide position) amplified by RT-PCR was transferred into the pcDNA4/HisMax©TOPO® TA expression vector (Thermo Fisher, USA) by direct ligation to form pcDNA4-G. pcDNA4-G was then transformed into TOP10 competent cells. The orientation of the pcDNA-G construct and the absence of unwanted mutations were checked by sequence analysis.

Expression of recombinant glycoprotein

The pcDNA4-G plasmid was transfected into Vero-E6 cells in six-well plates using lipofectamine 2,000 (Thermo Fisher, USA). Fresh medium was supplied 24 hr after transfection, and the expression of glycoprotein was assessed by immuno-peroxidase tests and Western blotting at 72 hr after transfection. At the same time, transiently transfected Vero cells were treated with Zeocin (Thermo Fisher, USA) (100 µg/ml, the lethal dose tested in Vero cells) to obtain a stable cell line.

Immunoperoxidase test

The cells were then treated with 1 ml of 0.1% Triton X-100 for 15 min to allow permeabilization at room temperature. Blocking was performed at room temperature for 1 hr with 5% skimmed milk powder prepared by dissolving in 0.05% PBST-20 solution [with PBS containing 0.05 % (v/v) Tween 20]. Once washed with PBS (Phosphate-buffered saline), the cells were treated with 3% H_2O_2 for 1 hr to eliminate the endogenous peroxidase effect. Following two washes with PBS, BEFV G1 specific monoclonal antibody (EMAI, Australia, diluted 1/50 in PBS) was added to each well.

Western blotting

Purified proteins from the transfected cell lysate were obtained using the Ni-NTA Spin Kit (QIAGEN, Germany) purification system, used for Western blot analyses. Purified proteins were separated on 10% resolving and 5% stacking SDS-PAGE gel in a mini electrophoresis unit (Bio-Rad, USA) for 120 min at 130 Volts. The transfer of proteins in the acrylamide gel to the PVDF membrane (Millipore, USA) was carried out using the mini-transblot transfer system (Bio-Rad, USA) for 60 min at 100 Volts. The membrane was blocked with 5% skimmed milk in PBS. Following blocking, the membrane was washed several times with wash solution (PBS with 0.05 Tween 20) and incubated at room temperature for 2 hr in BEFV G1 specific monoclonal antibody (diluted 1/100). The membrane was washed again and incubated in HRP (Horseradish peroxidase)-labeled anti-mouse IgG conjugate (Sigma-Aldrich, USA, diluted 1/1,000) for 1 hr. After washing, the membrane was incubated for 15 min in the chromogen-substrate solution (0.05 M Tris pH 7.2, 1 mg/ml diaminobenzidine, and 0.3% H_2O_2). After washing the membrane in distilled water, the size of the proteins was estimated relative to a peptide molecular weight marker (MW-SDS 200, Sigma-Aldrich, USA).

Immunization preparation

Two-three µl of glycerol stocks carrying pcDNA4-G recombinant plasmid and control plasmid (pcDNA4-lacZ) were grown in LB Medium (Ampicillin,

60 µg/ml, 50 ml). The tubes were incubated overnight in a shaking incubator (37 °C, 180 rpm). The next day, the tubes were centrifuged (4,500 rpm for 15 min) and plasmid purification was performed using the PureLink™ Expi Endotoxin-Free Maxi Plasmid Purification Kit (Thermo Fisher, MA, USA).

Plasmid DNA pellets (100 µg) were diluted with 40 µl of sterile PBS. Ten microliters of lipofectamine 2,000 were then added to the plasmid-PBS solution. A 50-µl aliquot of the plasmid-PBS-lipofectamine mixture was prepared for injection into the mice assigned to the plasmid-immunised group. The mouse brain adapted BEFV (1,000 PFU/500 µl) was prepared for injection into the mice assigned to the BEFV-immunised group.

