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**Original Article**
Özgün Araştırma

# Determination of the effects of Enrofloxacin, Linco-Spectin and Florfenicol Antibiotics on BHK-21 cell culture and FMD 146S virus particles-infective titers

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**Abstract:** Enrofloxacin, linko-spectin and florfenicol antibiotics were intended to be used in the BHK-21 An30 cells and the foot and mouth disease virus (FMDV) culture during the vaccine production process. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and cell cultures assays were performed in the BHK-21 An30 cells treated with the antibiotics that took place in this study to determine the concentration that inhibits cell proliferation and adverse effects on cell morphology. Virus cultures were performed by inoculating of FMDV serotypes (A/TUR/11, O/TUR/07, Asia-1/TUR/15) to the treated cell cultures with the antibiotics. 146S and infective titres of the obtained virus suspensions were determined. The non-toxic upper limit was determined to be 15 µg /ml for enrofloxacin and 300 µg/ml for linco-spectin in terms of cell morphology and cell numbers versus positive control (gentamycin, penicillin-streptomycin) and negative control (antibiotic-free medium) as a result of MTT and cell culture tests on BHK cells. It was observed that florfenicol also inhibited cell proliferation and induced cell degeneration, even at a concentration of 5 µg/ml. The mean 146S values of FMD virus cultures containing enrofloxacin and linco-spectin were determined to be 0.49, 0.46, 0.53, 0.47 and 0.30, 0.28 µg/ml for serotype A/TUR/11, O/TUR/07 and Asia-1/TUR/15 respectively. The mean values of the infective titres were $10^{7,04}$, $10^{7,25}$, $10^{6,04}$, $10^{6,59}$ and $10^{7,26}$, $10^{7,6}$ pfu/ml for serotype A/TUR/11, O/TUR/07 and Asia-1/TUR/15 respectively. In the control group used gentamycin, penicillin-streptomycin and antibiotic-free medium, the mean 146S FMD virus particles were obtained as 0.51, 0.50, 0.50; 0.52, 0.55, 0.52 and 0.36, 0.33, 0.30 µg/ml for A/TUR/11, O/TUR/07 and Asia-1/TUR/15 respectively. The mean values of the FMD infective virus titres were $10^{7,35}$, $10^{7,40}$, $10^{7,11}$; $10^{6,24}$, $10^{6,46}$, $10^{6,62}$, and $10^{7,70}$, $10^{7,75}$, $10^{7,77}$ pfu/ml for A/TUR/11, O/TUR/07 and Asia-1/TUR/15 respectively.

As a result, the infective FMDV titer and 146S results obtained in the control group (gentamicin, penicillin-streptomycin) and FMD virus cultures using enrofloxacin (15 µg/ml) and lincospectin (300 µg/ml) were very close to each other. According to these data, it was concluded that enrofloxacine and lincospectin can be used up to the upper limit in the BHK-21 An30 cell and FMD virus cultures. However, Florfenicol should not be used in cell and virus cultures.

**Key words:** Antibiotic, BHK cell culture, contamination, FMD virus,

## Enrofloxacin, Linco-Spectin ve Florfenicol antibiyotiklerinin BHK-21 hücre kültürü ve FMD 146S virus partikülü-infektif titreleri üzerine etkilerinin belirlenmesi

**Özet:** Bu proje ile FMD aşısı üretimi sürecinde, hücre ve virus kültürlerinde kullanılan gentamisin, penisilin ve streptomisin antibiyotiklerine ek olarak; enrofloxacin, linco-spectin ve florfenicol antibiyotiklerinin kullanıma alınması amaçlandı. Bu amaçla projede yer alan antibiyotiklerin BHK-21 An30 hücresinde, hücre morfolojilerine olumsuz etki eden ve hücre çoğalmasını inhibe eden konsantrasyonunu belirlemek için MTT (Methylthiazolyldiphenyl-tetrazolium bromide) testleri ve hücre kültürleri yapıldı. Bu hücre kültürlerine FMD virüslerinin (serotip A/TUR/11, O/TUR/07, Asia-1/TUR/15) inokulasyonu ile virüs kültürleri gerçekleştirildi. Elde edilen virüs süspansiyonlarının 146S ve infektif titre değerleri tespit edildi. BHK-21 An30 hücresi ile yapılan MTT testleri ve hücre kültürleri sonucunda hücre sayıları açısından toksik olmayan üst sınır enrofloxacin için 15 µg/ml ve linko-spektin için 300 µg/ml olarak belirlendi. Florfenikolün ise 5 µg/ml konsantrasyonda dahi, hücre üremesini inhibe ettiği ve hücrede dejenerasyona yol açtığı gözlendi. Enrofloxacine (15 µg/ml) ve linco-spectinin (300 µg/ml) kullanılan FMD virüs kültürlerinde ortalama 146S değerleri sırası ile A/TUR/11 için 0.49, 0.46 µg/ml, O/TUR/07 için 0.53, 0.47 µg/ml ve Asia-1 /TUR/15 için 0.30, 0.28 µg/ml olarak; infektif titre değerleri ortalaması ise A/TUR/11 için $10^{7,04}$, $10^{7,25}$ pfu/ml. O/TUR/07 için $10^{6,04}$, $10^{6,59}$ pfu/ml ve Asia-1/TUR/15 için $10^{7,26}$, $10^{7,6}$ pfu/ml olarak tespit edildi. Kontrol grubunda gentamisin, penisilin-streptomisin ve antibiyotiksiz vasat ile hazırlanan virüs kültürlerinde 146S viral partikül değerleri ortalaması sırası ile A/TUR/11 için 0.51, 0.50, 0.50 µg/ml, O/TUR/07 için 0.52, 0.55, 0.52 µg/ml ve Asia-1/TUR/15 için 0.36, 0.33, 0.30 µg/ml olarak

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bulundu. Aynı kültürlerin infektif titre değerleri ortalaması ise A/TUR/11 için $10^{7,35}$, $10^{7,40}$, $10^{7,11}$ pfu/ml, O/TUR/07 için $10^{6,24}$, $10^{6,46}$, $10^{6,62}$ pfu/ml ve Asia-1/TUR/15 için $10^{7,70}$, $10^{7,75}$, $10^{7,77}$ pfu/ml olarak saptandı. Sonuç olarak kontrol grubu (gentamisin, penisilin-streptomisin) ile enrofloxacin (15 µg/ml) ve linco-spectinin (300 µg/ml) kullanılan FMD virüs kültürlerinde elde edilen infektif FMDV titresi ve 146S sonuçları birbirine çok yakın değerlerde tespit edilmiştir. Elde edilen bu verilere göre, BHK-21 An30 hücre ve FMD virüs kültürlerinde enrofloxacin (15 µg/ml) ve linco-spectinin (300 µg/ml) üst sınırına kadar kullanılabilmesine karşın florfenicolün ise kullanılmaması gerektiği kanaatine varılmıştır.

