Etlik Vet Mikrobiyol Derg, 2020; **31 (1):** 62-69
doi: 10.35864/evmd.699742

Original Article
Özgün Araştırma

Adaptation of Contagious ecthyma vaccine strain to MDBK cell culture and stability-immunity studies in lambs

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Geliş Tarihi / Received: 13.03.2020, **Kabul Tarihi** / Accepted: 12.05.2020

Abstract: Contagious ecthyma (CE) is a common viral infection in lambs and kids, and still maintains its importance in sheep and goat breeding. Attenuated, live and lyophilized CE vaccines adapted to cell culture are widely used in the fight against CE. The aim of this project was to adapt the CE vaccine strain to Madin Darby Bovine Kidney (MDBK) cell culture and to produce the vaccine, as well as determining the shelf life, stability, innocuity, and immune response in lambs.The titer of the vaccine virus adapted to the MDBK cell culture was determined to be $TCID_{50}$ $10^{6.5}$/ml. In the innocuity study on mice and guinea pigs, no local or general reactions were observed. Body temperature of 10 lambs vaccinated by scarification was found to be between normal values (38.2-39.1°C). Neutralizing antibodies belonging to CE virus with VNT could not be detected in blood sera taken on 30th day following the vaccination. As a result of challenge performed with pathogen (E(P)CK_5) CE virus, the protection was observed against CE infection in vaccinated lambs. In the stability study, the titers of lyophilized CE vaccine were found to be $TCID_{50}$ $10^{6.5}$/ml from 1 to 15 months and $10^{6.0}$/ml at 18, 21 and 24 months during the 24-month storage period at +4/+8°C. It was determined that the vaccine virus remained as stable at $TCID_{50}$ $10^{6.5}$/ml titer for 12 hours at 30, 33, 37 and 40°C and after 12 hours, the virus titer regressed to $TCID_{50}$ $10^{5.75}$/ml at 42°C and 45°C. This study concluded that CE vaccines could be produced in MDBK cell cultures by adapting CE virus to MDBK cell culture.

Key words: Contagious ecthyma, immunity, stability, vaccine, virus

Ecthyma contagiosa karşı MDBK hücre kültüründe aşı üretimi ve bağışıklık-stabilite çalışmaları

Özet: Contagious ecthyma (CE), kuzu ve oğlaklarda yaygın viral bir enfeksiyondur ve hala koyun ve keçi yetiştiriciliğinde önemini korumaktadır. Hücre kültürüne adapte canlı ve liyofilize CE aşıları mücadelede yaygın olarak kullanılmaktadır. Bu proje CE aşı suşunun Madin Darby Bovine Kidney (MDBK) hücre kültürüne adapte edilmesi ve MDBK hücre kültüründe üretimi yapılan CE aşısının raf ömrü, stabilite ve kuzularda bağışıklık düzeyinin saptanması amacıyla gerçekleştirildi. MDBK hücre kültürüne adapte edilen aşı virüsünün titresinin $DKID_{50}$ $10^{6.5}$/ml olduğu belirlendi. Fareler ve kobaylarda yapılan zararsızlık çalışmalarında lokal veya genel reaksiyonlar gözlenmedi. Scarifikayon yoluyla aşılanan 10 kuzunun vücut ısılarının normal değerler (38.2-39.1°C) arasında olduğu tespit edildi. Aşılamayı izleyen 30. günde alınan kan serumlarında VNT ile CE virüsüne karşı oluşan nötralize edici antikorlar tespit edilemedi. Patojen CE virüsü (E(P)CK_5) ile yapılan challenge sonucunda aşılanmış kuzuların CE enfeksiyonuna karşı korunduğu gözlendi.

