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Determination of antibody response after simultaneous administration of vaccines against foot and mouth disease (FMD) and bluetongue in sheep*

SEVIL SARDOĞAN[1], OYA BULUT[2], VELI GÜLYAZ[3]

[1]FMD (Sap) Institute, Ankara, Türkiye
[2]Selçuk University, Faculty of Veterinary Medicine, Department of Virology, Konya, Türkiye
[3]Harran University, Faculty of Veterinary Medicine, Department of Virology, Şanlıurfa, Türkiye

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Sardoğan S., Bulut O., Gülyaz V.

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Summary

Foot-and-mouth disease (FMD) and bluetongue disease (BT) are two of the most devastating viral diseases affecting domestic and wild ruminants. They cause significant economic losses, and vaccination is the most important method for combating both FMD and BT. Simultaneous vaccinations are crucial for animal welfare as well as labor and cost savings. No reports were found on the simultaneous administration of FMD and BT vaccines to sheep. This study evaluated the effect of the simultaneous administration of an inactivated FMD vaccine and an attenuated BT vaccine on the humoral immune response to both diseases. A total of 75 six-month-old Akkaraman-Kıvırcık crossbred sheep that did not have any vaccination history were used as research material. Before the experiment, the animals were tested for the absence of FMD and BT antibodies. The sheep were divided into three experimental groups and vaccinated with an FMD vaccine alone, a BT vaccine alone, and both. Blood samples were taken on days 30 and 60 post-vaccination. The blood samples were evaluated using liquid-phase blocking ELISA (LPBE) and virus neutralization test (VNT) for FMD antibodies, as well as competitive ELISA (c-ELISA) and VNT for BT antibodies. The study revealed that differences between the FMD and BT antibody titers of the group vaccinated simultaneously and the FMD and BT antibody titers obtained from the groups vaccinated separately were not statistically significant ($p > 0.05$). It was concluded that the antibody response these diseases was not altered when the FMD and BT vaccines were administered together.

Keywords: foot-and-mouth disease, bluetongue, simultaneous vaccination, sheep

Foot-and-mouth disease (FMD) is a highly contagious viral infection that affects cloven hoofed animals and causes economic losses. This virus, which belongs to the *Apthovirus* genus, *Picornaviridae* family, has seven serotypes: A, O, C, Asia 1, and SAT (Southern African Territories) 1, 2, and 3. The FMD virus is an RNA virus with a highly variable structure, and there is generally no cross-protection between its serotypes (24, 27).

FMD-free countries seek to prevent the introduction of the virus into the country by imposing restrictions on the importation of animals and animal products from countries where the disease is present. In addition, in the event of an epidemic in these disease-free countries, compulsory slaughter, quarantine, and vaccination are implemented. In endemic countries, precautions are taken to reduce the incidence of the disease by combining sanitation practices and preventive vaccinations with inactivated vaccines of the appropriate serotype (7).

Bluetongue (BT) is a viral disease of ruminants. It is transmitted by vectors and results in high morbidity and mortality rates. The causative agent of BT is the bluetongue virus (BTV), which belongs to the *Orbivirus* genus of the *Reoviridae* family (29). Recent studies have demonstrated virulence differences and extremely

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low cross-immunity between the 29 serotypes of BTV (28, 35). Live attenuated and inactivated vaccines are widely used to combat BT (10).

Vaccination campaigns against many pathogens in the field generally coincide within the same period, resulting in economic, labor, and time losses due to repeated visits of veterinarians to enterprises for each vaccination. Moreover, considering the stress caused by vaccination to animals, simultaneous/combined administration of vaccines is also important for animal welfare. Several studies were conducted in Turkey and abroad in which an inactivated FMD vaccine was applied simultaneously with different attenuated live vaccines. It has been reported that inactivated FMD vaccines and live attenuated vaccines can be administered together (2, 6, 10, 13, 15, 34). The present research was conducted to determine the level of immunity against both diseases that can be induced by the mandatory spring period administration of inactivated FMD vaccine and live attenuated BT vaccine in Turkey.

Material and methods

Vaccines. This study used TURVAC-OIL (Serial No. 12/12), manufactured by the FMD Institute in Ankara, Turkey, a tetravalent inactive double oil emulsion FMD vaccine containing A/NEP 84 (A/Asia/G-VII), A/TUR 16 (A/Asia/G-VII), O/TUR 07 (O Panasia II), Asia 1/TUR 15 (As/Sindh 08) vaccine strains, and Montanide ISA 206 adjuvant (Seppic, France). The FMD vaccine, with 6 PD_{50} per dose, contained 6 µg/ml antigens of the A serotype, 8 µg/ml of the O serotype, and 6 µg/ml of the Asia 1 serotype. The BT vaccine used in this study was BLU-T4 ETVAC (Serial No. 16/BT/1), manufactured by the Central Veterinary Control Institute in Ankara, Turkey. It is a lyophilized live attenuated BT vaccine containing SA/BT/4 with a titer of $TCID_{50}$ $10^{-4.5}$/ml.

Viruses. FMD viruses A/NEP 84 (A/Asia/G-VII), A/TUR 16 (A/Asia/G-VII), O/TUR 07 (O Panasia II), and Asia 1/TUR 15 (As/Sindh 08) and the BTV-4 (SA/BT/4) virus were used to evaluate blood serum samples with the virus neutralization test (VNT).

Cell cultures and preparation. BHK-21 An_{31} monolayer cell culture was used to detect FMD antibodies, and the African green monkey kidney (Vero) cell line was used to detect BT antibodies in VNT. Firstly, the medium in the cell culture flasks covered with BHK-21 An_{31} and Vero cells was discarded. Following the trypsinization process, the flasks were placed in an incubator at 37°C. Glasgow Minimum Essential Medium (GMEM) for BHK-21 An_{31} and Dulbecco's Modified Eagle's Medium (DMEM) for Vero cells (Gibco-12491015) containing 10% fetal calf serum (FCS) were added to the flasks when the cells were separated from the surface of the flask. The cells were then homogenized using a pipette and prepared as 6×10^5 cells/ml for BHK-21 An_{31} and 5×10^5 cells/ml for Vero cells.