**Anahtar kelimeler:** Antibiyotik , BHK-21 An30, hücre kültürü, kontaminasyon, şap virüsü

## Introduction

Foot and mouth disease (FMD) is the most contagious disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus (FMDV), namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1 (OIE, 2017). The serotypes of FMDV are not distributed uniformly around the world. The serotype O, A and C viruses have had the widest distribution and have been responsible for outbreaks in Europe, America, Asia and Africa. The FMDV particle is roughly spherical in shape and about 25-30 nm in diameter. It consists of the RNA genome surrounded by a protein shell or capsid (Jamal and Belsham, 2013) and causes heavy economic losses to the livestock industry such as high morbidity in adult animals, treatment costs, reduced milk production, loss of working ability in draught animals of developing countries, reproductive disorders and high mortality in young ones (Grubman and Baxt, 2004; Nawaz et al, 2019). The control of FMD is a national and regional responsibility and, in many countries, the vaccine may be used only under the control of the veterinary authority. Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IB-RS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. The sensitivity of any cells used should be tested with standard preparations of FMDV (OIE manuel, 2017; Or and Fidancı, 2009). The FMD vaccine production is carried out by producing of BHK-21 cells in suspended conditions in high capacity reactors (1000, 3000 and 5000 Lt) and infecting with the required FMD vaccine strains. As seen in other cell cultures and vaccine production processes, the biggest problem encountered in FMD vaccine production stages is bacterial and fungal contaminations due to antimicrabial resistance.

Bacterial, yeast, and fungal contamination are hazards to those studying various phases of metabolism and growth of mammalian cells in vitro (Fleckenstein et al, 1994). Although we have used antibiotics for more than 30 years to eliminate or suppress unwanted microbial contaminants, many who add them to tissue cultures for this purpose are unfamiliar with the products used, the limitations for their use, and the practical value of this strategy (Akkan ve Karaca, 2003; Perlman, 1979). Antibiotics are called bacteriostatic and bactericidal in terms of preventing the growth and reproduction of bacteria and causing their death. Values indicating the potency of antibiotics are MIC (minimum inhibitor concentration) and MBK (minimum bactericidal concentration). In terms of effect spectra, they are called narrow and broad spectrums. As seen in penicillin-streptomycin combinations, the antibacterial spectra can be expanded by making combinations of antibiotics that produce a synergetic effect (Reeves, 2012).

Antimicrobial drugs are divided into 5 groups cell wall synthesis inhibitors (Penicillins and cephalosporins), protein synthesis inhibitors (Macrolides, linkosamides and chloramphenicol), nucleic acid synthesis inhibitors, antimetabolites (Trimethoprim), membrane integrity (polymyxin and nystatin) (Campos et al. 2012; Saran and Karahan, 2010; Reeves, 2012).

It is a big problem that bacteria develop resistance against antibiotics in both invitro and invivo applications. Indicator of resistance development is the gradual increase of minimum inhibitory concentration and minimum bactericidal concentration values of the drug in microorganism (Yarsan, 2012). In addition, bacteria that are resistant to an antibiotic may lose their sensitivity to another antibiotic of similar structure, which is called cross-resistance.

The use of antibiotics in the cell culture medium is a valuable preservative. Gentamicin, which is widely used in cell cultures and is one of the most widely used antibiotics in vaccine production in our Institute; It has a broad spectrum bactericidal activity because of containing an aminoglycoside group. It enters the bacterial cell and irreversibly binds to a receptor in the 30 S subunit, preventing the formation of m-RNA, formyl-methionine and tRNA complexes. It inhibits protein synthesis. Another aminoglycoside group antibiotic, Streptomycin, shows a stronger Gram (+) activity by binding to

30S and inhibits protein synthesis, and has a narrower spectrum than other antibiotics in the same group. Penicillin; on the other hand, it is effective by inhibiting cell wall synthesis, and in combination with streptomycin, their spectrum broadens for many Gram (+) and Gram (-) bacteria. However, antibiotics have many important disadvantages (Freshney, 2005). Antibiotics cause the development of resistant organisms while at the same time hiding the presence of low levels of contamination, causing the formation of resistant strains when the culture conditions change. It also has negative effects on a cell basis. Antibiotics can have adverse effects on cell growth and functions. They can reduce cell yield, growth rate and shorten the life of the cell (Spier and Grifits, 1985). In this way, they can remove the cell from its original state by creating toxic effects on the cell. Therefore, it should not be used in routine cultures; It is recommended for use in limited primary culture development or in large-scale, labor-intensive and costly studies (Freshney, 2005). Antibiotics to be used in mammalian cell cultures are desired to have some properties. Antibiotics with bactericidal effects should be preferred in order to eliminate microbial contaminants. Antibiotics should not suppress mammalian cell metabolism and growth in tissue cultures. It should not affect the intended use of mammalian cells, such as virus production or antigen preparation. The antibiotic should be non-toxic and suitable for use by laboratory personnel. It should also be compatible with other culture media components. Antibiotics to be used in mammalian cell cultures should have cytotoxicity tests performed and certain data should be obtained (Perlman, 1979). With this project, it was aimed to use enrofloxacin, linco-spectin and florfenicol antibiotics instead of gentamycin, penicillin and streptomycin antibiotics due to reasons such as insufficient spectrum or bacterial resistance formation in FMD vaccine production in Turkey.

## Materials and Methods

***Cell culture:*** BHK-21 An30 cell culture was obtained from FMD (SAP) institute cell bank laboratory.

***Culture media (Applichem-A-1321):*** Glasgow Minimum Essential Medium (GMEM) medium was used in cell culture and FMD virus production.

***Fetal calf serum (Biochrom):*** It was inactivated at 56°C for 30 minutes and used by adding 10 and 2 % to the cell and virus production medium respectively.

***Trypsin EDTA solution 0.25% (Sigma T-5775):*** The commercial trypsin-EDTA solution was used during the passaging of cells.

***Viruses:*** FMD serotype A, O and Asia-1 type viruses (A/TUR/11, O/TUR/07, Asia-1/TUR/15) were obtained from the FMD (SAP) institute virus bank laboratory.

***Methylthiazolyldiphenyl-tetrazolium bromide (MTT) test dye (Sigma-5655):*** MTT dye (was dissolved in RPMI-1640 medium without phenol red and filtered at 0.2µm filter and kept at -20 °C as 5 ml.

***Gum Tragacanth*** *(Sigma 9000-65-1):* Gum was used as a covering medium in determining infective titer, it was dissolved 1.3% in distilled water. After autoclaving at 121°C for 20 minutes, it was mixed 50%+50% (v/v) with 2XGMEM medium. It was used after the addition of 1% sodium bicarbonate.

***Antibiotics:***

**1.** ***Gentamycin*** (Applichem-A4854), ***Penicillin*** (Applichem-A1837), ***Streptomycin*** (Applichem-A1852): Powder antibiotics were dissolved with distilled water and sterilized through a 0.22 µm porous filter. After preparing 10.000 ppm stock solutions, it was added to cell and virus production media according to the working concentrations.

**2.** ***Enrofloxacin*** **(Deva-1000025),** ***Lincomycin*** **(Deva-1000091),** ***Spectinomycin*** **(Deva-1000692):** Enrofloxacin and linco-spectin were dissolved in 0.1% formic acid and distilled water respectively. Following, 10,000 ppm stock solutions of both antibiotics were prepared.

**3. Florfenicol (Teknovet-201401037):** The antibiotic supplied as the powder was dissolved with distilled water and a stock solution of 1,000 ppm was prepared. All stock solutions were sterilized by passing through a 0.22 µm porous filter. It was used by adding to cell and virus production media according to the study concentrations.