Stabilite çalışmasında, +4 /+8°C'de 24 ay süreyle muhafaza edilen liyofilize CE aşısı titrelerinin 1-15. aylar arasında $DKID_{50}$ $10^{6.5}$/ ml ve 18, 21 ve 24 aylarda ise $10^{6.0}$/ml olduğu tespit edildi. Aşı virüsünün 30, 33, 37 ve 40°C'lerde $DKID_{50}$ $10^{6.5}$/ml titrede 12 saat stabil kaldığı ve 12 saat sonra virüs titresinin 42 ve 45°°C'lerde $DKID_{50}$ $10^{5.75}$/ml'ye gerilediği tespit edildi. Bu çalışma sonucu, CE aşı virüsünün MDBK hücre kültürüne adapte edilmesi ile CE aşılarının MDBK hücre kültürlerinde üretilebileceği sonucuna varmıştır.

Anahtar kelimeler: Aşı, bağışıklık, contagious ecthyma, stabilite, virus

Introduction

Also known as Orf, Ecthyma contagiosum, Sore mouth, Scabby mouth, Contagious pustular dermatitis, dermatitis pustulosa of small ruminants, ulcerative dermatosis of sheep, lip and leg ulceration is a viral disease caused by Parapoxvirus ovis (Rabinson and Balassu (1981). Parapoxvirus infections (PPV) are seen in sheep, goats, deer and humans (Mazur et al. 2000; Haig and Melnnes 2002). Contagious ecthyma (CE) is seen in South and North America, New Zealand, Netherlands, Germany, Norway, Japan, Italy, UK, Western Australia, South Asia, and our country (Nettleton et al. 1996; Hosamani et al. 2006). Contagious ecthyma virus (CEV) is morphologically and immunologically in close relationship with cattle's

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parapoxviruses and has a diameter of about 300-350 nm x 200-250 nm (Buddle et al. 1984; Mazur et al. 2000; Chan et al. 2007). As a result of genomic studies with DNA restriction enzymes, heterogeneity has been detected between strains. CE virus reproduces in ovine, bovine and human cell cultures. The best titer was obtained in the ovine primary cell lines derived from sheep for multiplication of the virus (Ivanov et al. 2016). The primary lamb kidney (PLK) and primary lamb testis (PLT) are the most commonly used cells for virus isolations (Ergin and Köklü 1973). Other cell lines include ovine testis cells (OA3.Ts), Vero, ovine organotypic skin culture, ovine fetal turbinate (OFTu) cells, BHK-21, and Madin-Darby ovine kidney cells (Scagliarini et al. 2005). The permanent cell lines also favor the multiplication of the virus, but titer yield is comparatively lower. Within 2-3 days, the virus titer reaches its maximum level and typical cytopathic effects such as rounding, pyknosis, ballooning and detachment of cells, are observed at the same time. (Karki et al. 2019)

The virus remains viable for many years especially in dried skin lesion scabs. In fact, it may remain viable for 17 days at room temperature in purified tissues. Virus is inactivated in 2 minutes at 64°C. Effective disinfectants include creolin and chloramine (Rabinson and Balassu 1981; Hussain and Burger 1989).

Small ruminants, wild ruminants, chamois, llamas and humans are sensitive to CE virus. There is no evidence of calves contracting the disease. The transmission of the disease can be by direct contact or indirectly by potable water or feed. The disease is common in sheep and goats, especially in dry seasons during the feeding period with dry herbs. The CE virus is an epitheliotropic virus that enters the damaged or scarified skin and reproduces in regenerative epidermal keratinocytes. The infection is usually acute and sometimes chronic infections have also been reported. In sheep and goats, CE is observed clinically in the form of macules, papules, vesicles, pustules and proliferative lesions in the mouth, nose, gums, oral mucosa, udders, coronary band of the feet and anus (Mc keever et al. 1988; Ameel et al. 1995; Nourani and Maleki 2006). Primary infections can be severe and common, and lesions usually resolve within 6-8 weeks. It can also be seen on the broken horn bottoms of animals (Haig and Melnnes 2002). Another feature of the disease is that it causes venereal balanoposthitis and vulvitis. CE infection is seen especially in spring and summer seasons, but it can be seen in lambs and kids in every season of the year. Its prevalence is low in old sheep and goats. This may be due to both the survival of the animals and the vaccination (Rabinson and Balassu 1981; Ergin and Köklü 1995). The reason for the disease to be seen in udders in sheep and goats can usually be the transmission of virus from mouth lesions while infected lambs and kids are breastfeeding. Incubation period varies from 24 to 72 hours in experimental infections. Morbidity is very high and can reach up to 100%. Mortality is up to 1% in cases where there is no complication. Mortality is 20-50% in case of secondary infections. Complications mostly involves Cochliomyia Americana and Fusobacterium Tunnicliffle (Rabinson and Balassu 1981; Sowmiya et al. 2018). In lambs, the disease occurs when the lambs that suck milk from infected udders receive the virus, and it spreads rapidly in the herd. Sheep are natural reservoirs of the disease. If sheep and llamas are kept together, llamas can get infected. For the diagnosis of the disease, virus isolation, PCR, electron microscopy, indirect immunofluorescence and other serological tests can be used in tissue cultures. Delayed hypersensitivity test is among sensitive tests (Ergin and Köklü 1973; Mondal et al. 2006).