Animals and vaccination. A total of 75 six-month-old Akkaraman-Kıvırcık crossbred sheep with no vaccination history were used as research material. The sheep was divided into three experimental groups and one control group (Tab. 1). Sheep in the experimental groups were vaccinated according to the manufacturer's recommendations. In Group 1, 1 ml of the FMD vaccine was administered intramuscularly to 20 sheep. In Group 2, 1 ml of the BT vaccine was injected subcutaneously into 20 sheep. In Group 3, 1 ml of the FMD vaccine was injected intramuscularly into hind legs of 20 sheep, and 1 ml of the BT vaccine was administered subcutaneously into their front legs. In Group 4 (control group), 8 sheep were vaccinated intramuscularly with 1 ml of PBS, and 7 sheep were vaccinated subcutaneously with 1 ml of PBS.

Tab. 1. Experimental design and vaccination groups

Group No	Number of animals (n)	Vaccine administered
Group 1	20	FMD vaccine
Group 2	20	Bluetongue vaccine
Group 3	20	FMD + Bluetongue vaccine
Group 4 (control)	15	PBS

Blood sampling. Blood samples were taken from the sheep three times: before vaccination (day 0) and on the 30^{th} and 60^{th} days post-vaccination (dpv). Then they were centrifuged at 3000 rpm for 10 minutes. The serum samples obtained by centrifugation were transferred to sterile Eppendorf tubes, inactivated at 56°C for 30 minutes, and then stored in a deep freezer at –20°C until tests.

The liquid-phase blocking ELISA (LPBE). The liquid-phase blocking ELISA (LPBE) was used to detect FMD antibodies and was prepared in-house by the FMD Institute. FMD antibodies were determined using LPBE according to Hamblin et al. (12). Briefly, ELISA plates were coated with rabbit antibodies against anti-FMDV 146S antigens. In the meantime, 1/16 dilutions of test and control sera were added to the carrier microplate. The plate was then inoculated with FMDV serotypes O, A, and Asia 1. The ELISA and carrier plates were incubated overnight at 4°C. On the second day of the test, 50 µl of serum/antigen mixture was transferred from the carrier microplate to the ELISA microplate after washing the ELISA plate. The plates were incubated at 37°C with continuous shaking for 1 hour. After washing, 50 µl of FMDV serotype-specific guinea pig antibodies against anti-FMDV 146S antigens were added and incubated at 37°C for 1 hour. Then, a 50 µl of the working dilution (1 : 2000) of the conjugate (DAKO, P0141) was added to the wells and incubated at 37°C for 1 hour. A total of 50 µl of chromogen OPD/substrate (H_2O_2) (o-Phenylenediamine dihydrochloride, SIGMA P8412) was added to each well and then incubated at room temperature for 15 minutes. The reaction was stopped with 1.25 M sulphuric acid, and the absorbance was measured at 492 nm with a microplate reader (VersaMax, Molecular Devices, USA).

Competitive ELISA (c-ELISA). A commercially available c-ELISA (Bluetongue Virus VP7 Antibody Test Kit (Cat. No. P00450-5; IDEXX Montpellier, France) was used for the detection of BT group-specific antibodies. The test was performed according to the manufacturer's instructions. Competitive ELISA was used to determine BTV antibody titers in accordance with a method reported by the manufacturer. According to the test protocol, 80 µl of dilution buffer solution was added to each well of the plates. The

wells were filled with 20 µl of two positive and two negative control sera. Each well except the control wells received 20 µl of the serum samples. The plate was then covered and incubated at 18-26°C for 45 minutes. After incubation, 100 µl of the conjugate was added to each well. The plate was covered and re-incubated at 18-26°C for 45 minutes. After incubation, the plate was emptied and washed three times by adding 300 µl of washing solution to each well. After washing, 100 µl of the substrate was added to each well, and the plates were kept in the dark for 10 minutes. Then, 100 µl of stop solution was added to all wells, and the absorbance was measured at 450 nm with a microplate reader. According to the test protocol, samples with %SN (sample/negative control) ≤ 70 were considered BT virus antibody-positive.

Determination of infective titers of FMD and BT viruses ($TCID_{50}$/ml). Microtitration was performed according to a method reported by Frey and Liess (1971) for determining the titers of FMD and BT viruses that were used in VNT. FMD virus titration plates were examined under a tissue culture microscope (Olympus, Japan) and evaluated by staining with crystal violet. Virus titers (tissue culture infective dose 50-$TCID_{50}$) were calculated according to a method reported by Kearber (1964).

Determination of FMD antibody titers by VNT. VNT was used for the determination of FMD antibody titers. First, 50 µl of GMEM was added to all wells. Then, 50 µl of serum samples were placed in the first two wells of the microplate. From the first well, 50 µl of the serum sample was transferred to the following wells, and dilutions of the serums ranging from 1 : 4 to 1 : 2048 were prepared. Then, 50 µl of 100 $TCID_{50}$ diluted FMD virus (A/NEP 84, A/TUR 16, O/TUR 07, and Asia 1/TUR 15) was added to each well and incubated at 37°C in a 5% CO_2 chamber for 1 hour. After incubation, 50 µl of BHK-21 cell suspension (6×10^5 cells/ml) was added to each well. The microplates were incubated at 37°C for 72 hours, and the cells were stained with crystal violet dye. The titer values were determined by observing the cytopathic effect (CPEs) forms in the wells.

Determination of BT antibody titers by VNT. VNT was used to determine BT antibody titers. First, 1/5 dilutions of serum samples were placed in the wells of microtitration plates in the first row. After adding DMEM medium to all wells, 50 µl of the serum sample from the first well was transferred to the following wells, and dilutions of the serums ranging from 1 : 5 to 1 : 1280 were prepared. The plates were incubated for 1 hour at 37°C in a 5% CO_2 chamber. After incubation, 50 µl of Vero (5×10^5) cell suspension was added to each well. The microneutralization plates were covered and incubated at 37°C in a 5% CO_2 chamber. The titers were determined by observing the formation of the cytopathic effect.

Statistical analyses. The mean antibody titers of the groups in which the FMD and BT vaccines were administered alone were compared with the FMD and BT antibody titers of the group in which the FMD and BT vaccines were administered simultaneously at 30 and 60 dpv. The groups were compared using the independent sample *t*-test. Statistical analyses were conducted using the SPSS statistical software (3), and p-value ≤ 0.05 was accepted as statistical significance level.