**Bürker cell count:** The Bürker chamber has 9 large squares (1 $mm^2$ each), divided by double lines (0.05 mm apart) into 16 group squares. The double lines form small 0.0025 $mm^2$ squares. The Chamber depth is 0.1 mm. The cells were counted. Briefly, both operators take 10 µl of cell suspension with a micropipette and put them in the cell count chamber and then count the cells in each of the 4 large squares. At the end of the procedure the operators calculate the average of the 4 readings (from 4 large

squares) and calculate the cell concentration as follows: (Gunetti et al, 2012).

$$\left[\left[\frac{\text{Cell}}{\text{ml}}\right]=\left[\frac{\Sigma\text{cell counted in 4 large squares}}{4}\right]\text{x(dilution factor)x1x10}^4\right]$$

## Determination of cytotoxicity of antibiotics in BHK-21 An30 cell culture by MTT test

MTT test was performed to determine the concentration ranges of antibiotics that negatively affect on cell morphology and proliferation. For this purpose, the stock solutions of the antibiotics of the control and experimental groups were diluted with cell culture medium containing 10% serum to obtain final concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml.

BHK-21 An30 cells were prepared as monoleyer in 96 well plates with $4x10^4$ cells/ml. The medium was removed from all wells after incubation at 37°C with 5% $CO_2$ for 48 hours. The cell culture media with antibiotics and without antibiotics were added to the plates as 100 µl/well and incubated at 37°C with 5% $CO_2$. Eight eyes from top to bottom were used for each concentration of control and trial group antibiotics. In order to evaluate as a cell control and negative control, 16 wells were used for the medium without antibiotics. For each antibiotic, 5 plates were prepared to evaluate at 24, 48, 72, 96, and 120 hours. At the end of the incubation periods, a plate prepared for each antibiotic was taken and observed under a microscope and then the media were removed. Serum free medium without antibiotics was placed as 100 µl in to the all wells. Later, MTT dye (13 µl/well) was dissolved at the time of use and added in the dark place. All the plates were covered with aluminum foil and left in incubation at 37°C with 5% $CO_2$ for 4 hours. At the end of the incubation period, 100 µl of isopropyl alcohol was added to each well by observing the formazan crystals. It was read at 570 nm in the spectrophotometer and the results were recorded. The results were recorded at the end of 48, 72, 96, and 120 hours for each antibiotic.

Considering the first MTT test results, a new test was designed for the experimental group antibiotics by determining new concentration ranges. For this purpose, stock solutions of antibiotics of the control and experimental groups were used. Enrofloxacin and linco-spectin were added to cell culture media containing %10 serum with final concentrations of 10, 50, 80, 100, 200, 400, 500, 600, 800, and 1000 µg/ml. Florfenicol at final concentrations of 10 µg/ml, 30, 50, 80, 100, 120, 140, 160, 180, and 200 µg/ml were prepared in cell culture media containing %10 serum. MTT tests of antibiotics at new concentration ranges were performed with the same procedure.

Results were evaluated according to FMD (SAP) Institute protocol; Cytotoxicity test (with MTT) formula was used as % viability = $test_{absorbansort}$ / Cell Control absorbansort X 100. According to formula; the results were evaluated as non-toxic (>%90 cell viability), low toxic (60-90% cell viability), moderately toxic (30-59% cell viability) and toxic (<%30) (Holst-Hansen and Brünner, 2000).

## Determination of antibiotic concentrations that negatively affect the morphology and proliferation of BHK-21 An30 cells

### *Determination of the toxic level of antibiotics:*

***Enrofloxacin:*** Cell culture media were prepared in final concentrations of 10, 30, 50, 80, 100, 200, and 400 µg/ml using stock solutions of antibiotics of enrofloxacin and control group (gentamicin and penicillin-streptomycin). BHK-21 An30 cells were trypsinized and resuspended with the antibiotic-free medium and divided into 22 flasks (25 $cm^2$) with $4x10^5$ cells/ml. Cell culture media prepared with antibiotic solutions of enrofloxacin and control group (gentamicin and penicillin-streptomycin) were added in to the flasks and incubated in incubator with %5 $CO_2$ at 37°C for 48 hours. At the end of the 48 hour, the monolayer ratio of the cells was observed. The media in the flasks were poured and made trypsinization. The cells in each flask were resuspended with their own media and the cell numbers and morphological features were recorded with the Burker slide method.

According to the toxicity results obtained by the first dilution set, a second dilution range was determined. Another set of concentrations were prepared again for the final concentrations of 10, 15, 20, 25, and 30 µg/ml enrofloxacin in cell culture media. These cell culture media were added to 16 flasks (25 $cm^2$) containing BHK-21 An30 cells and incubated for 48 hours in a 37°C incubator with 5% $CO_2$. At the end of the $48^{th}$ hour of each subculture, the confluency of the cells was observed, and the used medium was discarded and cells were harvested by trypsin. Each flask was resuspended with its medium and the cell counts and morphological properties were recorded with the Burker slide method. The cells ($4,0x10^5$ cell/ml) were transferred to flasks and subcultured three times with its medium. The same procedure was repeated three times.

***Linco-Spectin:*** The cells in each flask were re-suspended with their media and the cell numbers and various concentrations linco-spectin and control group of antibiotics (gentamicin and penicillin-streptomycin) were prepared in cell culture media from stock solutions. The concentrations were 50, 80, 100, 200, 400, 600, and 800 µg/ml. Monolayer BHK-21 An30 cells from the same line as the cell used in the MTT test were grown. BHK-21 An30 cells were harvested by trypsin and re-suspended with the antibiotic-free medium and divided into 22 flasks (25 $cm^2$) each contained $4x10^5$ cells/ml. The cell culture media prepared with antibiotic solutions of linco-spectin and control group (gentamicin and penicillin-streptomycin) were added into the flasks and incubated at 37°C with 5% $CO_2$ for 48 hours. At the end of the 48 hours, the confluency of the cells was observed by a microscope. The media in the flasks were discarded and the cells were harvested by trypsin and morphological features were recorded with the Burker slide method.

According to the toxicity results obtained by the first dilution set, a second dilution range was determined. Another set of concentrations was prepared again for the final concentrations of 100, 200, 300, and 400 µg/ml linco-spectin in cell culture media. These cell culture media were added to 16 flasks (25 $cm^2$) containing BHK-21 An30 cells and incubated for 48 hours in a 37°C incubator with 5% $CO_2$. At the end of the 48$^{th}$ hour of each subculture, the confluence of the cells was observed, and the used medium was discarded and cells were harvested by trypsin. Each flask was resuspended with its medium and the cell counts and morphological properties were recorded with the Burker slide method. The cells ($4,0x10^5$ cell/ml) were transferred to flasks and subcultured three times with its medium. The same procedure was repeated three times. A control flask without antibiotics was kept in the same conditions.

***Florfenicol:*** Various concentrations florfenicol and a control group of antibiotics (gentamicin and penicillin-streptomycin) were prepared in cell culture media from stock solutions. The concentrations were 10, 30, 50, 80, and 100 µg/ml. Monolayer BHK-21 An30 cells from the same line as the cell used in the MTT test were grown. BHK-21 An30 cells were harvested by trypsin and re-suspended with the antibiotic-free medium and divided into 16 flasks (25 $cm^2$) each contained $4x10^5$ cell/ml. Cell culture media prepared with antibiotic solutions of florfenicol and the control group (gentamicin and penicillin-streptomycin) were added into the flasks and incubated at 37°C with 5% $CO_2$ for 48 hours. At the end of the 48 hours, the confluency of the cells was observed by a microscope. The media in the flasks were discarded and the cells were harvested by trypsin. The cells in each flask were re-suspended with their media and the cell numbers and morphological features were recorded with the Burker slide method.

