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Original Article
Özgün Araştırma

The use of rabbits in studies of immunity and safety of Contagious Ecthyma (CE) vaccine

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Abstract: Contagious ecthyma (CE) is a zoonotic viral infection and common localized cutaneous infection of young sheep and goats caused by a Parapoxvirus with worldwide distribution. The aim of this study was to determine whether rabbits will be used instead of lambs and kids in immune and safety studies after the production of CE vaccine. The titres of Pendik CE pathogen strain isolated from lamb ($E(P)CK_4$) and attenuated vaccine strain originated from lamb ($E(P)CK_{22}$) used in the study were $TCID_{50}$ $10^{6.50}$ and $10^{7.00}$/ml, respectively. In the study, to determine the pathogenicity of CE virus ($E(P)CK_4$) in rabbits, it was found that CE virus-specific hyperemia, vesicles and pustules were not observed and there was no increase in body temperature, and CE virus was not detected by PCR in the scabs located in the skin of back-waist regions. In the blood sera of rabbits treated with pathogen CE virus and vaccinated with CE vaccine virus, no antibodies were detected against CE virus. In the immunity study in lambs and kids vaccinated with CE vaccine ($E(P)CK_{22}$), it was found that the vesicles, pustules and scabs appeared on day 3 and the lesions healed on the 15th day seen that CE vaccine virus protects lambs and kids against pathogen CE ($E(P)CK_4$) strain.

Key words: Contagious ecthyma, immunity, kid, lamb, rabbit, vaccine.

Contagious Ektima (CE) aşısının bağışıklık ve zararsızlık çalışmalarında tavşanların kullanılması

Özet: Contagious Ektima (CE) hastalığı, genç koyun ve oğlaklarda lokalize kutanöz enfeksiyonlara sebebiyet veren, dünyada yaygın olarak bulunan Parapoxvirusların neden olduğu zoonotik viral bir enfeksiyondur. Bu çalışmanın amacı, CE aşısının üretilmesinden sonra bağışıklık ve güvenlik çalışmalarında kuzu ve oğlakların yerine tavşan kullanmaktı. Çalışmada kullanılan kuzulardan izole edilmiş patojen Pendik CE suşu ($E(P)CK_4$) ve attenüe olan ($E(P)CK_{22}$) aşı suşunun titrelerinin sırasıyla $DKID_{50}$ $10^{6.50}$ ve $10^{7.00}$/ml olduğu belirlendi. Tavşanlarda CE virusunun ($E(P)CK_4$) patojenitesini belirleme çalışmasında, CE virusuna özgü hiperemi, veziküller ve püstüller gözlemlenmedi, vücut sıcaklıklarında artış olmadı ve bel bölgesinde bulunan yara kabuklarında CE virusu PCR ile tespit edilmedi. Patojen CE virusu uygulanan ve CE aşı virusu ile aşılanan tavşanların kan serumlarında, CE virusuna karşı antikor tespit edilmedi. CE aşısı ($E(P)CK_{22}$) ile aşılanmış kuzu ve oğlaklarda yapılan bağışıklık çalışmasında, skarifikasyon yapılan deri bölgesinde veziküllerin, püstüllerin ve kabukların 3. günde ortaya çıktığı ve lezyonların 15. günde iyileştiği tespit edildi. CE aşı virusunun kuzu ve oğlakları patojen CE ($E(P)CK_4$) virusuna karşı koruduğu saptandı.

Anahtar kelimeler: Aşı, bağışıklık, contagious ektima, kuzu, oğlak, tavşan.