For the prevention of disease, attenuated tissue culture and vaccines that are not fully attenuated and produced in sheep are used (Pye D 1990; Musser et al. 2012)

The aim of this study was to adapt the CE vaccine strain to Madin Darby Bovine Kidney (MDBK) cell culture and to produce the vaccine, as well as determining the shelf life, stability, innocuity, and immune response in lambs.

Materials and Methods

Contagious ecthyma (CE) virus strains: CE vaccine strain at 17th passage level ($EP(CK)_{17}$) and pathogen field virus ($EP(CK)_5$) propagated in primary calf kidney cell culture were obtained from the contagious ecthyma vaccine production laboratory in Pendik Veterinary Control Institute.

Cell culture: MDBK was obtained from Ankara foot and mouth disease (FMD) institute (Sap Enstitüsü). 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% for virus production (Burleson et al. 1992).

DNA Extraction Kit: Commercial viral DNA extraction kit was used in accordance with the protocol specified by the manufacturer (Roche, High Pure ViralNucleic Acid Kit, cat.no: 11858874001).

PCR: Pan-parapoxvirus primers were used to partially amplify the major envelope membrane protein gene (B2L) by semi-nested PCR (Table 1) (Inoshima et al. 2000; Sullivan et al. 1994). As positive control, CE vaccine virus was obtained from Viral Vaccines Production Laboratory at Pendik Veterinary Control institute. 2xPCR master mix by Norgen was used for semi-nested PCR (Norgen, cat.no: 28007).

Table 1. Primers used to partially amplify the B2L gene (Sullivan et al. 1994; Inoshima et al. 2000)

Primers	Primer sequence (5'-3')
PPP1	GTCGTCCACGATGAGCAGCT
PPP4	TACGTGGGAAGCGCCTCGCT
PPP4	GCGAGTCCGAGAAGAATACG

DNA extraction and PCR: 200 µl of the prepared inoculum was used for DNA extraction. DNA extraction was performed according to the kit protocol using reference CE vaccine virus DNA as positive control. To partially amplify the B2L gene, the master mix was done as shown in tables 2 and 3, and the temperature-time cycles were done as shown in table 4 (Inoshima et al. 2000).

Table 2. Master mix content round 1

1 Sample	Quantity (µl)
Enzyme	10
PPP1	0.5
PPP4	0.5
Nuclease-free water	6
Sample DNA quantity	3
TOTAL	**20 µl**

Table 3. Master mix content round 2

1 Sample	Quantity (µl)
Enzyme	10
PPP3	0.5
PPP4	0.5
Nuclease-free water	6
PCR product from the first round	3
TOTAL	**20 µl**

In the first and second round of amplification, a product of 594 bp and 235 bp was obtained respectively.

Preparation of MDBK cell culture: MDBK cell culture stored in nitrogen tank was rapidly dissolved in water bath at 37°C and diluted with DMEM-Ham's F-12 medium containing 10% FCS at 37°C to have $3x10^5$/ml cells. 7.5 ml of it was then placed in 25 cm^2 flasks**.** The flasks were incubated at 37°C and in 5% CO_2 medium for the formation of monolayer cell culture and the cells were checked for reproduction daily. MDBK cell culture produced was used as working cell culture (Ergin and Köklü 1975; Burleson et al. 1992).