Results and discussion

Titers of FMD and BT viruses. Titer values for FMD viruses (A/NEP 84, A/TUR 16, O/TUR 07, and Asia 1/TUR 15 strains) ranged from $TCID_{50}$ $10^{-5.5}$ to $10^{-7.2}$/0.1 ml, and the BT virus (BTV-4 strain) titer was found to be $DKID_{50}$ $10^{-4.5}$/0.1 ml.

Evaluation of pre-vaccination blood serum samples. Before vaccination, all sheep were negative for FMD and BT antibodies.

Liquid phase blocking ELISA (LPBE). The protective cut-off value was previously determined by the potency test of the FMD vaccine. The cut-off value for LPBE was determined to be ≥ 1/96 for serotypes A/NEP 84, O/TUR 07, and Asia 1/TUR 15. The antibody titers of both Group 1 (FMD vaccine administered alone) and Group 3 (FMD and BT vaccines administered together) were positive (≥ 1/96) at 30 and 60 dpv.

Antibody titers ranged from 1/96 ($\log_{10}$ 1.98) to 1/4096 ($\log_{10}$ 3.61), with titers in the control group being negative (< 1/96). In Group 1, antibody titers against the serotypes of FMDV ranged from 1/96 to 1/1024 at 30 dpv and from 1/192 to 1/4096 at 60 dpv. In Group 3, antibody titers against all FMDV serotypes ranged from 1/96 to 1/3096 at 30 dpv and from 1/96 to 1/4096 at 60 dpv.

In both groups, the highest titer was found for the A/NEP 84 strain at 30 dpv, followed by O/TUR 07 and Asia 1/TUR 15. At 60 dpv, the highest titer was determined for A/NEP 84 in both groups. While the mean titer of the O/TUR 07 serotype was higher than that of the Asia 1/TUR 15 strain in the group vaccinated against FMD alone, the Asia 1/TUR 15 titer was higher than that of O/TUR 07 in Group 3 at 60 dpv. In both groups, the titers at day 60 were found to be higher than those at 30 dpv (Fig. 1, Fig. 2).

Comparing the LPBE titers of different vaccination groups (Group 1 and Group 3), the mean antibody titers against FMDV A/NEP 84, O/TUR 07, and Asia 1/TUR 15 strains at 30 dpv and the mean antibody titers against O/TUR 07 and Asia 1/TUR 15 strains at 60 dpv were found to be higher in Group 3 than in Group 1 (Fig. 1, Fig. 2). This difference between mean antibody titer ($\log_{10}$) values in the two groups at 30 and 60 dpv was found to be statistically insignificant for all serotypes ($p > 0.05$) (Tab. 2).

Competitive ELISA (c-ELISA). In accordance with the manufacturer's instructions, serums with an optical density (OD) value of ≤ 70 were considered positive. At 30 dpv, 17 (85%) out of 20 sheep in Group 2 (BT vaccine alone) and Group 3 (FMD and BT vaccines together) were positive, and 3 of them were negative. At 60 dpv, 17 (85%) out of 20 sheep were positive, and 3 were negative in both groups. The mean OD value at 30 dpv was 42.81 in Group 2, whereas the mean at 60 dpv was 44.69. In Group 3, the mean OD value at 30 dpv was 48.24, while at 60 dpv, it was 49.73.

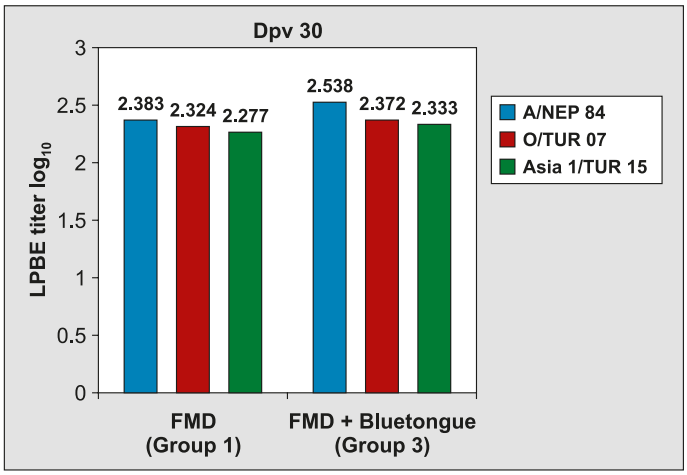


Fig. 1. The post-vaccinal arithmetic means of LPBE antibody titers against FMD serotypes A/NEP 84, O/TUR 07, Asia 1/TUR 15 on the 30th Dpv

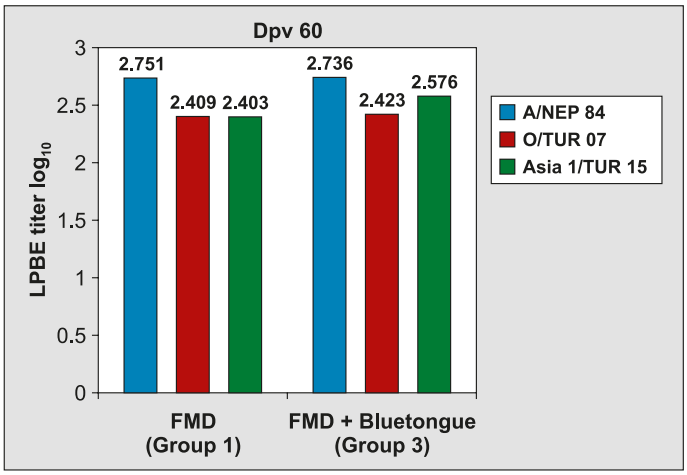


Fig. 2. The post-vaccinal arithmetic means of LPBE antibody titers against FMD serotypes A/NEP 84, O/TUR 07, Asia 1/TUR 15 for the experiment groups on the 60th Dpv