Ethical statement

All animals used in the study were obtained from the Experimental Research Center of Firat University. The Firat University Animal Experiments Local Ethics Committee approved the use of experimental animals (Protocol number: 2016/50).

Experimental study

Female BALB/c mice (4 weeks old) were divided into four groups (n = 7, each). The experimental mouse groups were designed as two study groups (pcDNA4-G-, BEFV-immunised) and two control groups (pcDNA4-lacZ- and PBS-immunised). The mice in the vaccine group were sedated with ketamine (200 mg/kg/IP) and, after about 10 min, the injection site was disinfected with 70% ethanol and injections were performed. All injections were performed on the quadricep muscle (except the BEFV immunised group given IP) on days 0, 14, and 21. Blood was collected from the tail vein on days 0, 13, 20, and 30 from all animals in the experimental groups.

The tubes were incubated overnight at 4 °C for centrifugation (4,000 × g for 15 min) and kept in a - 20 °C freezer until use.

Antibody response

ELISA

In the experimental group, antibody response was determined using a modified version of a commercially available BEFV ELISA (EMAI, Camden Park, Australia). All wells in the BEF ELISA plate were first blocked overnight with 5% (w/v) skimmed milk diluted in 0.5% PBST-20. After blocking, the wells were washed twice with wash solution [0.05% (v/v) PBST-20]. Non-immune and immune blood sera from pcDNA4-G-, BEFV-, PBS-, and pcDNA4/lacZ-immunised mice were diluted 1/100. Aliquots (100-µl) of these serum samples were transferred into the wells of a ELISA-plate, which was incubated for 2 hr at room temperature and shaking at 50 rpm. After incubation, the wells were washed seven times with wash solution. After washing, 100 µl of 1/5,000 diluted streptavidin HRP (Thermo Scientific, MA, USA) was added to each well. The plate was incubated for 1 hr at room temperature. After washing, a solution of tetramethylbenzidine (TMB, Sigma Aldrich, MA, USA) substrate was prepared, and 100 µl of TMB substrate was added to all wells. The plate was then incubated in the dark for 10 min. The reaction was stopped with 100 µl of 1M H_2SO_4. The plate was read at 450 nm absorbance in the ELISA reader.

Plaque Reduction Neutralization Test (PRNT)

T75 flask-cultured cells were trypsinized and passaged to a 24-well plate (0.02×10^6 cells/well). All mouse serum samples were removed from the freezer (- 20 °C), thawed at room temperature, and then inactivated by incubating at 56 °C for 30 min. Aliquots (20 µl) of each serum sample were added to a microcentrifuge tube containing 180 µl of DMEM-F12. A 100-µl aliquot of the vortexed mixture was serially diluted with DMEM-F12 (Sigma-Aldrich, MA, USA) (1:10, 1:20, 1:40, and 1:80). Inactivated serum samples were studied individually in 24-well cell culture plates, and different plates were used for each experimental group.

In addition to the sera from the experimental group, four wells of plasmid control, two wells of virus control, and two wells of Vero E6 cells were included on each plate. Mouse brain-adapted BEFV stock was removed from the freezer (- 80 °C) and diluted with DMEM-F12 to contain 50 PFU/100 µl of the virus. A 100-µl aliquot of this diluted virus was transferred to serum tubes with serial dilutions. The mixture was vortexed and incubated at 37 °C for 1 hr. After incubation, this mixture (200 µl) was added to the medium-removed cells in a 24-well plate and seeded for 1hr by adsorption. The plates were mixed at 15-min intervals. During the incubation, 0.3% overlay medium (1:1 mixture of 2x DMEM-F12 and 0.6% agarose) was prepared.

After 1 hr, the inoculum was removed from the plate wells, and the cells were coated with 1 ml of overlay medium. The plates were incubated at 37 °C, 5% CO_2 for 4 days. Cells were examined under the microscope and scored for CPE (Cytopathic effect) foci.