Table 4. PCR temperature-time cycle

	Temperature (ºC)	Time	Number of cycles
Denaturation	94	2 min	1
PCR Amplification (a total of 40 cycles)			
Denaturation	94	20 sec	30
Binding	56	20 sec	
Elongation	72	1 min	
Final elongation	72	5 min	1

Adaptation of CE vaccine virus (EP)CK_{17}) to MDBK cell culture: For this purpose, the MDBK cell culture surfaces produced as monolayer in flasks of 75 cm^2 were washed 3 times with PBS, and CE vaccine strain (E(P)CK_{17}) (2.5 ml) at $TCID_{50}$ $10^{4.0}$/ml titers was inoculated in to 2 flasks. The flasks were incubated at 37°C for the absorption of the virus into the cells. One hour later, the inoculum was taken with a pipette and DMEM-Ham's F-12 medium with 2% FCS was placed in each flask. When 80-90% cytopathic effect (CPE) was observed in cells (within 4–8 days), flasks were frozen and thawed 3 times at -70°C (Ergin and Köklü 1973; Burleson et al. 1992; Musser et al. 2008). Serial passages of Ecthyma (EP)CK_{17}) vaccine virus were performed 5 times in MDBK cell culture (E(P)CK_{17}-$MDBK_5$). Following the serial passages, the E(P)CK_{17}-$MDBK_3$ passage of the ecthyma vaccine virus was named and stocked as master seed virus and the E(P)CK_{17}-$MDBK_4$ passage was named and stocked as the working seed virus (Ergin and Köklü 1975; Burleson et al. 1992).

Production of ecthyma (E(P)CK_{17}-$MDBK_5$) vaccine

Production of MDBK cell culture: MDBK cell culture stored in nitrogen tank was rapidly dissolved in water bath at 37°C and diluted with DMEM-Ham's F-12 medium containing 10% FCS at 37°C to have $3x10^5$/ml cells. The cells was then placed in 25 cm^2 flasks. The flasks were incubated at 37°C with 5% CO_2 for the formation of monolayer cell culture and the cells were checked for reproduction daily. Monolayer cell cultures were prepared by passaging MDBK cells produced in flasks of 75, 175 and into

roller bottles (2 liters capacity), respectively (Ergin and Köklü 1975; Burleson et al. 1992).

Production of Ecthyma working seed virus: One ml ($TCID_{50}$ $10^{4.0}$/ml) of E(P)CK_{17}-$MDBK_4$ working seed vaccine virus was applied to MDBK cell cultures produced as monolayer in disposable roller bottles, which were then incubated for 1 hour at 37°C for adsorption of the virus. At the end of the period, the surface of the cells was washed 3 times with PBS and the cell cultures were incubated at 37°C by adding DMEM-Ham's F-12 medium containing 2% FCS. When 80-90% cytopathic effect (CPE) was observed in cells (within 4–8 days), bottles were placed in the freezer at -20°C (Ergin and Köklü 1975; Burleson et al. 1992).

Collection of CE final product (E (P) CK_{17}-$MDBK_5$): Bottles containing virus suspension in the freezer were frozen and thawed 3 times in a 37°C water bath. The virus suspension was centrifuged at 3000 rpm for 30 minutes and collected for use as the upper liquid vaccine, and sterility, identity and titer control tests were performed (Ergin and Köklü 1975; Burleson et al. 1992; Musser et al. 2008).

Lyophilization: The CE virus suspension was mixed with an equal amount of preservative solution that was freshly prepared with 5% LAH and 10% sucrose. 2 ml aliquots of this mixture were put in 5 ml bottles and lyophilized. Titration of vaccine was performed by taking samples from these bottles. Log.0.5 drop per milliliter was considered normal (Ergin and Köklü 1975; Musser et al. 2008).