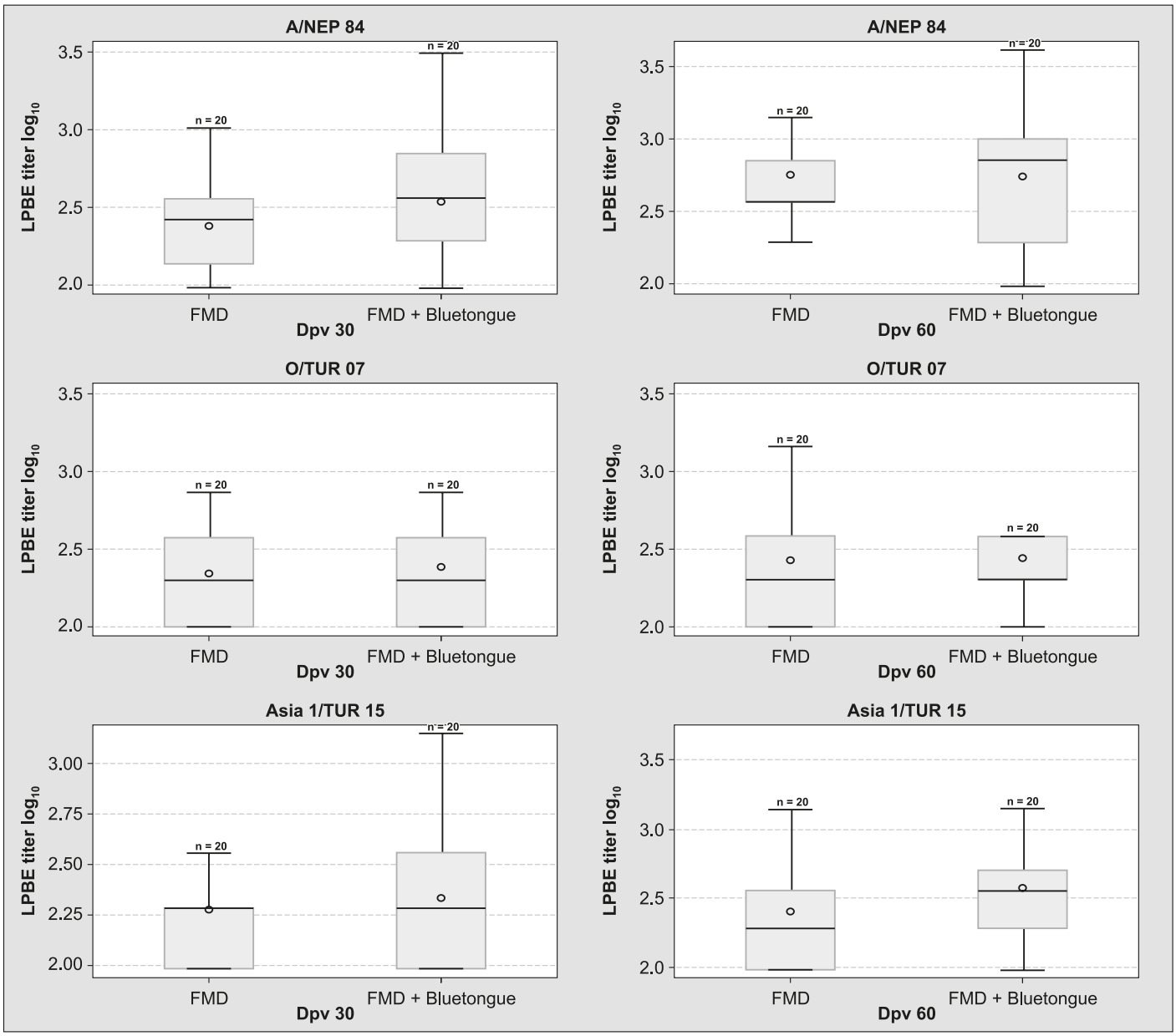


Fig. 3. Boxplots showing LPBE titers for FMD serotypes A/NEP 84, O/TUR 07, Asia 1/TUR 15. Boxplots show the median (horizontal line), interquartile range (box) and range (whiskers). Group sample sizes are shown above each box and individual data points are marked as grey vertical dashes. Titres below the detection threshold were given a value of zero

Tab. 2. Descriptive statistic and t-test result of the groups

FMD-LPBE	Group	Arithmetic mean	Standart deviation	p-value
Serotype A/NEP84 Dpv 30	FMD	2.383	0.300	0.192
	FMD+BT	2.538	0.426	
Serotype A/NEP 84 Dpv 60	FMD	2.751	0.399	0.910
	FMD+BT	2.736	0.436	
Serotype O Dpv 30	FMD	2.324	0.336	0.678
	FMD+BT	2.372	0.379	
Serotype O Dpv 60	FMD	2.409	0.443	0.917
	FMD+BT	2.423	0.378	
Serotype Asia1 Dpv 30	FMD	2.277	0.266	0.550
	FMD+BT	2.333	0.320	
Serotype Asia 1 Dpv 60	FMD	2.403	0.470	0.237
	FMD+BT	2.576	0.436	
FMD-VNT	**Group**	**Arithmetic mean**	**Standart deviation**	**p-value**
Serotype A/NEP 84 Dpv 30	FMD	2.423	0.423	0.999
	FMD+BT	2.423	0.491	
Serotype A/NEP 84 Dpv 60	FMD	2.618	0.435	0.705
	FMD+BT	2.664	0.313	
Serotype A/TUR16 Dpv 30	FMD	2.257	0.397	0.706
	FMD+BT	2.204	0.477	
Serotype A/TUR16 Dpv 60	FMD	2.167	0.475	0.076
	FMD+BT	2.408	0.352	
Serotype O Dpv 30	FMD	2.054	0.347	0.512
	FMD+BT	2.105	0.474	
Serotype O Dpv 60	FMD	2.054	0.395	0.714
	FMD+BT	2.122	0.297	
Serotype Asia 1 Dpv 30	FMD	1.635	0.367	0.839
	FMD+BT	1.612	0.349	
Serotype Asia 1 Dpv 60	FMD	1.795	0.263	0.167
	FMD+BT	1.918	0.289	
BT c-ELISA/VNT	**Group**	**Arithmetic mean**	**Standart deviation**	**p-value**
BT c-ELISA Dpv 30	BT	48.247	19.294	0.805
	FMD+BT	49.731	15.301	
BT c-ELISA Dpv 60	BT	42.813	20.039	0.799
	FMD+BT	44.696	21.987	
BT VNT Dpv 30	BT	1.737	0.4150	0.691
	FMD+BT	1.680	0.458	
BT VNT Dpv 60	BT	1.401	0.337	0.118
	FMD+BT	1.577	0.329	

At 30 and 60 dpv, the mean OD values in Group 2 (42.81-48.24) were higher than they were in Group 3 (44.69-49.73) (Fig. 7). This difference between the mean antibody titers in the two groups was found to be statistically insignificant ($p > 0.05$) (Tab. 2).

