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Original Article
Özgün Araştırma

Determining the potency of vaccines containing *Clostridium perfringens* epsilon toxoid via toxicity analysis in MDCK cell lines

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Abstract: The potency control of the clostridium vaccines containing epsilon toxoid is performed with the Toxin Neutralization Test (TNT). Although TNT is a standard method, it has some disadvantages in terms of animal welfare and method validation studies. For this reason, the development and application of alternative methods are encouraged. However, purification and standardization of antigen, antibody and reference standards in serological-based in vitro methods still remain a significant problem. In this respect, cell culture analyses performed using similar reagents are considered possible alternatives in potency tests of the clostridial vaccines. Based on this approach, epsilon antitoxin levels in the sera of vaccinated rabbits were determined by cytotoxicity assay in mouse TNT and Madin-Darby Canine Kidney (MDCK) cell cultures. The toxicity response in cell culture is similar to the response in mice. A high correlation and a good linear relationship were observed in the results of the two methods. There were no non-specific reactions. These results show that, in potency tests of vaccines containing *Clostridium perfringens* epsilon toxoid, in vitro MDCK cell line seroneutralization assay can be successfully used instead of in vivo mouse TNT.

Keywords: Clostridial vaccine, Epsilon toxin, MDCK, Potency, TNT

Clostridium perfringens epsilon toksoid içeren aşılarda MDCK hücre hatlarında toksisite analizi ile potensin belirlenmesi

Özet: Epsilon toksoid içeren klostridium aşılarının potens kontrolü Toksin Nötralizasyon Testi (TNT) ile yapılmaktadır. TNT standart bir metot olmakla birlikte hayvan refahı ve metot doğrulama çalışmaları açısından dezavantajları olan bir testtir. Bu nedenle alternatif metotların geliştirilmesi ve uygulanması teşvik edilmektedir. Ancak serolojik tabanlı in vitro metotlarda antijen, antikor ve referans standartların saflaştırılması ve standardizasyonu halen önemli bir sorun olarak durmaktadır. Bu bakımdan benzer reaktifler ile gerçekleştirilen hücre kültürü analizleri klostridial aşıların potens testlerinde olası alternatifler içinde görülmektedir. Bu yaklaşımla aşılanan tavşan serumlarında epsilon antitoksin düzeyi fare TNT ve Madin-Darby Canine Kidney (MDCK) hücre kültürü sitotoksisite analizi ile belirlenmiştir. Hücre kültüründeki toksisite yanıtı farelerdeki yanıta benzerdir. İki metodun sonuçları arasında yüksek bir korelasyon ve iyi bir lineer ilişki gözlenmiştir. Non-spesifik reaksiyon olmamıştır. Bu sonuçlar *Clostridium perfringens* epsilon toksoid içeren aşıların potens testlerinde, in vivo fare TNT yerine, in vitro MDCK hücre hattı seronötralizasyon analizinin başarıyla kullanılabileceğini göstermektedir.

Anahtar kelimeler: Epsilon toksin, Klostrial aşı, MDCK, Potens, TNT

Introduction

It is known that enterotoxemia, which is among the important diseases of farm animals, causes post-natal offspring deaths and heavy economic losses in the meat and dairy industry all over the world, including Turkey. However, due to the extensive availability of the microorganism in nature, its actual eradication is not possible. For this reason, prophylactic measures come to the fore in the fight against this disease. Vaccine-induced humoral immunity plays a role in protection from the infection. Inactivated bacterin-toxoid veterinary biological products are used for vaccination of target species (Prescott, 2013). Inactive toxoids used for immunization are expected to be non-toxic (i.e. the harmful effects of the toxin have been removed), do not contain live microorganisms (i.e. lacking cellular elements that can reproduce and produce toxins in the future), and potent (i.e. having the ability to create a protective immune response in vaccinated individuals) (Bonistalli, 2013).