According to the toxicity results obtained by the first dilution set, a second dilution range was determined. Another set of concentrations was prepared again for the final concentrations of 5 and 10 µg/ml florfenicol in cell culture media. These cell culture media were added to 16 flasks (25 $cm^2$) containing BHK-21 An30 cells and incubated at 37°C with 5% $CO_2$ for 48 hours. At the end of the 48$^{th}$ hour of each subculture, the confluency of the cells was observed, and the used medium was discarded and cells were harvested by trypsin. Each flask was resuspended with its medium and the cell counts and morphological properties were recorded. The cells ($4,0x10^5$ cell/ml) were transferred to flasks and subcultured three times with its medium. The same procedure was repeated three times. A control flask without antibiotics was kept in the same conditions.

**Virus culture in cells with media prepared with antibiotic concentrations below toxicity levels**

In the virus cultures of A, O and Asia-1 serotypes of FMD virus with known 146S particle amount and infective titers were used (A/TUR/11, O/TUR/07 and Asia/TUR/15). In cell culture, BHK-21 An30 cells (with media and antibiotics belonging to below the toxic concentration level) in 25 $cm^2$ flasks were inoculated 1 virus of A, O, and Asia-1 types of FMD virus per 50-100 cells. Media prepared with its antibiotic concentration were used in each flask. Flasks were incubated at 37°C with 5% $CO_2$ after virus inoculation, and the time during which 100% CPE (Cytopathogenic Effect) formation was recorded. Virus-grown flasks were frozen and thawed, centrifuged at 3000 rpm for 10 minutes. The same procedures were repeated three times.

***Detection of FMD 146S virus particles and infective titers:***

***146S virus particles*:** The determination of 146S virus particle size was done by SDG (Sucrose Density Gradient) method in FMD (ŞAP) Institute Protocol (2010). With the help of the gradient maker, a linear gradient was obtained in the ultracentrifuge tube by layering sucrose concentrations starting with 45% sucrose at the bottom and 15% sucrose at the top. 0.5 ml of virus sample was added slowly over

the gradients and the tubes were placed in the ultracentrifuge godet. Godet was placed in the rotor and centrifuged at 41.000 rpm for 2 hours at +4°C temperature in a vacuum condition. At the end of the centrifuge, to ensure that the samples pass through a computer-connected UV detector (254 nm), the device was started to be dispensed from the bottom of the tube and at a suitable flow rate of 60% sucrose base. When the sample started to pass through the detector, the appropriate absorbance width was set and the computer program (Chroma Simple) was started. After the peak occurred in the program, the starting and ending points of the peak are drawn; the program automatically calculated the amount of 146S particles (µg/ml) measured as the area of the peak.

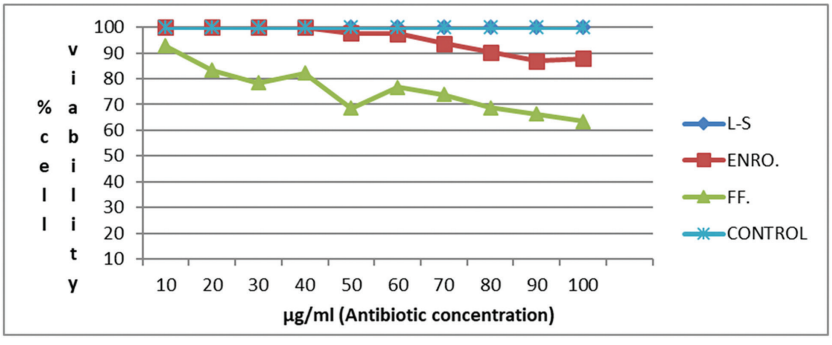
***Plaque Test (infective titer):*** In order to determine the infective titer of FMD viruses and to examine the plaque characters, dilutions of FMD virus (log.10) from $10^{-1}$ to $10^{-6}$ were prepared and inoculated as 100 µl per well into monolayer BHK-21 An30 cells produced in 6-well plates. For adsorption, the plates were left in the incubator at 37°C with 5% $CO_2$ for 1 hour and 3 ml medium (containing 50% GUM + 50% GMEM 2X) was added to each well at the end of this period. The Plates were incubated again for 48 hours at 37°C with 5% $CO_2$. At the end of the period, the media was poured and 2 ml of dyeing solution prepared with crystal violet were added to each well. Finally, the dyeing solution was spilled and all plates were washed with distilated water. In the evaluation, plaques formed by the fmd virus were counted at the latest dilutions and infective titers were calculated as PFU/ml (Baer and Kehn-Hall, 2014; Berg et al, 1963).

## Statistical Analysis

All data were analyzed with Shapiro-Wilk and Levene Statistical tests. According to results of these tests, One-Way Analysis of Variance (ANOVA) statistical test was performed to detect the differences between average values of groups. In addition to distinguish the significance of the differences between groups for levels of FMD 146S viral particles and infective titers of FMD virus serotypes, Duncan multiple range test was used. All the datas were evaluated on the 95% confidence interval and SPSS 22.0 (Inc., Chicago II, USA) software was used.

## Results

Cytotoxicity Assay (MTT): According to the results of the MTT test performed after the 5-day incubation of cells in the first prepared concentration ranges of the antibiotics used in the study, cell viability was measured as 100% and above absorbances in all dilutions in the control group (gentamicin, penicillin-streptomycin, and medium without antibiotics). In linco-spectin, one of the experimental group antibiotics, it was determined that the cell viability equivalent to the control group was measured, while 80 µg/ml in enrofloxacin, and then cell viability dropped below 90%. In Florfenicol, on the other hand, determined cell viability at a concentration of 20 µg/ml as 83.31% and continued to decrease at subsequent concentrations, and absorbance of 63.38% at 100 µg/ml was measured. The second concentration studies of florfenicol were not carried out since florfenicol caused high cell death even at 20 µg/ml concentration. In light of the first MTT test results, the second test was designed for experimental group (Linco-spectin and Enrofloksasin) antibiotics by determining new concentration ranges (Graphic 1).