Calculations and Statistical analysis

PRNT50 (the serum titer required to reduce viral plaques by 50%) was used to calculate the

neutralization titers. The critical value in the neutralization test was 1:10.

Cut-off values for the ELISAs were calculated by adding three-fold of the standard deviation to the mean of the negative control sera. Statistical analyses were performed using IBM SPSS 22 software, and graphs were generated using the GraphPad Prism 5 package program (GraphPad, San Diego).

The ELISA values were tested for normality, and a One-way ANOVA test was preferred because the values between the compared groups showed a normal distribution. Tukey (if homogeneous) and Tamhan T2 (if not homogeneous) tests were used according to whether the variances were homogenous in the post-hoc tests. The Kruskal Wallis H test was preferred for the neutralization test. The Mann Whitney U test was used to determine which groups were responsible for the difference.

Results

RT-PCR and Sequence analysis

The BEFV G gene was amplified in full length (1871 bp) by RT-PCR. After the sequence analysis, there was no disorientation or undesired stop codon in the pcDNA4-G structure.

Immunoperoxidase test and Western blotting

The immunoperoxidase test was performed on transiently transfected Vero cells using BEFV G1 specific monoclonal antibody and cells expressing gene with DAB chromogen were imaged at 72 hr.

In the transfected Vero E6 cells, brown staining indicating the presence of G protein of BEFV was observed after microscopic examination, whereas staining did not occur in negative control cells (Figure 1).

Figure 2 shows Western blot analyses of the purified proteins obtained from pcDNA4-G transiently transfected Vero cells with G1 specific monoclonal antibody of BEFV as G protein of size 77 kDa.

ELISA and PRNT50

The serum samples of the last immunization of pcDNA4-G immune mice were studied using BEFV ELISA. When the OD (Optical density) values of the last sera were examined on an individual basis, it was determined that only mouse #1 and #5 exceeded the cut-off value, while the others were below this value. Despite this, higher IgG antibody results were obtained in all individuals in the pcDNA4-G immune group than in the negative groups. These results show that the humoral response is achieved in all