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The potency of enterotoxemia vaccines is measured via toxin neutralization test (TNT) which is a standard procedure. This test is an animal procedure based on the principle of determining the functional and neutralizing antibody level produced against the toxoid in immunized animals (rabbits) in the presence of a reference toxin administered to the test animals (mice) by comparing the antibody level to a standard antitoxin of known strength (CFR, 2019; EDQM, 2022a). The level of antitoxin in sera can be calculated by observing the degree of neutralization of the lethal effect of the toxin in test animals. However, TNT is a test that inherently requires the use of many experimental animals. Furthermore, similar to all in vivo methods, it is possible to obtain variable or invalid test results in TNT depending on many factors such as the particular condition of the animal, care and feeding conditions, hygienic circumstances, environmental factors and execution skills of the operator. These factors can lead to the repetition of tests and a further increase in the number of animals used. Besides, conducting such tests based on severe clinical findings such as lethality or paralysis does not comply with ethical principles (The 3Rs concept) that aim to improve developing animal welfare, avoid painful practices in animals, and reduce the number of animals used in testing. These tests are also disadvantageous in terms of time, cost and labour (Redhead et al., 2011; Romberg et al., 2012). Current technical difficulties and prominent bioethical approaches lead to the widespread use of alternative methods, which limit the use of experimental animals in determining the potency of such vaccines (Erbaş, 2011; Hill, 2011; Lang et al., 2018). In the veterinary field, it is emphasized that especially vaccines for rabies, *Leptospira sp.*, Foot and Mouth disease, Newcastle disease, and Clostridium in addition to poultry and fish vaccines should be considered priority issues (Kulpa-Eddy et al., 2011; Romberg et al., 2012).

Among alternative approaches to the potency testing of vaccines, there are serological methods based on monoclonal or polyclonal antibodies, methods based on the measurement of the amount of cellular or flagellar antigen in the vaccine and toxicity tests on cell cultures. Although successful results have been obtained in some vaccine groups with both antibody-based ELISA methods and methods based on the measurement of antigen amount, the preparation, purification and standardization of mono/polyclonal antibodies and antigen elution procedures in these methods still remain as essential difficulties in application. (Kulpa-Eddy et al., 2011; Stokes et al., 2011; Romberg et al., 2012). In addition, non-specific reactions are a significant risk in these tests. It is also known that deficiencies in antibody maturation or the presence of low-affinity antibodies can cause significant deviations in the results of antigen-antibody-based tests. (Crowthwer, 2009).

Titration methods based on the neutralization of the cytotoxic effect of toxins in cell lines stand out among alternative approaches because they are similar to the animal model and do not require specific purified antibodies or antigen separation procedures (Borrmann et al, 2006; Souza Junior et al, 2010; Sinitskaya, 2015). However, due to the lack of a standard methodology, cell-based neutralization tests are developed as in-house procedures and applied after a verification process. Furthermore, studies on the cytotoxicity of *Clostidium perfringens* epsilon toxin (ETX) in cell lines have shown that MDCK epithelial cell lines are very sensitive to this toxin. It is known that the toxin, which binds to the specific receptors on the cell surface of MDCK without entering the cell, causes damage to the cell membranes and by changing the ion balance of the cell, it causes the mitotic activity to be stopped in polarized cells and consequently, the cytopathic effect takes place. Therefore, it is accepted that the MDCK cell line constitutes a good model for studies on ETX (Petit et al., 1997; Borrmann et al., 2001; Soler-Jover et al., 2004).

This study aims to discuss the feasibility of using the neutralization test based on the cytotoxic effect on MDCK cell lines as an alternative to TNT in the potency tests of inactivated enterotoxaemia vaccines containing *C.perfringens* epsilon toxoid by comparing the MDCK neutralization test's results to the reference method and evaluating the correlation between the two methods.

Materials and Methods

Immunization procedure and obtaining test serums

In the present study, 31 vaccines (21 of which contain *C.perfringens* epsilon toxoid and 10 of which do not) from different manufacturers were used. Healthy rabbits (3-6 months old New Zealand breed) with no previous vaccination history were used for immunization. For each product, 8-10 rabbits were subcutaneously vaccinated twice 21-28 days apart at the recommended dose and 12-14 days after the second vaccination, blood was drawn from the rabbits and their serum was separated. Equal amounts

of pooled blood serum were aliquoted for TNT and MDCK neutralization tests and stored at -20ºC (CFR, 2019; EDQM, 2022a).