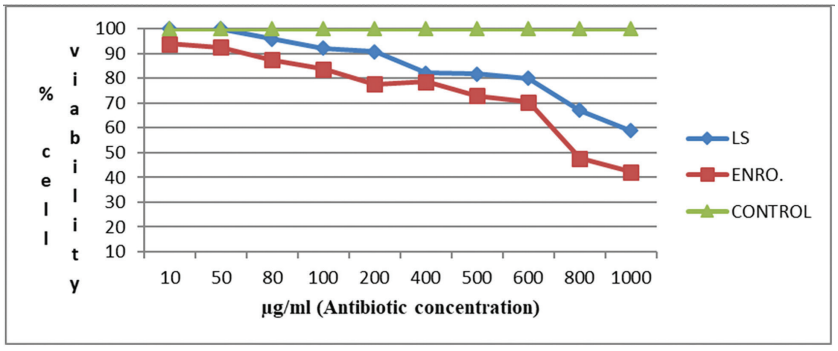

**Graphic 1.** MTT Test results-1

(L-S: Linco-spectin, Enro: Enrofloxacin, FF: Florfenicol)

In the control group (gentamicin, penicillin-streptomycin, and medium without antibiotics), the average of the test values obtained for 5 days; Cell viability was measured at 100% and above absorbances at all dilutions. Over 90% of cell viability was observed at 200 µg/ml linco-spectin, one of the experimental group antibiotics. The cell viability, measured as 82.2% at 400 µg/ml of the antibiotic,

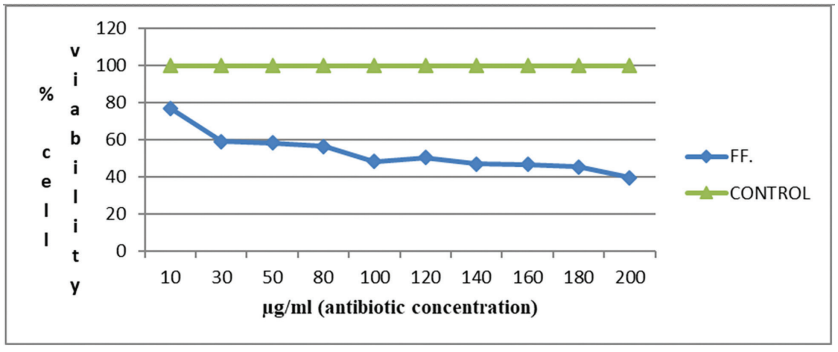
continued to decrease in subsequent concentrations, and 58.94 % cell viability at the highest 1000 µg/ml concentration was measured. In enrofloxacin, the cell viability was measured at the last concentration of 42.23 %, again with 80 µg/ml and 90 % cell viability below 90 %, and the subsequent decline continued (Graph 2).

Looking at the average of test values obtained for 5 days in the study with Florfenicol; Cell viability was measured in the control group (gentamicin, penicillin-streptomycin, and medium without antibiotics) as 100 % and above absorbances in all dilutions. Florfenicol decreased cell viability to 76.89 % at a concentration of 10 µg/ml and to 39.63 % at the final concentration (100 µg/ml). (Graphic 3).




**Graphic 2.** MTT Test results-2 (Group-1)

(L-S: Linco-spectin, Enro: Enrofloxacine)




**Graphic 3.** MTT Test results-3 (Group-2)

(FF: Florfenicol)

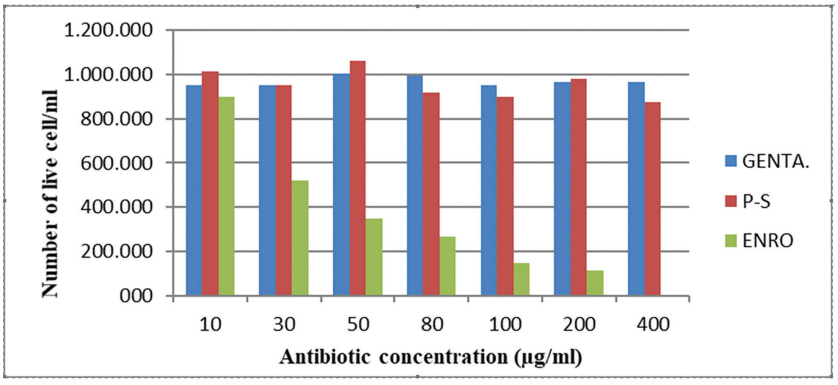
## Effect of antibiotics on BHK-21 An30 cell reproduction

***Enrofloxacin:*** As a result of passages on BHK-21 An30 cells at 10 µg/ml concentration of enrofloxacin, it was observed that the cell numbers were equivalent to the control group cell numbers in each passage. Cell concentrations decreased at 30 µg/ml and above concentrations, numbers started to decrease and degenerations were observed in cell morphology. In the rest of the passages, it was found that the cells were not attached to the flask's surface (Graph. 4). With the narrowing of the concentration ranges, enrofloxacin at 10 µg/ml and 15 µg/ml concentrations were observed to be equivalent to cell numbers of the control group at each passage. However, at concentrations of 20 µg/ml and above, the cell confluency was decreased and gaps between cells were formed, cell numbers were decreased and degenerations in cell morphology were observed (Graph. 5). 15 µg/ml was determined as the upper limit and cells were propagated for virus inoculation at the 3rd passage.

***Linco-spectin:*** It was determined that the number of cells up to 200 mg/ml concentrations of linco-spectin was equal or even above the mean of the control group cell numbers in each passage. It was observed that the cell numbers-coating rate decreased and degenerations started in cell morphology at concentrations of 400 µg/ml and above (Graph. 6).

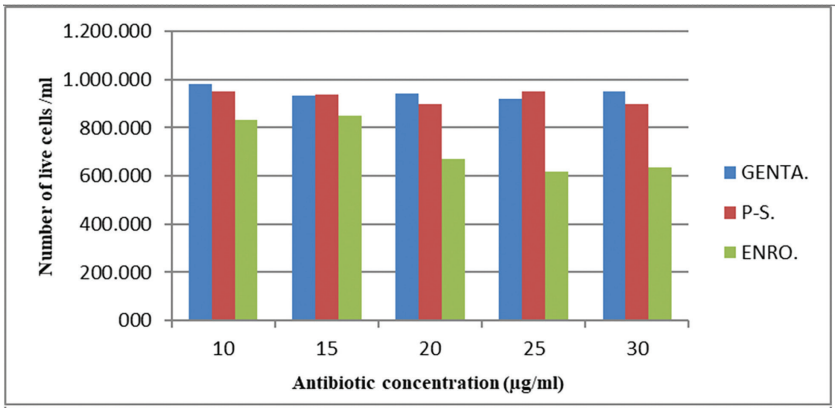
At the end of the repeated passages of BHK-21 An30 cell, it was observed that the number of cells propagated with medium containing linco-spectin at 300 µg/ml and lower concentrations were equiv-

alent to the mean cell numbers of control group at each passage. At the 400 µg/ml concentration, the cell coverage rate and the cell numbers decreased. Morphological cell degenerations were determined at concentrations above 300 µg/ml dilution of the Linco-spectin (Graph. 7).