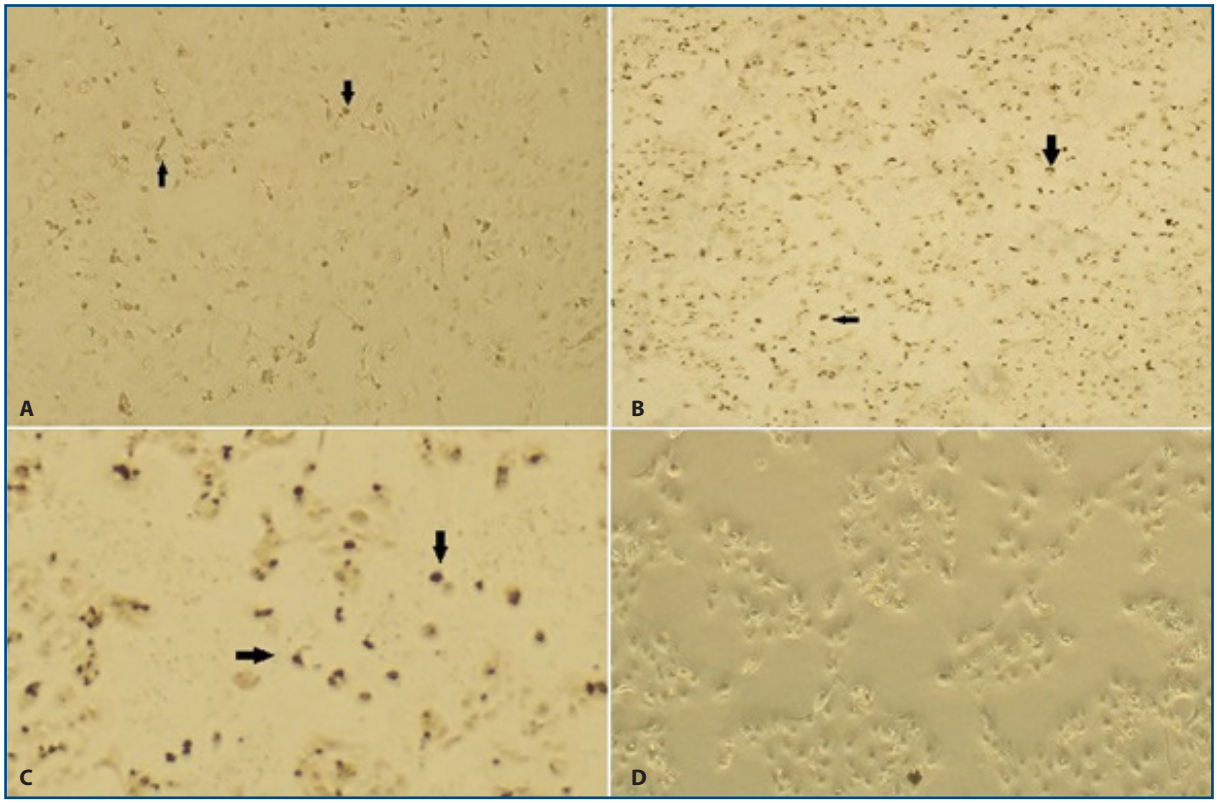

Figure 1. *Immunoperoxidase test using BEFV G1 spesific monoclonal antibody in pcDNA4-G-transfected Vero E6 cells.* **A.** *Vero E6 cells transiently transfected with the pcDNA4-G vector.* **B, C.** *Vero E6 cells stably transfected with the pcDNA4-G vector.* **D.** *Control Vero cell (40×).*

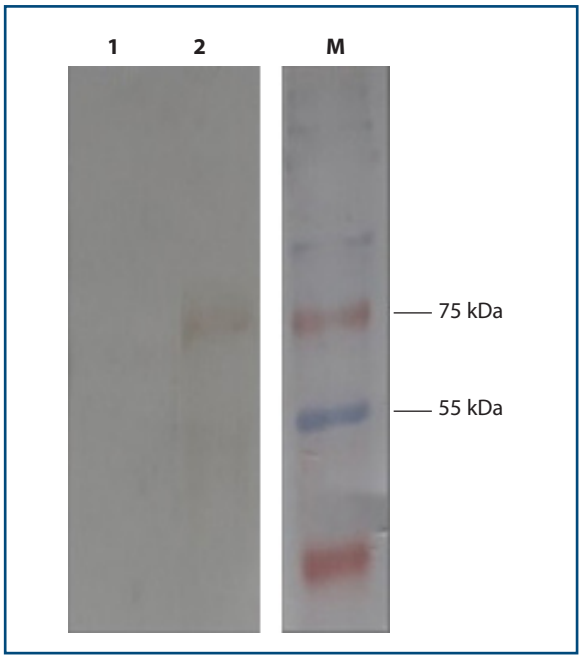

Figure 2. *Purified recombinant G protein visualized by Western blotting.* M = Protein ladder; Lane 1 = Control Vero E6 cell lysate; Lane 2 = Purified G protein.

mice immunised with pcDNA4-G. In the evaluation of the test, the cut-off value of the final serum samples was calculated as 0.965 (Figure 3).

The results were also evaluated on a group basis and group averages of the same day were calculated and compared with negative controls in pairs.

Accordingly, the mean OD value of the sera from the pcDNA4-G immune group was significantly different on day 30 when compared with the plasmid control group ($p < 0.05$) and PBS immune group ($p < 0.001$). The BEFV immune group revealed this difference on the 13th day ($p < 0.001$) (Figure 4).