Introduction

Parapoxvirus infections (PPV) occur in sheep, goats, deer and humans also known as contagious ecthyma, orf, sore mouth, scabby mouth, contagious pustular dermatitis, dermatitis pustulosa of small ruminants, ulcerative dermatosis of sheep, lip and leg ulceration, veneral balanoposthitis and vulvitis, lippengrind infection disease (Rabinson and Balassu, 1981; Mc keever et al., 1988; Nourani H and Maleki M, 2006). Orf virus (ORFV) is a parapoxvirus (subfamily Chordopoxvirinae,family Poxviridae) with ovoid-shaped virions of ~ 260 by 160 nm and a linear double-stranded DNA genome of 134-139 kb.(Da Costa et al. 2019). Small ruminants, wild ruminants, ibex, llama are susceptible to CE virus. The disease has a zoonotic potential particularly for those who are in close contact with animals such as veterinarians, farmers, animal attendants, and visitors (Bala et al. 2018).

The disease has been considered benign, the lesions are painful and can lead to anorexia and starvation. The outbreaks generate economic losses associated with a growth delay and deterioration of the animal's body condition (Nourani and Maleki,

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2006; Peralta et al. 2018). In case of secondary infections with other viruses and bacteria, the mortality rate increase from 10 to 20 % (Sowmiya et al. 2018). CE virus is transmitted through direct contact with infected animals and the virus which is highly resistant and can survive in the environment for more than one year (Sadıq et al, 2017; Al Saad et al. 2017; Tedla et al. 2018). In case of secondary infections, mortality rate is between 20-50%. There are genetic variations between CE goat and lamb field strain (Lin et al. 2015).

Within the scope of the fight against CE disease, newly purchased animals should be kept under quarantine before being brought to the farm. Animals should be disease free in clinical examination. Animals should be vaccinated with CE vaccines in the fight against CE disease. There are some kind of vaccine such as live attenuated in tissue culture and not fully attenuated and also produced in sheep are used for the prevention of CE disease, (Pye D, 1990; Musser et al. 2012).

The aim of this project was to investigate the possibility of using rabbits instead of lambs and kids in immunity and harmlessness studies of post-production CE vaccine.

Materials and Methods

***Contagious ecthyma (CE) virus strains*:** CE vaccine strain (E(P)CK_{22}) and pathogen strain (E(P)CK_4) were obtained from Pendik Veterinary Control Institute.

Cell culture: Madin Darby Bovine Kidney (MDBK) cell culture used for the production of viruses and serum neutralization tests were obtained from the cell culture centre of The Foot and Mouth Disease Institute. In order to propagate MDBK cells, fetal calf serum inactivated at 56°C for 30 minutes (BIOCHROM-cat no:S-0125) was added to DMEM/Ham's-F-12 (BIOCHROM-cat no:F 4815) medium as 10% and 2% (Burleson et al. 1992).

DNA isolation and cleaning kits: DNA isolation of CE virus was performed with the DNeasy Blood & Tissue Kit (QIAGEN, cat no: 69506) and in accordance with the protocols specified in the kit. The PCR Purification kit (Min Elute®, QIAGEN, cat no. 28004) was used for clearance of pre-sequence PCR products according to the protocols specified by the company (Chan et al. 2007).

Primers: For the identification of the CE vaccine and field (challande) virus, sequence primers belonging to the CE virus were selected from the reference genes in the NCBI gene bank (Table 1) and designed for DNA sequencing of the entire B2L gene using the Prim Select mode of the DNASTAR gene analysis program (Nettleton et al. 1996).

Table 1: Primers used for DNA sequencing of the B2L gene.

Primary name	Sense	Primary sequencing (5-3)	MER	Product length
B2LF1	+	AGA ACT CGC CCG CCT GCT AAA AGA	24	660 bp
B2LR2	-	CCC CGG AGT GGT CGA GGT GGA AGT	24	
B2LF2	+	CAA GCA CCT GGC CTG GGA CCT CAT	24	713 bp
B2LR3	-	GCT TGC GGG CGT TCG GAC CTT C	22	
B2LF3	+	GCA CCG CAT CGA GAA CGC CAA GAA	24	574 bp
B2LR4	-	AGG GAC GCC GCC GCA CAC C	19	
B2LF4	+	GAA GAA CTC GCC CGC CTG CTA AAA	24	346 bp
B2LR5	-	GTC CGC GTC CTT GTC CTT GCT CTG	24	

Propagation of cells: MDBK cell culture was produced with DMEM/Ham's-F-12 medium containing 10 % FCS in 25 cm^2 flask at 37°C and 5 % CO_2 as monolayer.