VNT results for FMD. The protective cut-off value for serotypes A/NEP 84, A/TUR 16, O/TUR 07, and

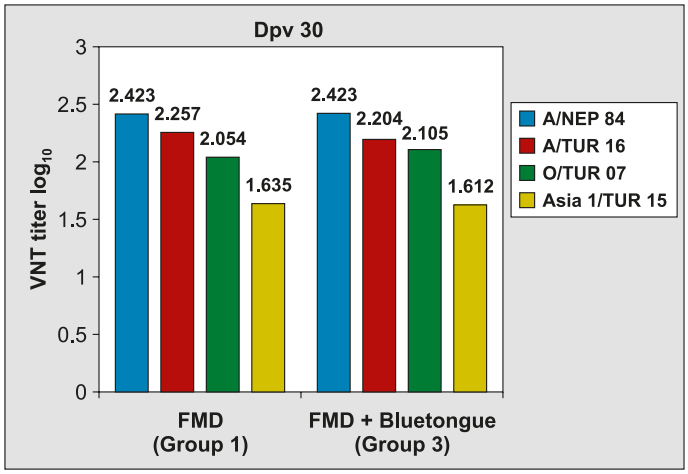


Fig. 4. The post-vaccinal arithmetic means of VN antibody titers against FMD serotypes A/NEP 84, A/TUR 16, O/TUR 07, Asia 1/TUR 15 for the experiment groups at 30th Dpv

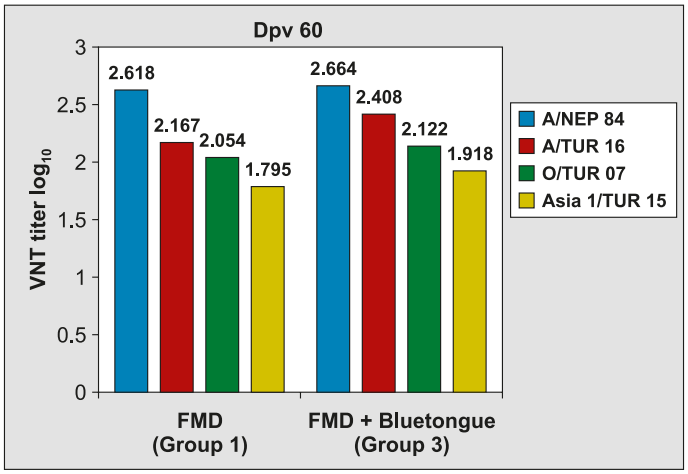


Fig. 5. The post-vaccinal arithmetic means of VN antibody titers against FMD serotypes A/NEP 84, A/TUR 16, O/TUR 07, Asia 1/TUR 15 for the experiment groups at 60th Dpv

Asia 1/TUR 15 was accepted to be ≥ 1/22. Antibody titers in Group 1 and Group 3 were found to be positive (≥ 1/22) at 30 and 60 dpv. The titers ranged from 1/22 ($\log_{10}$ 1.34) to 1/1445 ($\log_{10}$ 3.15), and all animals in the control group were negative (< 1/22). Antibody titers against all FMDV serotypes ranged from 1/22 to 1/1445 at 30 and 60 dpv in Group 1. In Group 3, the antibody titers of all FMDV serotypes ranged from 1/22 to 1/1445 at 30 dpv and from 1/45 to 1/1445 at 60 dpv.

In both groups, at 30 and 60 dpv, the A/NEP 84 and A/TUR 16 titers were higher than those of the other serotypes, followed by O/TUR/07 and Asia 1/TUR 15. VNT titers in Group 3 were higher at 60 dpv than titers against all FMDV serotypes at 30 dpv. Furthermore, in Group 1, A/NEP 84 and Asia 1 serotype antibody titers increased at 60 dpv, while O/TUR 07 serotype titers remained the same in both groups at 30 and 60 dpv (Fig. 4, Fig. 5).

VNT titer results varied between the two groups. The A/NEP 84 titer was found to be equal in both groups,

and the A/TUR 16 titer was higher in Group 1 at 30 dpv. Group 3 had higher FMDV O serotype titers than Group 1 at 30 dpv. At 60 dpv, VNT titers in Group 3 were higher than in Group 1 for all serotypes (Fig. 4, Fig. 5). This difference between the mean antibody titers (log_{10}) in the two groups was statistically insignificant for all FMDV serotypes ($p > 0.05$) at 30 and 60 dpv (Tab. 2).