Table 1 shows the PRNT50 results and percentages of neutralizing antibodies inhibiting BEFV under cell culture conditions. According to these results, in mice #1 and #5 in the pcDNA4-G immune group, a 1:20 serum dilution inhibited BEFV by approximately 70%. Therefore, the antibody titer was accepted as 1:20. Other mice in the same group exceeded the 50% inhibition value at a 1:10 dilution and remained below this ratio at a 1:20 dilution. Therefore, the neutralizing antibody titers of these mice were considered 1:10.

According to the PRNT50 results, five of the BEFV-immunised mouse sera were 1:80 and the remaining two inhibited BEFV by more than 50% at a serum dilution of 1:40. Dilution of BEFV immune sera at 1:160 produced less than 50% inhibition. Accordingly, antibody titers were calculated to be 1:80 in five of the BEFV-immunised mice and 1:40 in the remaining two mice (data not shown).

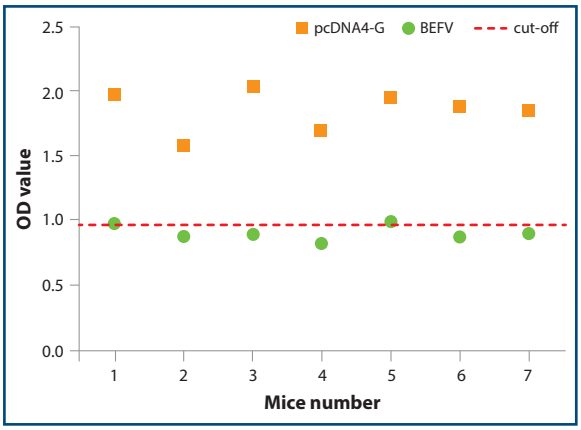

Figure 3. *Individual serum ELISA antibody measurements of BEFV- and pcDNA4-G-immunised mice.* The cut-off value indicates the final serum OD mean of all negatives plus three-times the standard deviation, calculated as 0.965.

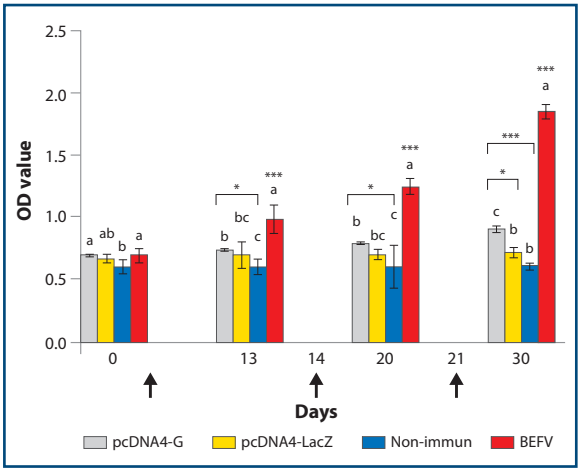

Figure 4. *Antibody measurements (BEFV ELISA kit) of sera from immunised mice.* The graph shows the mean OD value of the individuals in each experimental group with standard errors. Different letters (a, b, c) for the same day indicate differences between the groups. The significance of the difference is indicated by *. A single star (*) indicates $p \leq 0.05$ and three stars (***) indicate $p < 0.001$. The arrows indicate the days of immunization.

Discussion

BEFV vaccines show a protective immune response between 6 months and 1 year. Live vaccines in areas with dense mosquito populations might pose a risk if the vaccine strain regains virulence. In addition, contamination during the preparation of live vaccines poses a risk to animal health (Tzipori and Spradbrow 1973, Spradbrow 1975, Aziz-Boaron *et al.* 2013). Only repeated inoculations of attenuated vaccines induce a protective response against BEFV. This indicates that the virus loses its antigenicity as well as pathogenicity during attenuation (Spradbrow 1975). Therefore, some researchers have aimed to increase vaccine efficacy by using adjuvants with

Table I. *PRNT50 data from sera collected from pcDNA4-G-immunised mice on day 30.*