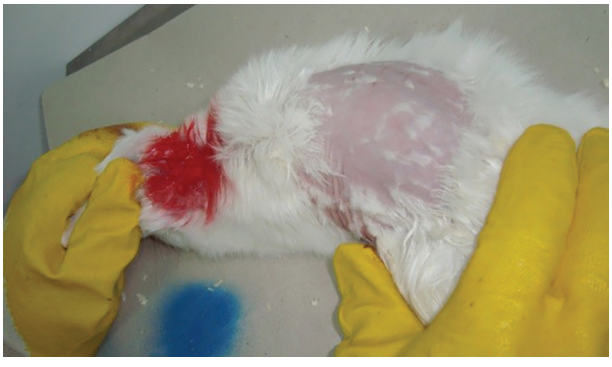
Propagation of pathogen CE virus (E(P)CK_4): 100µl CE virus at 4th passage level was inoculated in to the monolayer MDBK cell culture produced in 75 cm^2 flask and was incubated at 37°C and 5 % CO_2. Cell culture was observed daily for CPE formation and at 90 % CPE formation, the flasks were frozen and thawed at -70°C. The cell debris was removed by centrifugation at 3000 rpm for 30 minutes and stored at -70°C until use (Burleson et al. 1992).

Detection of infective titer of pathogen CE virus: Microvirus titration test was performed to determine the $TCID_{50}$/ml of the produced CE virus. Ten-fold dilutions of the CE virus were made with PBS from log_{10} 10-1 to 10-6. The 100 µl of each dilution was

placed in four wells of 96-well plate. 50 µl of MDBK cell culture (3-5x10^5 cells/ml) was added to all dilutions. 100 µl of medium and 50 µl of cell suspension were placed in the last four wells of the plate for cell control. The plate was incubated at 37°C with 5 % CO^2 for 10 days and checked daily. Formation of CPE due to virus growth was observed. $TCID_{50}$ values of the produced virus were determined by the Spermen-Kaerber method (Ergin and Köklü 1974; Nashirudddullah et al. 2016).

Pathogenicity study with pathogen CE virus (E(P) CK_4) in rabbits, lambs, and kids: 1 ml of CE virus field isolate was taken and diluted with dilution liquid containing 9 ml of 50% glycerin+50% PBS used for this purpose.

Pathogenicity study in rabbits: For this purpose, 10 rabbits were shaved at the waist skin and CE virus field isolates were inoculated 8 rabbits by scarification method (Picture 1).



Picture 1. Skin appearance of rabbits after challenge

Pathogenicity study in lambs and kids: Six lambs and kids were shaved on the inside of the hind legs and 4 lambs and 4 kids were given CE virus field isolates by scarification method (Yirrell et al. 1989; Housawi et al. 1993; Zamri et al. 1994). As a control group, two rabbits, 2 kids and 2 lambs were inoculated only with PBS + glycerin mixture by the scarification method.

Vaccination of rabbits, lambs and kids with CE vaccine: Before the study, blood samples were taken from 22 kids aged 2-3 months which had no CE disease in the previous years and did not show any signs of CE disease and they were checked by serum neutralization test (SNT) for carrying antibodies against CE virus. The kids were quarantined for 10 days before being tested. Their daily body temperature was recorded. Kids were observed during the quarantine for clinical signs of CE and other diseases (Buddle and Pulford, 1984; Pye, 1990; Musser et al. 2012).