Mouse toxin neutralization test procedure

TNT was performed on mice (18-22 g Swiss Albino breed). For this purpose, *C.perfringens* type D epsilon standard toxin and standard antitoxin preparations (*USDA-APHIS*) were diluted at a lot-specific amount, toxin doses of 10 L_0 and 10 L_+ per milliliter were prepared, and the antitoxin was adjusted to be in a certain international unit (IU) per milliliter. Sample sera were diluted taking into account the tested IU antitoxin level. For the test, mixtures of 10 Lo and 10 L_+ doses of standard toxin with standard antitoxin or sample sera were prepared separately and administered intravenously to mice after incubation under appropriate conditions. Mice kept under observation for 48-72 hours after injection were evaluated for signs of lethality (CFR, 2019).

Toxin neutralization method in MDCK cell culture

MDCK epithelial cells (*ATCC CCL-34*) were plated at a concentration of approximately $3x10^5$ cells/ml. After lot-specific dilutions of standard toxin and antitoxin (*USDA-APHIS*) were made, further dilutions specific to the cell culture study were made. Test sera were diluted in parallel to the target IU and antitoxin dilution. On each plate, toxin-antitoxin control, cell control and toxin control wells along with unknown test samples were placed (Figure-1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard Antitoxin					Blank		Unknown Serum 1				
B	Std. Toxin 1/2100	Std. Toxin 1/1800	Std. Toxin 1/1500	Std. Toxin 1/1200	Std. Toxin 1/900			Std. Toxin 1/2100	Std. Toxin 1/1800	Std. Toxin 1/1500	Std. Toxin 1/1200	Std. Toxin 1/900
C						Cell Control						
D												
E	Unknown Serum 2					Toxin Control		Unknown Serum 3				
F	Std. Toxin 1/2100	Std. Toxin 1/1800	Std. Toxin 1/1500	Std. Toxin 1/1200	Std. Toxin 1/900			Std. Toxin 1/2100	Std. Toxin 1/1800	Std. Toxin 1/1500	Std. Toxin 1/1200	Std. Toxin 1/900
G						Blank						
H												

Figure 1. Plate layout in MDCK cell culture analysis

Gradually diluted standard toxin (Figure 1) was added to standard antitoxin or unknown serum samples and left for incubation. EMEM (*Sigma M4655*) was added to the live cell control wells, and standard toxin was added to the toxin control wells (Figure-1). After the completion of the incubation process, the mixtures in this plate were transferred in the same order to the cell culture plate (where cell growth was 90-100% in all wells) removed from the CO_2 incubator. After the incubation, the plate was stained with crystal violet, passed through isopropyl alcohol for resuspension of viable cells and optical densities were read at 590 nm via an ELISA reader (*BioTek Epoch*).

In the interpretation of the test results, the mean OD of the live cell control wells was found and the 50% cut-off value was calculated. In addition, the mean ODs of standard antitoxin and unknown serum samples were found. Wells with a mean OD greater than the live cell control 50% cut-off value were considered viable, and wells smaller than 50% cut-off were considered dead. The epsilon antitoxin level in unknown serum samples was determined by comparison with known antitoxin columns (USDA, 2016).

Statistical Evaluation

Statistical evaluations were made using SPSS *(Version 24)*. The difference between mouse and cell culture toxin neutralization test results was evaluated by t test (two-tailed, $p<0.05$). Correlation between results was calculated using the Pearson correlation method (99% confidence level, two-tailed). In addition, the linear regression between the results of the two methods was shown.

Results

In MDCK cell analyses, it was observed that depending on the antitoxin level in positive (vaccinated with *C.perfringens* toxoid) serum samples, different con-

centrations of the toxin were neutralized, the cells remained alive in proportion to the degree of neutralization, and the cells that remained viable were stained dark blue with crystal violet and gave a high OD (Figure 2, Test serum samples II and IV). It was decided that the cells in the wells with an OD above the cut-off value remained alive (Figure 3). It was observed that the staining intensity and, thus, the size of the OD values gradually decreased in proportion to the decrease in the density of viable cells due to the increasing toxin concentration.