Serum dilution rates	Neg. control	Cell culture plate 1								Cell culture plate 2	
		Mouse 1		Mouse 2		Mouse 3		Mouse 4		Neg. control	Mouse 5
	Plaque number	Plaque number	% inhib.	Plaque number	% inhib.	Plaque number	% inhib.	Plaque number	% inhib.	Plaque number	Plaque number
01:10	28	0	100	2	**92.8***	8	**71.4***	4	**85***	26	0
01:20	34	10	**70.5***	20	41.1	26	23.5	19	44.1	29	8
01:40	32	24	25	34	-	29	-	29	-	27	21
01:80	35	33	-	32	-	36	-	32	-	30	28

(-) Indicates inhibition values below 10%; (*) Expresses the highest serum dilution rates that inhibit BEFV by 50% or more. These ratios were accepted as neutralizing antibody titer.

attenuated vaccines (Vanselow *et al.* 1985, Vanselow *et al.* 1995) Although it is not known how much of the virus can remain active after adjuvantization, it is estimated that it is 99.9% aggregated with Quil A saponin (Walker and Klement 2015). This may adversely affect the proliferation of the vaccine strain in the host.

Subunit vaccine studies, which initially focused on the protective antibody response of the G protein separated from the native virus (Uren 1994), at present continue with focus on recombinant protein production (Hansoongnern *et al.* 2019). Some researchers have demonstrated that the recombinant BEFV G protein, which has the potential to be used in vaccines or diagnostic kits, is expressed *in vitro* using the baculovirus expression system (Johal *et al.* 2008, Kanpipit *et al.* 2018). Hansoongnern and colleagues (Hansoongnern *et al.* 2019) reported that the transmembrane domain-deleted G protein (100 µg) adjuvanted with Montanide ISA 206 displays a neutralizing antibody response in guinea pigs. In such expression systems, it is critical that the protein produced is conformationally similar to the native virus. In addition, the need for purification and the conformational change of the protein during the purification or adjuvantisation may be a disadvantage for these systems.

Inactive vaccines are safe. However, the virulent live virus in the inactive vaccines should be completely inactive. Further, the adjuvants in these vaccines can lead to cytotoxicity (Spradbrow 1975) or undesirable reactions (Tzipori and Spradbrow 1973). Inactive BEFV vaccines produce a short-term antibody response in cattle, and a positive correlation between the level of antibody response and the protection offered by the vaccine is not always observed (St George 1988, Aziz-Boaron *et al.* 2014). In a previous study, although an emulsified inactive Israeli vaccine stimulated an antibody response at a ratio of 1:256 after the third vaccination, the protective efficacy of the vaccine against natural infection was found to be 50%, which was disappointing (Aziz-Boaron *et al.* 2013, Aziz-Boaron *et al.* 2014).

Some researchers have noted that humoral immune response alone is not sufficient to protect against BEFV and cellular immune response is also important (Aziz-Boaron 2014, Della-Porta and Snowdon 1979). Therefore, researchers have focused on recombinant vaccines (which are both safe and potent) rather than inactive or subunit vaccines (Wallace and Viljoen 2005, Zhang *et al.* 2017).

Alternatively, viral vector vaccines expressing the recombinant BEFV G protein have been studied by some researchers. Hertig and colleagues (Hertig *et al.* 1996) found that a vaccinia vector vaccine expressing the BEFV G protein protected cattle from experimental infection after the second inoculation; however, the virulence of the virus in the experiment was low. Zhang and colleagues (Zhang *et al.* 2017) reported that a recombinant Newcastle disease virus vaccine (La Sota strain) expressing the BEFV G protein produced a 1:64-1:128 neutralizing antibody titre in cattle after double vaccination (days 0 and 21). Wallace and colleagues (Wallace *et al.* 2005) reported that a lumpy skin disease virus vaccine expressing the BEFV G protein did not yield satisfactory results even after four inoculations in cattle. Viral vector-based vaccines developed against BEFV also failed to provide the expected results (Wallace *et al.* 2005, Zhang *et al.* 2017). In viral vector-based vaccines, antibodies resulting from pre-existing immunity to the vector virus can prevent vector proliferation. Therefore, repeated inoculations of such vaccines can also be ineffective (Leitner *et al.* 1999, Griffiths and Khader 2014).