Vaccination of rabbits: The CE vaccine was diluted with 40 ml of PBS + glycerin diluent. The waist area of 8 rabbits was shaved and the skin was scarified diagonally by 3 lines of 0.5-1 cm diameter with a needle deep enough to cross the first layer of the skin. 3 drops of diluted CE vaccine were applied to the scarified areas and waited for 3-4 seconds to complete the vaccination. Two rabbits were used as control (mock) by dropping PBS + glycerin mixture to the scarified skin.

Vaccination of lambs and kids: Lyophilized CE vaccine was diluted with 40 ml of PBS + glycerin diluent. The shaved skin area on the inside of the hind legs of 4 lambs and 4 kids was scarified diagonally by 3 lines of 0.5-1 cm diameter with a needle deep enough to cross the first layer of the skin and 3 drops of diluted CE vaccine were applied to the scarified areas. As for 2 lambs and 2 kids (Mock) used as controls in challenge studies, 3 drops of PBS + glycerin mixture were applied to the scarified areas and were waited for 3-4 seconds before releasing the animals.

Immune controls in vaccinated rabbits, lambs and kids: Body temperatures of animals vaccinated with CE vaccine were measured for 15 days. The vaccination site was checked for signs of necrosis, hyperemia and CE disease.

Determination of antibody titers against CE virus in lambs and kids: Thirty (30) days after the CE vaccination by scarification method, blood was taken from vaccinated lambs, goats, and rabbits, as well as the controls, to investigate antibody titers against CE virus (Mc Keever et al. 1987).

Serum Neutralization test (SNT): In order to determine the antibody titer levels against CE virus by SNT, 50 µl of DMEM /F12 Ham's medium was placed in all wells of 96-well plates. 50 µl of the blood serum was placed in the first two wells of the plate, and 50 µl of the first wells were transferred to the lower wells to make the two-fold dilutions of the serum. Standard CE virus (E(P)CK_4) strain in 50 µl of 100 $TCID_{50}$ titer was added to two-fold dilution of the serum samples in the wells and let them to neutralize for one hour at 37°C. At the end of the incubation period, 50 µl of MDBK cell culture was added to all wells and incubated in 5% CO_2 medium for 10 days at 37°C. Cells were checked daily for CPE formation (Nashirudddullah et al. 2016).

Determination of immunity to CE virus after vaccination in rabbits, lambs and kids: 10 ml of pathogen CE strain was diluted with 10 ml of PBS + glycerin diluent. Thirty days after the CE vaccina-

tion, 8 vaccinated rabbits from their back, 4 vaccinated lambs, 4 vaccinated kids, and 2 for each of Mock rabbits, lambs and kids were challenged with 3 drops at their previously shaved and scarified skin. Body temperature of rabbits, lambs and kids were measured daily for 15 days. Following the challenge, control animals challenged simultaneously with vaccinated lambs were examined for vesicles, pustules and later crustation in the scarification areas of their skins (Buddle and Pulford, 1984; Pye, 1990; Musser et al. 2012).

Investigation of CE virus by PCR in skin lesions after scarification in rabbits.

DNA isolation: Following the isolation of reference CE vaccine strain E(P)CK_5 in the cell culture as positive control and the inoculation to rabbits, DNA isolation from crustation on the skin was performed using High pure viral nucleic acid kit (Roche, cat no: 11796823001) and in accordance with the protocols specified in the kit. Briefly, 200 µl of cell culture supernatant, 200 µl binding buffer and 40 µl of Proteinase K mixture were incubated for 10 min at 70°C (rabbit's skin scrapings were kept in 200 µl of binding buffer for 2 hours at 70°C, then the protocol was continued), 100 µl isopropanol was added, and the whole was transferred to filtered columns. It was centrifuged at 8000 g for 1 minute, 500 µl inhibitor removal solution was added to the column and it was centrifuged again in the same way. 500 µl of washing solution was added and centrifuged, and finally, 200 µl of elution solution was added to the column and centrifuged in the same way to obtain DNA samples and stored at -20°C until use. The primers used in the study (Table 1) were selected and optimized from the B2L gene of CE virus.