Detection of titer of lyophilized CE vaccine (E(P)CK_{17}-$MDBK_5$): Microvirus titration test was performed to determine the $TCID_{50}$/ml of lyophilized CE (E(P)CK_{17}-$MDBK_5$) vaccine virus. Using phosphate buffered saline (PBS), 10-fold dilutions of CE virus from 10-1 to 10-6. The 100 µl of each dilution was put in four wells of the 96-well plate. 50 µl of MDBK cell culture (3-5x10^5 cells/ml) was added to all dilutions. For the purpose of cell control, 100 µl of medium and 50 µl of cell suspension were placed in the last four wells of the plate. The plate was put in to incubator at 37°C with 5% CO_2 and incubated for 10 days. CPE formation due to virus multiplication was observed. The titer of CE vaccine virus were determined according to the Spermen-Kaerber method (Ergin and Köklü 1974; Burleson et al. 1992; Nashirudddullah et al. 2016).

Determination of stability of CE vaccine strain at different temperatures: For this purpose, 10 lyophilized CE vaccines for each temperature were diluted with 10 ml of PBS and incubated at 30, 33 37, 40, 42 and 45ºC for 1, 2, 4, 6, 8, 10 and 12 hours to determine their titers.

Innocuity and immunity studies of CE (E(P)CK_{17}-$MDBK_5$) vaccine virus

Innocuity test in mice and guinea pigs: For this test, 6 guinea pigs weighing 250-350 g and 10 mice weighing 17-22 g were used. At least 5 lyophilized vaccines were diluted with PBS (0.5 ml/one dose). Two guinea pigs were injected intramuscularly with 0.5 ml and 2 other guinea pigs were injected intraperitoneally with 0.5 ml. Two guinea pigs were separated as controls (mock). In addition, 6 mice were injected intravenously with 0.1 ml of CE vaccine suspension and 4 mice remained as controls. Mice and guinea pigs were observed for 1 week. At the end of the observation period, experimental animals were necropsied (Ergin and Köklü 1973).

Immunity studies in lambs: Before the study, the lambs aged, 2-3 months old, which had no contagious ecthyma disease and had no clinical symptoms of contagious ecthyma disease, blood was collected and blood serums were obtained to check antibody titers against the contagious ecthyma virus by VNT. The lambs were kept in quarantine for 10 days before the trial and their daily body temperatures were recorded.

Vaccination of lambs: *L*yophilized CE (E(P) CK_{17}-$MDBK_5$) vaccine was diluted with dilution fluid containing 40 ml PBS (50%) + glycerin (50%). Scarification was performed diagonally by 3-4 scratches with a diameter of 0.5-1 cm with a needle deep enough to pass the first layer in the hairless skin region of the hind legs of 10 lambs. Three drops of the diluted CE vaccine were applied to the scarified areas and the vaccination was completed by waiting for 3-4 seconds. Four lambs to be used as controls (mock) were also scarified diagonally by 3-4 scratches with a diameter of 0.5-1 cm with a needle deep enough to pass the first layer in the hairless skin region of the hind legs. Three drops of PBS (50%) + glycerin (50%) were applied to the scarified areas and the animals were released after 3-4 seconds (Ergin and Köklü 1973; Buddle and Pulford, 1984; Pye 1990; Musser et al. 2012). Body temperature of the animals were measured 2 times a day for 15 days. The skin area to which the vaccine was applied was checked for necrosis, hyperemia and contagious ecthyma disease symptoms.

Challenge studies in vaccinated lambs: The blood samples were collected from the vaccinated and control lambs at 30th day after the CE vaccina-

tion and separated to determine the antibody titers against CE virus**.** Ten lambs vaccinated with the CE vaccine and 4 non-vaccinated control (mock) lambs were challenged by the scarification of inguinal region with E(P)CK_5 pathogen CE virus at a titer of at least $ID_{50}10^{3,2}$/ml. For this purpose, 10 ml of E(P)CK_5 pathogen CE virus was mixed equally with 10 ml of PBS (50%) + glycerin (50%) and vaccinated lambs were challenged by applying 3 drops into the scarified skin inside the hind legs. Body temperatures of lambs were daily measured for 15 days. Following the challenge, vesicles, pustules and subsequent scabbing caused by virus reproduction were observed in the scarification areas on the skin of the control lambs, which were challenged at the same time with vaccinated lambs (Ergin and Köklü 1973; Buddle and Pulford, 1984; Pye, 1990; Musser et al. 2012).