DNA vaccines are considered safe because there is no contamination with infectious agents. After DNA vaccination, an immune response develops only against the antigen targeted by the vaccine so that vaccinated animals can be easily distinguished from infected animals. (Dhama *et al.* 2008). After viral infection, viral proteins are produced and processed in the cells. Similarly, after the plasmid DNA enters the cell, the transcription and translation of the encoded genes occur in the cell. Thus, viral proteins having the native structure can be produced in

the cell (Alonso and Leong 2013). This feature is important in antigenic regions in the targeted protein. DNA vaccines can be rapidly produced, purified and combined with different types of vaccines or different gene regions. In the presence of maternal antibodies, which play an important role in protecting against high-risk diseases, the effect of these vaccines can continue (Dhama *et al.* 2008). In addition, DNA vaccines are known to elicit a strong cellular response and may be a good alternative to BEFV vaccines (Beláková *et al.* 2007).

Pasandideh and colleagues (Pasandideh *et al.* 2018) showed that anti-BEFV-neutralising antibodies were induced in mice injected with the pcDNA3.1-G1 construct. Similarly, we investigated the humoral immune response to the construct expressing the BEFV G gene in BALB/c mice in this study. We found that the humoral immune response was lower and later in pcDNA4-G-immunised mice than in BEFV-immunised mice. Although the humoral immune response was expected to occur later in case of DNA vaccines, the antibody titres measured on day 30 in the pcDNA4-G-immunised group were very low. This may be due to the route of administration of the vaccine. We preferred intramuscular injection because it is widely used and practical. After intramuscular administration, myocytes are often transfected. This region is not equipped with dendritic cells, and myocytes do not have the ability to deliver the antigen to CD4+ T cells via MHC II; therefore, the cellular response was dominant compared to the antibody response (Belakova *et al.* 2007, Dupuis *et al.* 2000). In addition, the degradation of plasmid DNA by serum proteins in intramuscular vaccinations might reduce the expression level, resulting in a delayed antibody response (Faurez *et al.* 2010). Some researchers have preferred intra-lymph node vaccination, electroporation or particle-mediated bombardment to avoid this disadvantage. These techniques can increase transfection and significantly increase immune responses; however, their application is difficult, particularly in large animals, and they have not yet progressed to routine use in animals. (Dhama *et al.* 2008, Van Drunen Littel-van den Hurk *et al.* 2004). In this study, a chemical liposome (lipofectamine 2000), a plasmid DNA transfer system, was used to prevent plasmid DNA degradation in the blood. Liposomes are synthetic vesicles consisting of a phospholipid bilayer structure and are now one of the most important agents used in the transfer of genetic material into cells.

Liposomes containing plasmid DNA can easily pass through cell membranes after transfer and release of the plasmid DNA content following fusion with the endosomal membranes. At the same time, liposomes rapidly escaping from endosomes are protected from degradation in this manner. This increases gene expression and contributes to immunogenicity (Pereira *et al.* 2014, Wang *et al.* 2011). Although it is not known whether liposomes positively contributed to humoral immune response in this study, their effect is predicted to be beneficial. The number of experimental studies using chemical transfer systems should be increased in the future, and the results should be compared with those of other transfer systems. According to minimum tissue damage and cost-effectiveness analysis, the most suitable system should be preferred.

In this study, the cellular response was not determined. Further, the fate of the long-term response remains unclear. Although the antibody response is low in case of DNA vaccines, the cellular response might be superior to that observed in case of subunit or inactive vaccines. Future studies investigating the cellular and long-term responses are planned.

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