PCR: In our PCR studies for isolation of target regions, 5 µl template DNA, 10 µl 10X buffer, 1 µl dNTP (0.8µM), 1 µl primer F and primer R (20pmol), 0.5 µl Taq DNA Polymerase (2.5U), and ddH_2O was added to obtain a total volume of 50 µl. Following 3 minutes at 94°C; 1 minute at 94°C, 45 seconds at 53°C, and 1 minute at 72ºC for 30 cycles; and finally 7 minutes at 72°C in the thermal cycler, the PCR products were electrophoresed below 100V in 2% agarose gel and DNA bands were observed in UV medium.

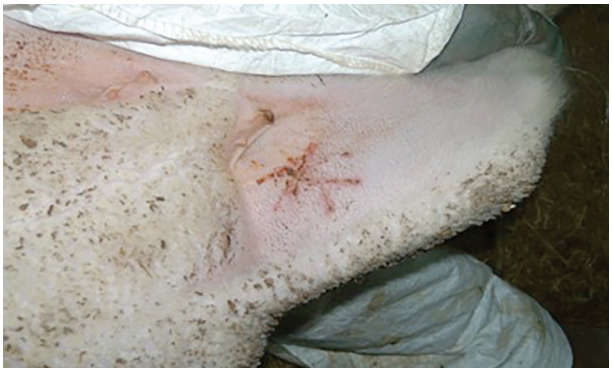
Results

The pathogen CE virus (E(P)CK_4) titer $TCID_{50}$ was found to be $10^{7.0}$/ml, while the vaccine virus titer was $TCID_{50}$ $10^{6.5}$/ml. No antibodies against CE virus were detected in the blood sera taken from the lambs and kids before the inoculation.

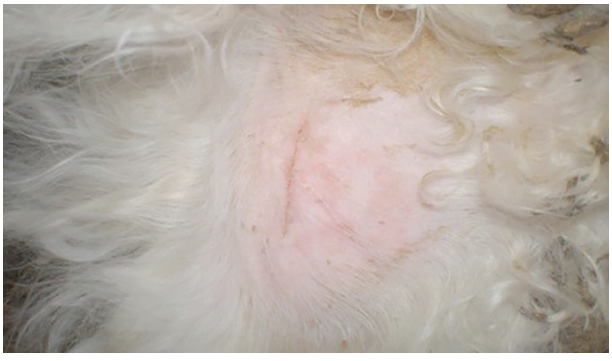
Pathogenicity study with pathogen CE virus in rabbits, lambs, and kids:

Pathogenicity study with pathogen CE virus in rabbits (Group 1): No hyperemia, pustule and crustation was detected due to CE virus growth in scarified skin areas after 15 days of clinical observation of 8 rabbits shaved at the back-waist area and scarified to administer CE virus field isolate and 2 control (mock) rabbits. In addition, there was no increase in body temperature for 15 days and no antibodies against CE virus were detected in blood sera.

Pathogenicity study with CE virus in lambs and kids (Group 2): Four lambs and 4 kids eprüvated with CE virus field isolates were detected not to have increase in body temperature (38.5-39.5°C) and to develop hyperemia, vesicles and pustules in scarified skin areas as from 2-3 days following the administiration in subsequent days (Picture 2, Picture 3). The lesions in lambs and kids were detected to heal in days 32-37. It was observed that the thin and small scabs were shed in 4-6 days as from day 2 and no lesion was remained on the scarified skin in lambs and kids used in two for each.