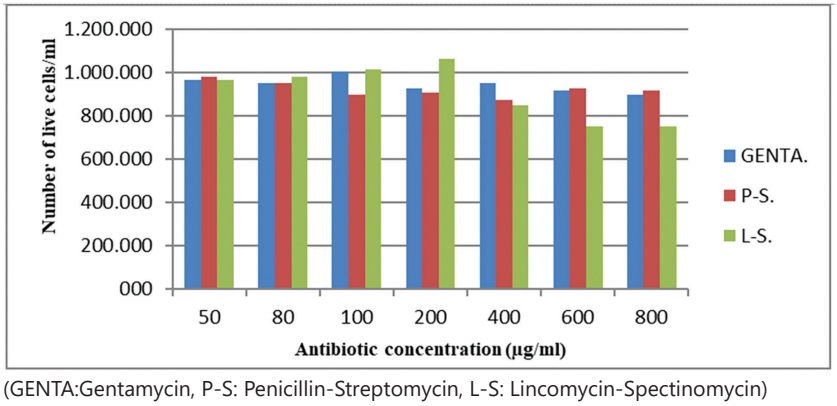

**Graphic 4.** Evaluation of the first monolayer cell cultures prepared with enrofloxacin media

(GENTA:Gentamycin, P-S: Penicillin-Streptomycin, ENRO: Enrofloxacin)



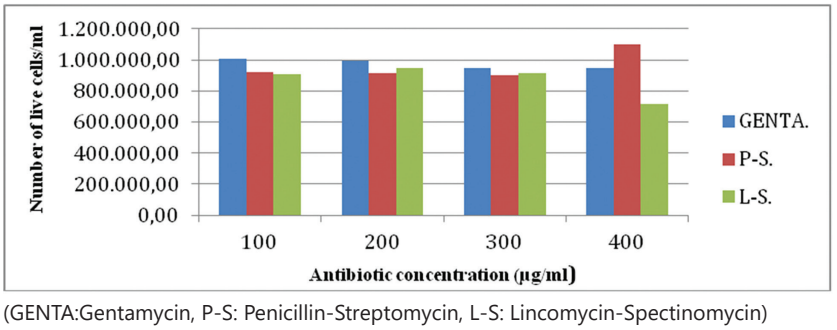

**Graphic 5.** Evaluation of second monolayer cell cultures prepared with enrofloxacin media

(GENTA:Gentamycin, P-S: Penicillin-Streptomycin, ENRO: Enrofloxacin)




**Graphic 6.** Evaluation of first monolayer cell cultures made with media prepared with linco-spectin

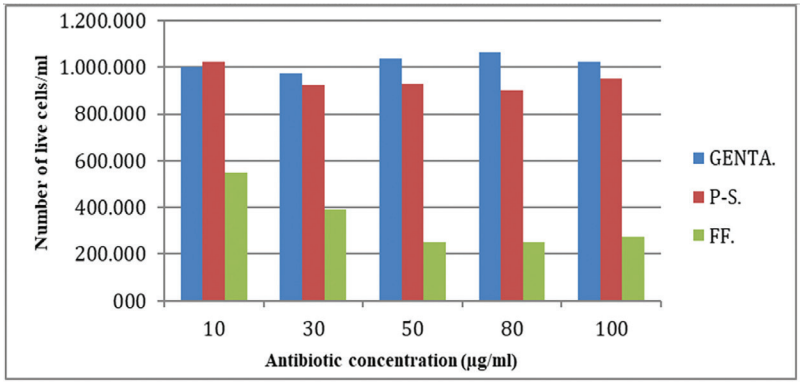
(GENTA:Gentamycin, P-S: Penicillin-Streptomycin, L-S: Lincomycin-Spectinomycin)



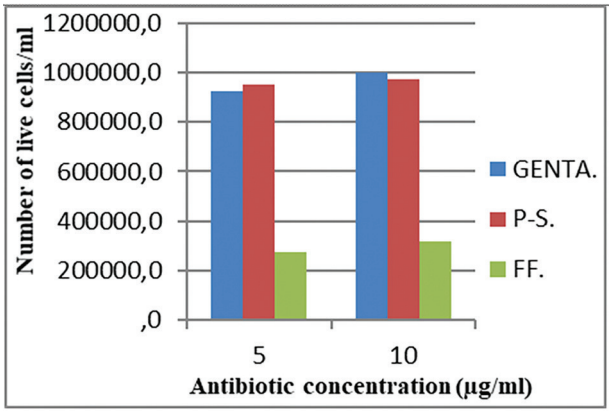

**Graphic 7.** Evaluation of second monolayer cell cultures prepared with linko-spectin-media

(GENTA:Gentamycin, P-S: Penicillin-Streptomycin, L-S: Lincomycin-Spectinomycin)

***Florfenicol:*** At the end of the passages of the BHK-21 An30 cell propagated with media at the concentrations of florfenicol at 5 µg/ml and 10 µg/ml, the number of cells was too below according to average number of control cells (Graph. 8). Even in the first passage of cell at concentrations of florfenicol at 30 µg/ml and above, the cell morphologies were disrupted and cells could not adhere into the flask surface. In addition, the degenerations increased in the following passages (Graph. 9).




**Graphic-8.** Evaluation of first monolayer cell cultures prepared with florfenicol media (GENTA:Gentamycin, P-S: Penicillin-Streptomycin, FF: Florfenicol)




**Graphic-9.** Confluent ratio and cell numbers in second monolayer cell cultures made with florfenicol media (GENTA:Gentamycin, P-S: Penicillin-Streptomycin, FF: Florfenicol)

***Effects of antibiotics on the FMD 146S virus particles:*** Following inoculation of FMD A, O and ASIA-1 serotypes into cell cultures within 25 $cm^2$ flasks at the 3rd passage level, the CPE formation belonging to FMD virus and pH of the medium was observed in the infected flasks during the incubation period. Serotypes A/TUR/11, O/TUR/07 and Asia-1/TUR/15 of the FMD viruses reached 100% CPE levels approximately at the 30, 21 and 24th hours of incubation, respectively.

The average amount of the FMD 146S virus particles in suspensions produced with control group antibiotics (Gentamicin, penicillin-streptomycin, medium without antibiotics) were obtained as 0.51, 0.50, 0.50 µg/ml for A/TUR/11; 0.53, 0.55, 0.53 µg/ml for O/TUR/07 and 0.34, 0.32, 0.30 µg/ml for ml and Asia-1/TUR/15. FMD 146S viral particles in virus suspensions produced containing enrofloxacin were determined to be 0.49 µg/ml, 0.53 µg/ml and 0.30 µg/ml for A/TUR/11, O/TUR/07, and Asia-1/TUR/15 respectively (Table 1).

**Table 1.** Amounts of FMD 146 S viral particles in virus suspension produced with control group and media with enrofloxacin.

| Treatment Groups | Levels of 146S viral particles FMD serotypes (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A Tur.11 | | | | O Tur. 07 | | | | Asia-1 Tur.15 | | | |
| | Mean value (X) | SX | SEM | P value | Mean value (X) | SX | SEM | P value | Mean value (X) | SX | SEM | P value |
| Gentamicin | 0,510 | 0,02 | 0,008 | | 0,530 | 0,04 | 0,015 | | 0,340[a] | 0,03 | 0,012 | |
| Penicillin-Streptomycin | 0,504 | 0,02 | 0,008 | 0,61 | 0,554 | 0,02 | 0,011 | 0,44 | 0,321[ab] | 0,02 | 0,010 | 0,017 |
| Media without antibiotics | 0,502 | 0,02 | 0,008 | | 0,535 | 0,02 | 0,009 | | 0,304[ab] | 0,03 | 0,012 | |
| Enrofloxacin (15µg/ml) | 0,492 | 0,02 | 0,010 | | 0,53 | 0,02 | 0,010 | | 0,300[b] | 0,03 | 0,012 | |

SX: Standart Deviation, SEM: Standart Error Mean, p: Significance
a,b: Mean values within the same column carrying different superscripts are significantly different at p<0,05.