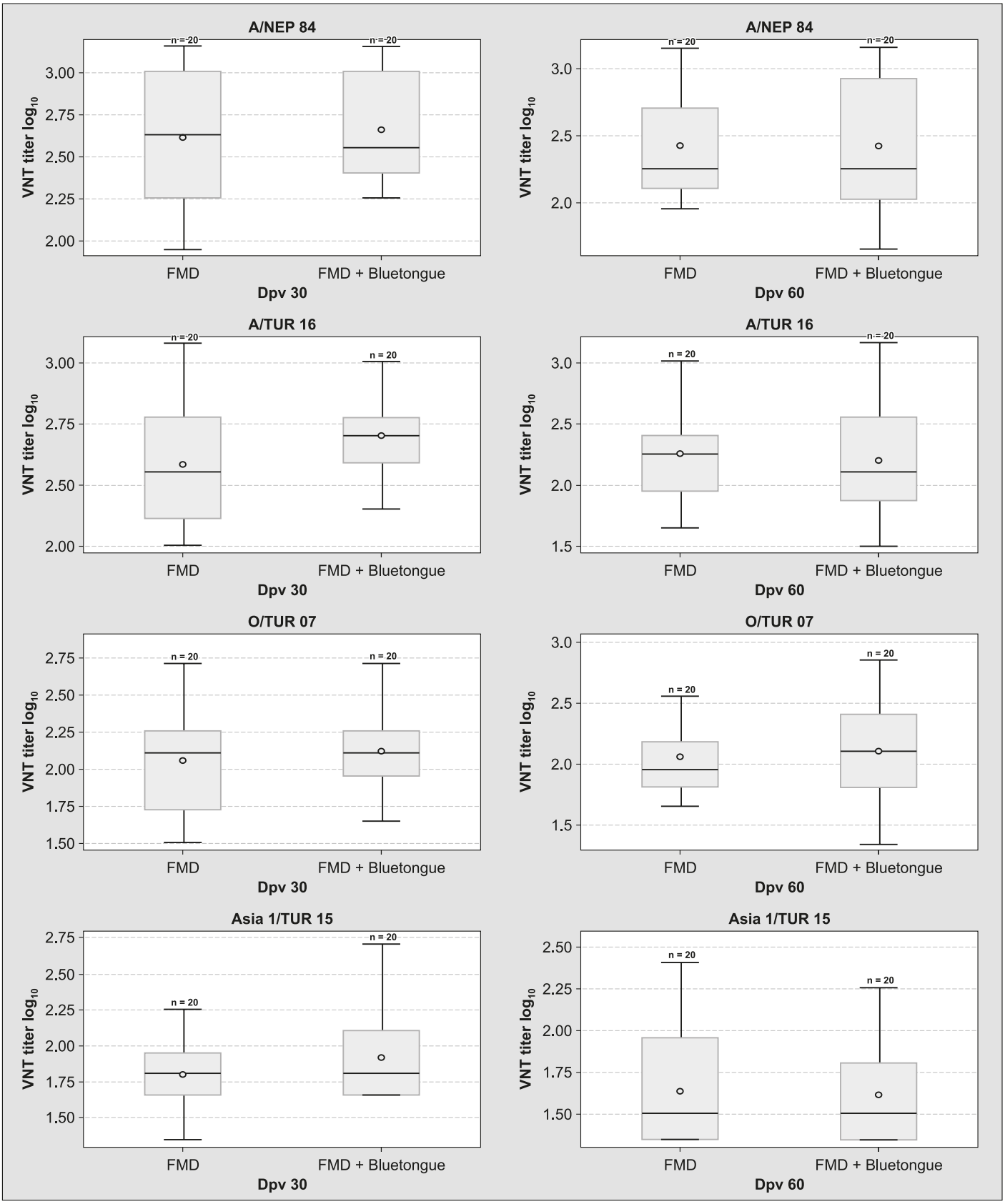


Fig. 6. Boxplots showing VN antibody titers for FMD serotypes A/NEP 84, A/TUR 16, O/TUR 07, ASIA 1/TUR 15. Boxplots show the median (horizontal line), interquartile range (box) and range (whiskers). Group sample sizes are shown above each box and individual data points are marked as grey vertical dashes. Titres below the detection threshold were given a value of zero

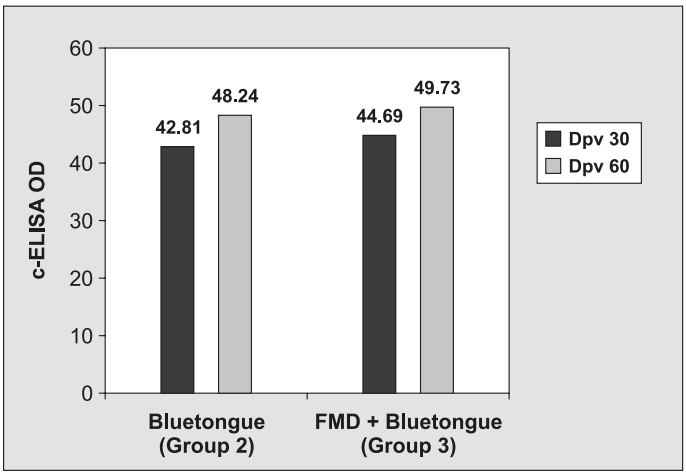


Fig. 7. The post-vaccinal competetive ELISA (c-ELISA) OD titers against serotype BTV-4 on the 30th and 60th Dpv

FMD is a major concern in the cattle and sheep/goat breeding industries because it causes significant economic losses (approximately $15.6 per animal) and remains endemic in Turkey and other countries (24, 31). In Turkey, a monovalent live attenuated lyophilized BT vaccine prepared from the BTV-4 strain is used for the control of BT disease. In order to reduce or minimize the incidence of the disease, quarantine methods and a regular and strict program of vaccination with vaccines specific to the type and subtype of the virus circulating in the field should be applied. To date, many conventional inactivated and attenuated vaccines have been routinely used in vaccination campaigns (20, 22). Many programs of vaccination against different pathogens in the field coincide within the same period. Simultaneous administration

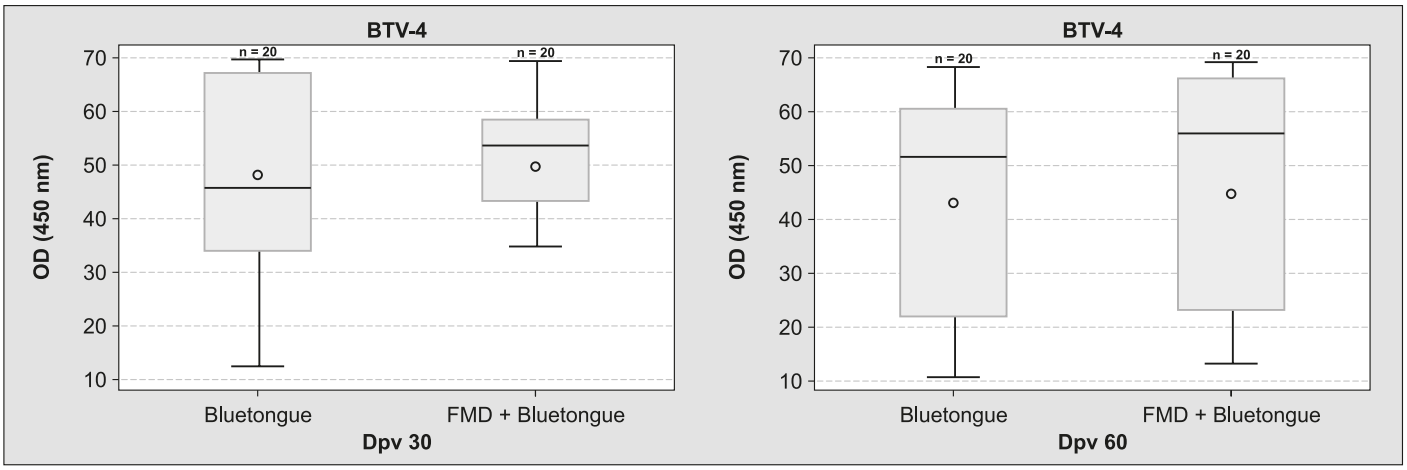


Fig. 8. Boxplots showing c-ELISA OD value. Boxplots show the median (horizontal line), interquartile range (box) and range (whiskers). Group sample sizes are shown above each box and individual data points are marked as grey vertical dashes. Titres below the detection threshold were given a value of zero