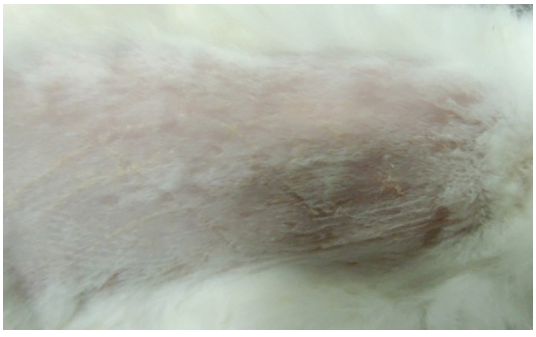


Picture 2. Skin lesions in lambs eprüvated with pathogen CE virus



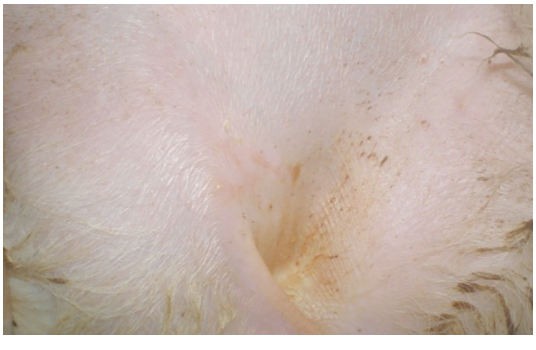
Picture 3. Skin lesions in kids eprüvated with pathogen CE virus

Determination of immunity to CE virus after vaccination in rabbits. Eight rabbits vaccinated with CE vaccine and 2 Mock rabbits were detected not to have increase in body temperature for 15 days following the challenge after scarification at the waist-back region. No antibodies against CE virus were detected in the blood sera of rabbits. No vesicles, pustules and later crustation were detected in the scarification areas on the skins of control rabbits applied simultaneously with rabbits vaccinated with CE vaccine (Picture 4).

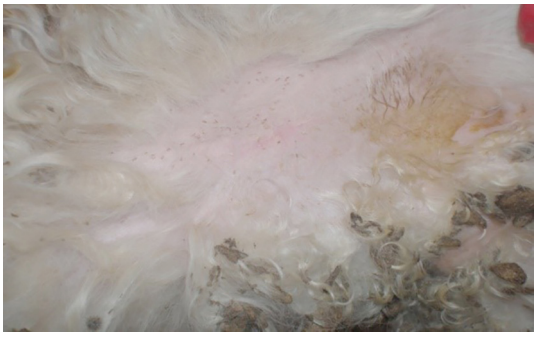


Picture 4. Skin appearance of rabbits eprüvated after CE vaccination

***Determination of immunity against CE virus after vaccination in kids and lambs*:** Following the challenge of vaccinated kids and lambs with pathogen CE virus (E(P)CK_4), they were detected not to have increase in their body temperature (38.1-39.2°C). Hyperemia was observed in scarified skin areas of kids and lambs as from the second day of the challenge and vesicles and pustules developed in subsequent days but healed in 9-11 days (Picture 5) (Picture 6). In 2 challenged control kids and lambs, skin lesions started to heal on day 28 and the scabs were shed on day 35. In negative control kids, it was observed that scabs occurred in day 2 and these thin and small scabs were shed in day 4 and no lesion was remained on the scarified skin

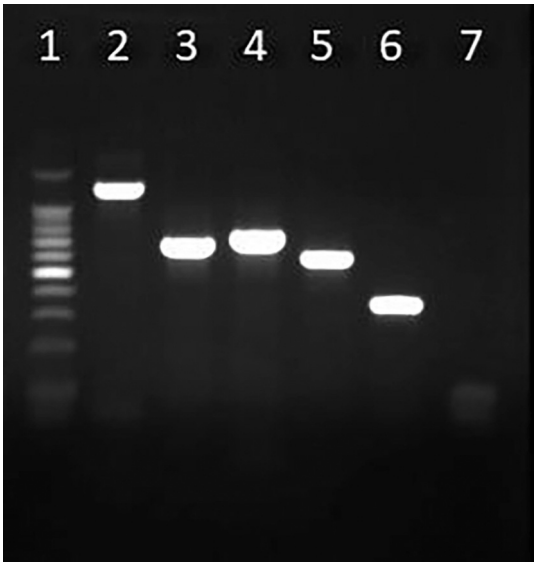


Picture 5. Skin appearance following eprüvated with CE field isolates in lambs vaccinated with CE vaccine