Virus neutralization test (VNT): Antibody titer levels against CE virus were determined by microvirus neutralization test. For this purpose, 50 µl of DMEM/F12 Ham's medium was placed in all wells of 96-well plates. 50 µl of blood serum of each lamb and kid was placed in the first four wells of the plate. 50 µl from the first wells were transferred to the bottom wells to make two-fold dilutions of the serum. To the serum samples, which were diluted two-folds in the wells, were added 50 µl of standard CE virus (E(P)CK_5) strain at 100 $DKID_{50}$ titers and they were let to neutralize at 37°C for one hour. At the end of the incubation period, 50 µl of MDBK cell culture was added to all wells and incubated at 37°C with 5% CO_2 incubator for 10 days. Cells were checked daily for CPE formations (Ergin and Köklü 1973; Nashirudddullah et al. 2016).

Results

The titer of the E(P)CK_{17}-$MDBK_5$ vaccine virus adapted to the MDBK cell culture (Figure 1) was determined to be $TCID_{50}$ $10^{6.5}$/ml (Figure 2). In the innocuity study with mice and guinea pigs, no local or general reactions were observed and no abnormal findings were detected in the necropsy. Body temperature of 10 lambs vaccinated by scarification was found to be between normal values (38.2-39.1°C). No neutralizing antibodies belonging to CE virus with VNT could be detected in blood sera taken on day 30 following the vaccination. As a result of challenge performed with pathogen E(P)CK5 CE virus, vaccinated lambs were observed to have no lesions caused by the reproduction of the pathogen ecthyma virus in their scarified skin areas. Thin small scabs caused by scarification have fell out within 3-4 days. On the other hand, it was observed that vesicles and pustules were formed due to CE virus reproduction in challenged skin areas of non-vaccinated control animals 2-3 days after scarification and that subsequent scabs started to fall out on day 24 (Figure 3).

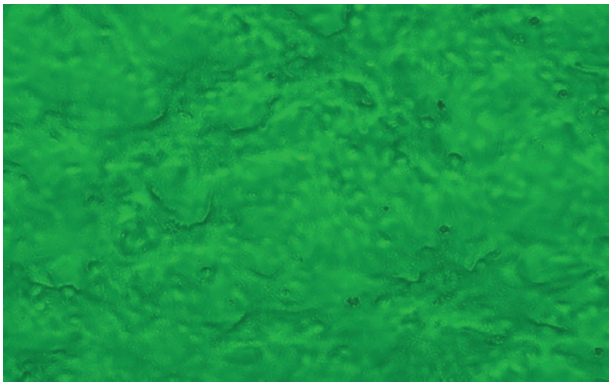


Figure 1. MDBK control cell culture

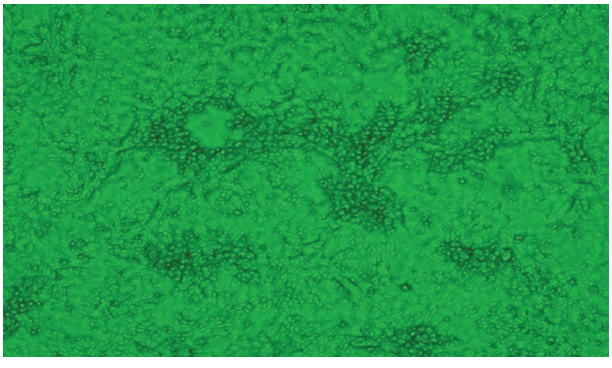


Figure 2. MDBK cell culture CPE (+)