VNT results for BT. The cut-off value was accepted as ≥ 1/7.5. The antibody titers of positive animals in Group 2 and Group 3 ranged from 1/7.5 ($\log_{10}$ 0.87) to 1/160 ($\log_{10}$ 2.20). At 30 dpv, antibody titers in Group 2 and Group 3 ranged from 1/10 to 1/160, and at 60 dpv the titers ranged 1/7.5 to 1/160.

According to the VNT results, antibody titers of 19 (95%) out of 20 animals were at or above the cut-off value in both Group 2 and Group 3 at 30 dpv. One animal in each group was found to be negative. At 60 dpv, 19 (95%) out of 20 animals were found to be positive in Group 2, whereas 18 (90%) out of 20 animals were positive in Group 3.

The mean antibody titer of Group 2 was higher than that of Group 3 at 30 dpv. However, the VNT titers in Group 3 were higher than those in Group 2 at 60 dpv (Fig. 9). This difference in mean antibody titers between the two groups was found to be statistically insignificant for all FMDV serotypes at 30 and 60 dpv ($p > 0.05$) (Tab. 2).

Statistical analyses. All comparisons revealed statistically insignificant differences between the groups ($p > 0.05$) (Tab. 2).

of different vaccines saves labor and improves animal welfare.

Simultaneous vaccination has been studied and implemented to combat animal diseases in Turkey and other countries (1, 4, 6, 13, 18, 21, 33, 34). This research was conducted to determine the level of immunity to FMD and BT after the simultaneous administration of an inactivated FMD vaccine and a live attenuated BT vaccine, which must be administered annually in vaccination programs in Turkey.

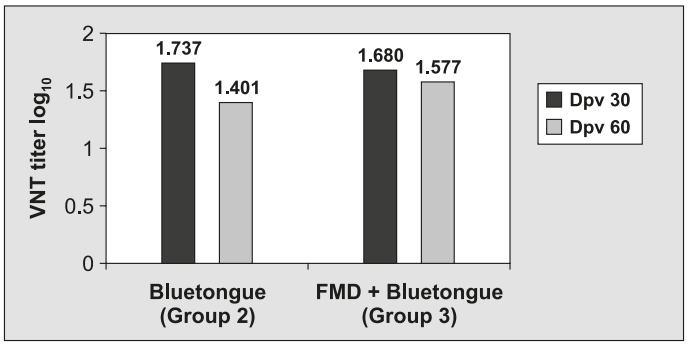


Fig. 9. The post-vaccinal arithmetic means of VN antibody titers against serotype BTV-4 on the 30th and 60th Dpv

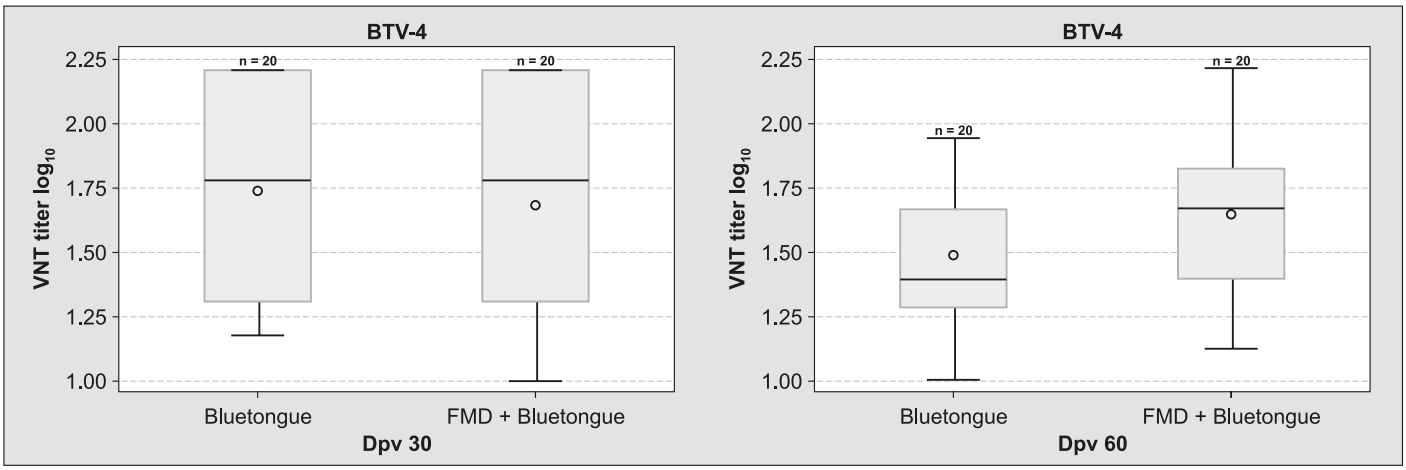


Fig. 10. Boxplots showing VNT titers for BTV-4 serotype. Boxplots show the median (horizontal line), interquartile range (box) and range (whiskers). Group sample sizes are shown above each box and individual data points are marked as grey vertical dashes. Titres below the detection threshold were given a value of zero

In this study, FMD antibody titer values in both LPBE and VNT (LPBE: 1 : 96-$\log_{10}$ 1.98; VNT: 1 : 22-$\log_{10}$ 1.34) obtained in Group 1 and Group 3 were found to be consistent with titer values ($\log_{10}$ $TCID_{50}$ 1.20 and 1.04 against serotypes A and O, respectively) reported by other researchers (5, 11, 30).