Picture 6. Skin appearance following eprüvated with CE field isolates in kids vaccinated with CE vaccine

Investigation of CE virus by PCR in scabs on the scarified skin after challenge in rabbits: No CE virus was detected by PCR test in the samples taken from the scabs on the skin of the rabbits after scarification with both CE field isolates and CE vaccine strain (Picture 7). The PCR products used in Samples 2-6 are PCR products of DNAs obtained from the E(P)CK_4 strain of CE virus (CEV). All primers had negative results with DNA isolated from rabbit scabs (Picture 7).



Picture 7. PCR results from rabbit scabs and different primers of the B2L gene region of the reference CEV strain

1: DNA ladder (Promega 100 bp), 2: CEV (Primers OVB2LF and OVB2LR, 1206 bp), 3: CEV (Primers B2LF1 and B2LR2, 660 bp), 4: CEV (Primers B2LF2 and B2LR3, 713 bp), 5: CEV (Primers B2LF3 and B2LR4, 574 bp), 6: CEV (Primers B2LF4 and B2LR5, 346 bp), 7: Rabbit crustation sample (primers OVB2LF and OVB2LR)

Discussion

Contagious ecthyma (CE) is a zoonotic viral infection of sheep and goats and live attenuated vaccines are used to protect animals from CE disease. This study was conducted to determine the usability of rabbits in the immunization studies of CE vaccine produced by public and private companies in our country for lambs and kids by conducting the immunization and harmlessness studies in rabbits together with lambs and kids.

In a study on the experimental infection of CE virus in rabbits and mice carried out by Cargnelutti et al. (2011) reported that ears, skin and labial commissure in rabbits, ears in mouse and labial commissure and inner hind legs in lambs were intradermally inoculated with $10^{8.5}$ $TCID_{50}$/ml of CE strain Iowa-82; animals were observed clinically, virologically, and pathologically; hyperemia, macule papules, vesicles, pustules and scabs occurred in 3-4 days and local lesions ended in 3-10 days; histological examination revealed focal proliferative dermatitis and balloon degeneration in keratinocytes; virus was recovered from lesions between days 2 and 14 pi.; intrastoplasmic inclusions were detected in eosinophils; similar lesions were detected in 5 out of 10 mice; clinical and histopathological lesions due to characteristic CE infection occurred in all lambs; and all rabbits were seropositive with ELISA but no neutralizing antibodies were detected on day 28. In our study conducted in rabbits, no hyperemia, macule papules, vesicles and pustules developed and no genomic material belonging to the CE virus was detected in scabs caused by scarification on the skin at the waist region of rabbits in pathogenicity tests performed with both pathogen and attenuated CE vaccine virus. Based on this data we can say that CE virus ($E(P)CK_4$) and ($E(P)CK_{22}$) could not adapt and grow in the skin cells of rabbits due to the application of scarification method administered to the scarified skin by dropping. In the study conducted by Cargnelutti et al. (2011), the pathogen CE virus isolate was directly injected intradermally into the skin and virus growth and skin lesions were observed on the skin of rabbits. In addition, the field virus used in our study is likely to be less pathogenic than the CE virus used by Cargnelutti (2011). It was concluded that it would be appropriate to repeat this study in rabbits by intradermal route reported by Cargnelutti et al. (2011).

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Author Contributions: VG and MH designed the experiments, VG, MH, SU and FS carried out the experiments, VG and MH analysed the data and wrote the paper.

Conflict of interest: The authors declare that they have no conflict of interest

Animal and Human Rights Statement This study was conducted under the supervision of the general directorate of agricultural research and policies of the ministry of agriculture and forestry. In addition, permission (85/09) was obtained from the ethics committee of experimental animals of Pendik Veterinary Control Institute.

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