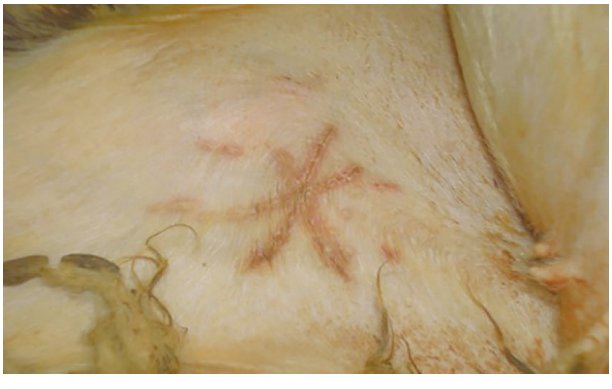


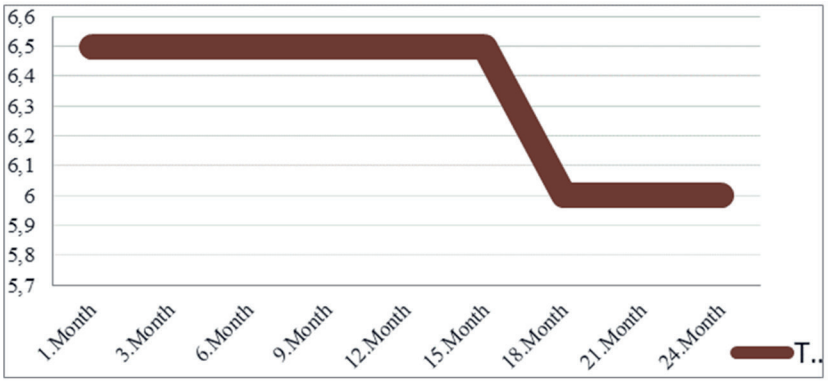
Figure 3. Skin lesion after challenge in control lambs (day 6)

The titer of Lyophilized CE vaccine was found to be as $DKID_{50}$ $10^{6.5}$/ml from 1 to 15 months and $10^{6.0}$/ml at 18, 21 and 24 months during the 24-month storage period at + 4/+8°C (Figure 1). In the stability study of the lyophilized CE vaccine it was determined that the vaccine virus remained the stable at a titer of $TCID_{50}10^{6.5}$/ml at 30, 33, 37 and 40˚C at 1, 2,

4, 6, 8, 10 and 12 hours. It was found that the titer, which declined to $TCID_{50}10^{6.0}$/ml at the 8th hour after incubation, decreased to $DKID_{50}10^{5.75}$/ml at 42˚C and 45˚C as from the 12th hour (Table 5).

Table 5. The results of heat stability study for CE vaccine strain

Temperature	Hour 1 (ml)	Hour 2 (ml)	Hour 4 (ml)	Hour 6 (ml)	Hour 8 (ml)	Hour 10 (ml)	Hour 12 (ml)
30˚C	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$
33˚C	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$
37˚C	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$
40˚C	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$
42˚C	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.0}$	$10^{6.0}$	$10^{5.75}$
45˚C	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.0}$	$10^{5.75}$	$10^{5.75}$



Month 1 Month 3 Month 6 ... Month 24

Graphic 1. Heat stability study results for Ecthyma vaccine

Discussion and Conclusion

Contagious ecthyma (CE) is a common viral infection in lambs and kids in our country, and still maintains its importance in sheep and goat breeding. Attenuated, live and lyophilized CE vaccines adapted to cell culture are used in the fight against this disease. The CE vaccine strain ($E(P)CK_{22}$) used in the fight against CE disease in our country was isolated from the lip lesions of a lamb infected with CE in 1975 and was attenuated by passaging 22 times in primary calf kidney cell culture.

In the production of CE vaccines, 1-3 month calves are slaughtered for each series of vaccine production, primary cell culture is prepared from the kidneys under sterile conditions, and vaccine is produced by cultivating the vaccine strain when a complete monolayer is formed. However, it is difficult to produce sufficient amount of vaccines because vaccine preparation with kidney cells obtained from calves has a contamination risk with especially pestivirus, blue tongue viruses, PPR virus, Parainfluenza 3, and akabane viruses to vaccinated animals. The aim of this project was to adapt the CE vaccine strain produced in primary calf kidney cell culture to MDBK cell culture and to produce the vaccine, as well as determining the shelf life, stability, innocuity, and immune response in lambs.