Average titers of FMD 146S virus particles propagated by the media, including the control group antibiotics (gentamicin, penicillin-streptomycin, median without antibiotics) were obtained at 0.51, 0.50, 0.50 µg/ml for A/TUR/11, 0.51, 0.55, 0.53 µg/ml for O/TUR/07 and 0.38, 0.34, 0.30 µg/ml for Asia-1/TUR/15. The mean amount of FMD 146S virus particles produced with media containing linco-spectin were determined to be 0.46, 0.47 and 0.28µg/ml for A/TUR/11, O/TUR /07 and Asia-1/TUR /15 respectively (Table 2).

**Table 2.** Amount of the FMD 146 S viral particles in virus suspension produced with control group and media with linco-spectin.

| Treatment Groups | Levels of 146S viral particles FMD serotypes (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A Tur.11 | | | | O Tur. 07 | | | | Asia-1 Tur.15 | | | |
| | Mean Value (X) | SX | SEM | p value | Mean Value (X) | SX | SEM | p value | Mean Value (X) | SX | SEM | p value |
| Gentamicin | 0.510$^{a}$ | 0.038 | 0.014 | 0.02 | 0.512$^{ab}$ | 0.037 | 0.014 | 0.03 | 0.380$^{a}$ | 0.02 | 0.010 | 0.001 |
| Penicillin-Streptomycin | 0.500$^{ab}$ | 0.035 | 0.013 | | 0.550$^{a}$ | 0.047 | 0.017 | | 0.340$^{ab}$ | 0.04 | 0.016 | |
| Media without antibiotics | 0.500$^{ab}$ | 0.035 | 0.035 | | 0.530$^{a}$ | 0.040 | 0.015 | | 0.300$^{bc}$ | 0.03 | 0.013 | |
| Linco-Spectin (300µg/ml) | 0.460$^{b}$ | 0.039 | 0.034 | | 0.470$^{b}$ | 0.030 | 0.011 | | 0.280$^{c}$ | 0.02 | 0.010 | |

SX: Standart Deviation, SEM: Standart Error Mean, p: Significance
a,b,c: Mean values within the same column carrying different superscripts are significantly different at p<0,05.

## The infective titer of the FMD virus

***Effect of enrofloxacin on FMD infective virus titer:*** The average infective titers of the control group antibiotics (gentamicin, penicillin-streptomycin, median without antibiotics) were found to be $10^{7.39}$, $10^{7.46}$, $10^{7.06}$ pfu/ml for A/TUR/11; $10^{6.24}$, $10^{6.42}$, $10^{6.49}$ pfu/ml for O/TUR/07 and $10^{7.69}$, $10^{7.73}$, $10^{7.84}$ pfu/ml for Asia-1/TUR/15 respectively. The mean infective titers of FMD serotype A/TUR/11, O/TUR/07 and Asia-1/TUR/15 propagated with medium containing enrofloxacin were determined to be $10^{7.04}$, $10^{6.04}$ and $10^{7.26}$ pfu/ml (Table 3).

**Table 3.** The infective titers of FMD virus serotypes propagated by media with Enrofloxacin

| Treatment Groups | Levels of the infective titers of FMD serotypes (pfu/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A Tur.11 | | | | O Tur. 07 | | | | Asia-1 Tur.15 | | | |
| | Mean Value (X) | SX | SEM | p value | Mean Value (X) | SX | SEM | p value | Mean Value (X) | SX | SEM | p value |
| Gentamicin | 7.392$^{a}$ | 0.034 | 0.01 | 0.001 | 6.240$^{b}$ | 0.151 | 0.05 | 0.001 | 7.69$^{a}$ | 0.22 | 0.08 | 0.002 |
| Penicillin-Streptomycin | 7.462$^{a}$ | 0.172 | 0.06 | | 6.420$^{a}$ | 0.124 | 0.04 | | 7.73$^{a}$ | 0.15 | 0.05 | |
| Media without antibiotics | 7.061$^{b}$ | 0.180 | 0.06 | | 6.490$^{a}$ | 0.160 | 0.06 | | 7.84$^{a}$ | 0.25 | 0.09 | |
| Enrofloxacin (15µg/ml) | 7.040$^{b}$ | 0.252 | 0.09 | | 6.042$^{c}$ | 0.184 | 0.06 | | 7.26$^{b}$ | 0.16 | 0.06 | |

SX: Standart Deviation, SEM: Standart Error Mean, p: Significance
a,b,c: Mean values within the same column carrying different superscripts are significantly different at p<0,05.

***Effect of Linco-Spectin on FMD infective virus titer*** Infective titers of FMD virus produced with control group antibiotics (gentamicin, penicillin-streptomycin, medium without antibiotics) of A/TUR/11, O/TUR/07 and Asia-1/TUR/15 were determined to be $10^{7.31}$, $10^{7.35}$, $10^{7.16}$ pfu/ml; $10^{6.24}$, $10^{6.51}$, $10^{6.74}$ pfu/ml and $10^{7.71}$, $10^{7.77}$, $10^{7.70}$ pfu/ml respectively. Infective titers of the FMD virus cultured containing linco-spectin were determined as Log $10^{7.25}$, $10^{6.59}$ and $10^{7.60}$ pfu/ml for A/TUR/11, O/TUR/07 and Asia-1/TUR/15 respectiveliy (Table 4).

**Table 4.** The infective titers of FMD virus serotypes propagated by media with linco-spectin

| Treatment Groups | Levels of the infective titers of FMD serotypes (pfu/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A Tur.11 | | | | O Tur. 07 | | | | Asia-1 Tur.15 | | | |
| | Mean Value (X) | SX | SEM | p value | Mean Value (X) | SX | SEM | p value | Mean Value (X) | SX | SEM | p value |
| Gentamicin | 7.301 | 0.113 | 0.04 | 0.32 | 6.241b | 0.06 | 0.02 | 0.002 | 7.71 | 0.16 | 0.06 | 0.59 |
| Penicillin-Streptomycin | 7.350 | 0.142 | 0.05 | | 6.510a | 0.261 | 0.09 | | 7.77 | 0.25 | 0.09 | |
| Media without antibiotics | 7.160 | 0.330 | 0.12 | | 6.741a | 0.185 | 0.07 | | 7.70 | 0.27 | 0.10 | |
| Linco-Spectin 300µg/ml | 7.250 | 0.093 | 0.03 | | 6.590a | 0.284 | 0.10 | | 7.60 | 0.21 | 0.07 | |

SX: Standart Deviation, SEM: Standart Error Mean, p: Significance
a,b: Mean values within the same column carrying different superscripts are significantly different at p<0,05.

## Statistical Analysis

In the comparison made according to Table 1, there was no statistical difference between the A/TUR/11 and O/TUR/07 serotypes FMD 146S virus particles average values and it was observed that they showed a similar effect with each other. In Asia-1/TUR/15 serotype; the highest values were obtained from the medium containing gentamicin. A statistically significant difference was found between the group containing gentamicin and the medium containing enrofloxacin (15 µg/ml), but it was observed that the average values of all four groups were quite close to each other (Table 1). According to Table 2, the A/TUR/11 and O/TUR/07 serotypes FMD 146S virus particles average values of Linco-Spectin (300 µg/ml) were determined as 0.46 and 0.47 µg/ml, respectively. In Asia-1/TUR/15 serotype, the mean value was found to be slightly lower at 0.28 µg/ml compared to the other two serotypes. Although there are some statistical differences in FMD 146S virus particle's average values of the four groups, it has been observed that the values are close to each other.