As reported in other studies (14, 17, 23, 26) that used a high antigen content and oil adjuvants in FMD vaccines, FMD antibody titers were higher at 60 dpv than they were at 30 dpv in both groups, as determined by LPBE, and in the group vaccinated simultaneously, as determined by VNT. In addition, in both groups, the parallel increase in FMD antibody titers at 30 and 60 dpv across all serotypes indicates that the BT vaccine had no negative effect on FMD antibody titers.

In both LPBE and VNT, the highest antibody titers were found for A serotypes (A/NEP 84 and A/TUR 16) in both groups, while other serotypes showed variability within the groups. Lombard (19), in a study investigating the efficacy of FMD vaccines, reported that the difference in antibody response between serotypes was due to varying amounts of antigen, and emphasized that the immunological potency of each serotype is different (7). In this study, it has been concluded that the reason for the different antibody responses of the serotypes is related to the use of different amounts of antigen in the FMD vaccine and the varying immunological potencies of the serotypes, as suggested by the researchers (7, 19).

The European Medicines Agency (EMA) (8) has reported that concomitant administration of two or more vaccines may result in an increase or decrease in immunogenicity compared to a single vaccine. In this study, at 30 dpv in LPBE and at 60 dpv in VNT, the titers of the A/NEP/84, A/TUR/16, O/TUR/07, and Asia 1/TUR/15 serotypes of FMD in the group vaccinated simultaneously against FMD and BT (Group 3) were higher than those in the group vaccinated against FMD alone (Group 1).

Hedger et al. (15) and Gullemin et al. (10) administered inactivated FMD and attenuated rinderpest vaccine simultaneously and separately to cattle. It was reported that antibody titers in the group vaccinated simultaneously were comparable with titers in the group in which the vaccines were administered alone.

Trotta et al. (34) used inactivated FMD and attenuated live anthrax vaccines simultaneously in cattle. According to the results of that study, total antibody response to the O_1 Campos strain was higher in the group vaccinated simultaneously. In another study, Çokçalışkan et al. (4) administered inactivated FMD and attenuated live anthrax vaccines simultaneously to sheep. In the group vaccinated simultaneously, FMD titers were significantly higher than they were in the group vaccinated against FMD alone at 7 dpv (4). Trotta et al. (34) commented that the cause of the increase in FMD antibodies was a cytokine increase due to the live anthrax vaccine.

In this study, the group in which the FMD vaccine was administered alone (Group 1) and the group in which the FMD and BT vaccines were administered together (Group 3) were compared in terms of FMD antibody titers. At 30 dpv in LPBE and at 60 dpv in VNT, the titers of the A/NEP/84, A/TUR/16, O/TUR/07, and Asia 1/TUR/15 serotypes in group vaccinated simultaneously were higher than those in the group vaccinated against FMD alone. In this study, it was found that FMD antibody titers were higher in the group in which FMD and BT vaccines were administered together. This result was interpreted as the live attenuated BT vaccine potentially eliciting a stronger immune response by stimulating cellular immunity, as suggested by other researchers (4, 34).

The differences between FMD antibody titers for the groups vaccinated against FMD alone and against both FMD and BT together were found to be statistically insignificant ($p > 0.05$). As reported in other studies (1, 2, 4, 13, 15), in which FMD antibody titers did not decrease when an FMD vaccine was administered with

other vaccines, in this study as well there was no loss in the FMD antibody titer when the FMD vaccine was administered together with the BT vaccine.

It has been reported that each serotype of BTV produced a different immunological response after the vaccination of sheep against BT (16). In a study by Savini et al. (31), 36 out of 44 animals vaccinated with bivalent attenuated BT vaccine containing BTV-2 and BTV-9 serotypes developed neutralizing antibodies against both serotypes, whereas neutralizing antibody response did not occur in 4 sheep. In our study, c-ELISA results showed that 17 (85%) out of 20 sheep vaccinated against BT alone (Group 2) and against FMD and BT together (Group 3) were positive at 30 and 60 dpv, while three were negative. VNT results showed that in both groups, 19 (95%) out of 20 sheep were positive at 30 and 60 dpv. In Group 2, 19 (95%) of the 20 sheep were positive for BT antibodies, while in Group 3, 18 (90%) of the 20 sheep were positive for BT antibodies. Individual immune responses can explain negative results in some animals, as reported by other researchers (31).

In a study conducted by Zhugunissov et al. (36) to determine the duration of the protective immune response after a single vaccination against BTV-4 and BTV-16 serotypes in sheep, antibody titers after vaccination were evaluated by serum neutralization (SNT) and c-ELISA tests. The researchers reported that the neutralizing antibody titers (log_{10}) ranged from 1.1 to 1.25 in all animals 1 week after vaccination, while the highest antibody titers (4.0-4.8 log_2) were noted 4 weeks after vaccination. In our study, BT antibody titers at 30 dpv (log_{10} 1.73-1.40) and 60 dpv (log_{10} 1.68-1.57) in Group 2 and Group 3 were similar to those reported by other researchers (36). In group comparisons for BT antibody titers at 30 and 60 dpv, antibody titers were higher with the c-ELISA test in Group 2. According to VNT, BT antibody titers were higher in Group 2 at 30 dpv, whereas at 60 dpv, BT titers were higher in Group 3.

Differences between BT antibody titers for the group vaccinated against BT alone and the group vaccinated against FMD and BT together were found to be statistically insignificant ($p > 0.05$). Since the difference in BT titers between the groups (Group 2 and Group 3) was statistically insignificant, it was concluded that the FMD vaccine did not have a negative effect on the level of BT antibodies.

In conclusion, when administered concurrently to sheep, the inactivated FMD and live attenuated BT vaccines did not inhibit the humoral immune response to either vaccine. The antibody values in the groups were found to be comparable, which suggests that the two vaccines could be safely administered together in sheep. The data obtained from this research will be useful for overcoming problems, such as economic losses and reduced animal welfare, arising from vaccinations at different times, especially in endemic regions where FMD vaccination campaigns are mandatory.

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Corresponding author: Veli Gülyaz, Prof. Dr. DVM, Harran University, Faculty of Veterinary Medicine, Department of Virology, Akçakale Caddesi Eyyübiye Yerleşkesi 255 AN, 63200 Eyyübiye/Şanlıurfa, Türkiye; e-mail: veli.gulyaz@harran.edu.tr, veligulyaz@yahoo.co.uk