In the production of CE virus, primary cell and cell line cultures are used and different virus titers (between $10^{4.2}$ and $10^{7.6}$ $TCID_{50}$/ ml) are obtained in these cell cultures (Mercante et al. 2008; Wang et al. 2019; Galante et al. 2019). A study by Wang et al. (2019) reported that, in the CE virus production, bovine sertoli cells are more sensitive than primary neonatal bovine testicular cells and Madin-Darby bovine kidney (MDBK) cell lines, and the CE virus titers were approximately 1 log higher in bovine Sertoli cells ($TCID_{50}$ $10^{6.55}$/ml) compared to MDBK ($TCID_{50}$ $10^{5.10}$/ml) and primary neonatal bovine testicular ($TCID_{50}$ $10^{5.50}$/ml) cells.

Mercante et al. (2008) reported that 10 serial passages of CE virus were performed to obtain attenuated CE working seed virus and CE virus was obtained at $10^{4.2}$ $TCID_{50}$/ml titers in primary chicken

embryo fibroblasts cell culture. In addition, the researchers found in the immunity studies of the lyophilized CE vaccine virus that sufficient immunity occurred in animals with CE virus at $10^{4.5}$ $TCID_{50}$ per dose. Nashirudddullah et al. (2010) reported that the titer of the reference CE vaccine virus produced in continuous fetal lamb testicular cell culture was found as $10^{5.75}$ $TCID_{50}$/ml. In this study, it was found that the post-production titers of CE virus produced in the MDBK cell culture ($DKID_{50}$ $10^{6.5}$/ml) were higher than the titers reported by other researchers. It has been reported that the amount of virus in lyophilized form CE vaccines should be at least $DKID_{50}$ $10^{4.5}$/ml for the protection of lambs against CE infection. This study determined that the titer of CE vaccine virus produced in MDBK cell culture after lyophilization was similar to the values reported by other researchers (Ergin and Köklü 1973; Ergin and Köklü 1975; Buddle and Pulford 1984; Nashirudddullah et al. 2010; Gülyaz et al. 2012). At the end of the challenge study, it was seen than there was not any lesions caused by pathogen CE virus on the skin of vaccinated lambs. According to this result, it was concluded that vaccinated lambs are immune to CE infection.

Following the production of veterinary vaccines, real-time shelf life is required before administration in the field. This study determined that the titers of ecthyma vaccines, which were kept at +4/+8°C for 24 months, decreased from $DKID_{50}$ $10^{6.5}$/ml to $DKID_{50}$ $10^{6.0}$/ml at the end of the 24th month, and the titers detected were sufficient to provide sufficient immunity in the lambs after vaccination.(Ergin and Köklü 1973; Ergin and Köklü 1975; Buddle and Pulford 1984; Gülyaz et al. 2012).

One of the most important criteria for insufficient immunity in animals is the reduction of live virus titers in lyophilized vaccines during the administration of vaccines in the field, especially due to environmental temperatures. Therefore, it is necessary to know the stability of live lyophilized vaccines in the field against environmental temperatures under field conditions. This study showed that the lyophilized CE vaccine remains stable at 45°C for 12 hours after the dilution means that it will remain at sufficient titers to immunize animals adequately in the field despite the high ambient temperatures.

In conclusion, this study shown that the vaccine produced in the MDBK cell culture provides sufficient immunity against CE virus in lambs. In addition, this study concluded that it would be appropriate to produce CE vaccines in MDBK cell cultures since the real-time shelf life of the lyophilized CE vaccine is 24 months, the vaccine virus remains stable at 42-45°C for up to 12 hours after dilution with the vaccine reconstitution fluid.

Acknowledgement

This project was supported by the Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policies (TAGEM/HSYGAD/14/A07/P02/42).

Author Contributions

VG and FS designed the experiments, FS, VG, SU, EA and FS carried out the experiments, VG and FS 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 Istanbul Pendik Veterinary Control Institute.

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