In infective titer values of enrofloxacin (15 µg/ml), slightly lower titer values were found compared to other groups (Table 3). There was no statistical difference for serotypes A/TUR/11 and Asia-1/TUR/5 in comparison of infective titer values of linco-Spectin (300 µg/ml) with other groups. For the serotype O/TUR/07, similar titer values were detected for Linco-Spectin, penicillin-streptomycin, and antibiotic-free medium, However, lower infective titer values were obtained in the group containing only gentamycin as an antibiotic (Table 4).

Consequently, in the virus growth media with enrofloxacin 15 µg/ml and linco-Spectin 300 µg/ml used in the study, it was statistically shown that they do not harm the FMD 146S virus particle and that they produce very close infective titer values with penicillin-streptomycin and gentamicin. With the obtained results, it was found that enrofloxacin 15 µg/ml and linco-Spectin 300 µg/ml levels can be used in cell growth media. It was concluded that florfenicol should not be used in cell and virus cultures because of its toxic effects even at the 5 µg/ml concentration on cells, and it was not included in the statistical analysis.

## Discussion and Conclusion

The most widely used antibiotics in cell cultures are; penicillin (50-100 IU/ml), streptomycin (50-100µg/ml), gentamicin sulfate (50-100 µg/ml). In addition, kanamycin sulfate (100 µg/ml), neomycin sulfate (50 µg/ml), ampicillin (100 µg/ml), carbenicillin (0.1-75 µg/ml), chloramphenicol (5 µg/ml), ciprofloxacin (10 µg/ml) and tetracycline (10 µg/ml) are commonly used antibiotics. These rates recommended for antibiotics were determined for serum-containing media. These rates are usually reduced by 50 % due to the increased cytotoxic effect of antibiotics in serum-free conditions. Besides, the effects of antibiotics are tried to be expanded by combining antibiotics to create a synergistic effect (Perlman, 1979).

FMD, one of the most devastating diseases of livestock, can cause significant economic losses worldwide and remains the most important constraint to international trade in live animals and animal products (Çokçalışkan et al, 2016). FMDV is divided into seven serotypes with no cross-protection conferred among the serotypes (Xiao et al. 2016). Vaccination is very important in combating FMD disease. Production of vaccines against FMD disease is carried out in suspended BHK cell cultures. One of the most important problems encountered during vaccine production is bacterial contamination of cell cultures. The use of antibiotics as a preservative

against bacterial contamination, which is one of the biggest problems in the production of cell cultures, has become imperative especially in large-scale systems. Gentamycin, penicillin, and streptomycin are routinely used for protective purposes against bacterial contamination in the production of large volumes of FMD vaccine. However, although these antibiotics (gentamicin, penicillin, and streptomycin are used in FMD vaccine production, bacterial contaminations due to bacteria resistant to these antibiotics are frequently observed. Therefore, this study was needed. This study was carried out to provide the use of enrofloxacin, linco-spectin, and florfenicol antibiotics as an alternative to antibiotics (gentamicin, penicillin, and streptomycin) for different times in current use. For this purpose, cytotoxicity tests were performed during the cell, and virus cultures were determined and results showed that the non-toxic upper limit of enrofloxacin was 15 µg/ml, for linco-spectin 200 µg/ml while florfenicol had a toxic effect even at very low concentrations.

Uphoff et al. (2012), used 25 µg/ml enrofloxacin (Baytril), for the treatment of mycoplasma contamination in cell culture. Liu et al. (2015) reported enrofloxacin related cytotoxic effects and induction of apoptosis for the treatment of Ctenopharyngodon idellus in the hepatic cell line. The cytotoxic effect was directed by apoptosis and this apoptosis occurs in a dose-dependent manner. Besides, in the cultures performed with the same researchers with different concentrations of enrofloxacin (12.5-200 µg/ml), apoptosis was induced at doses after 50 µg/ml and the cytotoxic effect was observed at continued doses, especially 200 µg/ml.

Lim et al. (2008), in their study on the effects of enrofloxacin on canine tendon cells and invitro chondrocyte proliferation, enrofloxacin was used in the concentration range of 10-200 µg/ml. enrofloxacin inhibited cell proliferation depending on dose and time. Also, the same investigators demonstrated that high concentrations of enrofloxacin (200 µg/ml) induce DNA degradation and apoptosis. Egerbacher et al. (2001), conducted another study showed the dose-dependent effect of enrofloxacin.

The effect of enrofloxacin and ciprofloxacin in dog and horse chondrocyte cells was investigated in vitro, enrofloxacin tested between 10 and 150 µg/ml concentrations. Quinolones have been reported to reduce cell adhesion to culture vessels at increasing concentrations, creating an irregular integrin signal, thereby affecting cellular changes (Egerbacher et al. (2001). In this study, the limit determined for enrofloxacin (15 µg/ml) below the toxic threshold reported by other studies.

Hu et al. (1989), reported that There was no negative effect of lincomycin on cell viability. In a study on human melanocytes (Naughton et al. 1983), spectinomycin was used in addition to gentamicin and penicillin as a preservative in cell production media. In our study, since the linco-spectin did not harm cell viability at a higher concentration (300 µg/ml) compared to other antibiotics, the results are consistent with other studies.

The concentration of highly immunogenic and resistant 146S particles in raw materials is of great importance for FMD vaccine production (Doronin et al. 2019). Vaccine doses to be given to animals in FMD vaccine production are calculated in µg/ml of FMD 146S virus particles. In this study, the average FMD 146S virus particle amounts obtained in the vaccine suspension produced by the virus-producing medium containing gentamicin, penicillin-streptomycine) (A/TUR/11, O/TUR/07 and Asia-1/TUR/15; were 0.46 µg, 0.47 µg/ml and 0.28 µg/ml respectively and 146S FMD virus particle amounts with 300 µg/ml linko-spectin containing medium produced A/TUR/11, O/TUR/07 and Asia-1/TUR/15; were 0.46 µg/ml, 0.47 µg/ml, 0.28 µg/ml respectively. The results were very close to each other.

The amount of 146S FMD virus particles obtained with medium containing enrofloxacin (15 µg/ml) and the amount obtained with the medium of penicillin-streptomicine (0.44 µg/ml) was close regarding 146S (0.45 µg/ml) amount. No negative effect of enrofloxacin on the formation of 146S virus particles of the FMD virus was seen. Besides, the infective titers of FMD virus serotypes produced with medium contained Enrofloxacin (15 µg/ml) were similar to the gentamicin and penicillin-streptomycin-contain medium's FMD virus titers in this study. Results showed that enrofloxacin did not have an inhibitory effect on the production of the FMD virus at a dose of 15µg/ml.

As a conclusion, the use of enrofloxacin and linco-spectin up to the concentrations of 15 µg/ml and 300 µg/ml in media, for BHK-21 An30 cell culture in the FMD vaccine production process is safe, contaminations from bacteria in the spectrum of these antibiotics can be prevented in cell cultures. In this way, it has been concluded that the economic burden caused by contaminations would be prevented; so the antibiotics will contribute to minimizing the loss of labor.

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**Author Contributions:** VG and BBO designed the experiments, BBÖ, VG, NT, ŞY, YG, AU and FS carried out the experiments, VG, MH and GÖ 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, ethical committee permission was not obtained due to lack of study on experimental animals.

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