In the toxicity analysis of negative (unvaccinated with *C.perfringens* toxoid) samples in MDCK cells, since there was no antitoxin in the serum, death occurred in the cells due to the toxin, and in the staining performed with crystal violet, because these wells did not take dye (Figure 2, Test serum sample III), it was seen that they gave low ODs below the cut-off value (Figure 3).

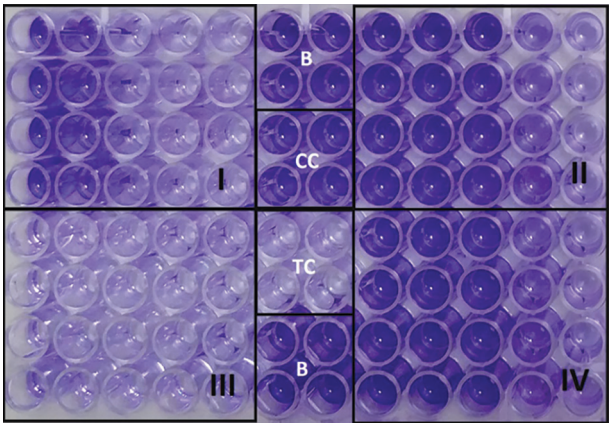

Figure 2. Plate appearance in MDCK cell culture toxicity analysis (CC: cell control, TC: toxin control, B: Blank, I: Toxin-antitoxin control, II, III and IV: Test samples (Serum II and IV indicate the presence of antitoxin at the tested level, serum III indicates the absence of antitoxin at the tested level)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.713 (LC)	0.884 (DC)	0.622 (DC)	0.605 (DC)	0.570 (DC)	Blank		3.0388 (LC)	2.862 (LC)	1.8603 (LC)	0.84 (DC)	0.5258 (DC)
B												
C						2.950						
D												
E	0.683 (DC)	0.524 (DC)	0.475 (DC)	0.452 (DC)	0.407 (DC)	0.196		2.9985 (LC)	1.977 (LC)	0.920 (DC)	0.3935 (DC)	0.2978 (DC)
F												
G						Blank						
H												

Figure 3. Determination of viable cell wells (above the 50% cut-off value of 1.475) in the MDCK cell culture toxicity assay (1-5/A-D: standard toxin-standard antitoxin control (I), 8-12/A-D: test serum (II), 1-5/E-H: test serum (III), 8-12/E-H: test serum (IV), 6-7/C-D: EMEM cell control, 6-7/E-F: toxin control; LC: live cell, DC: dead cell

When the cells were evaluated microscopically, it was noted that the epithelial cells forming the healthy MDCK cell line were formed as monolayer, spindle-shaped cells attached to each other without leaving any spaces between them, and exhibited a uniform appearance (Picture 4-A). In epithelial cells treated with different concentrations of toxin, it was seen that the uniform appearance and cellular borders disappeared, the monolayer structure was distorted, the cells became rounded and separated from each other, wide spaces appeared between them, and their nuclei showed a pycnotic appearance (Picture 4-B). It was observed that the extent of cellular destruction increased depending on the toxin concentration.

The results obtained from in vivo mouse TNT and in vitro MDCK cell culture toxicity analyses of clostridial serum samples containing Epsilon antitoxin are shown in Table-1. The results show that close titration data were obtained with TNT and MDCK cell culture analyses and there was no statistical difference between the results (t test, $p>0.05$). A high correlation ($r=0.952$, $p<0.01$, two tailed) between the two analyses was found (Table-1). A good linear relationship ($y=1.007x+0.994$, $R^2=0.906$) was observed between methods by the analysis of sera at different concentrations (2-20 IU/ml) (Figure-5). No protective response was observed in the TNT and MDCK toxicity analysis of 10 vaccines that did not contain epsilon toxoid (Table-1).

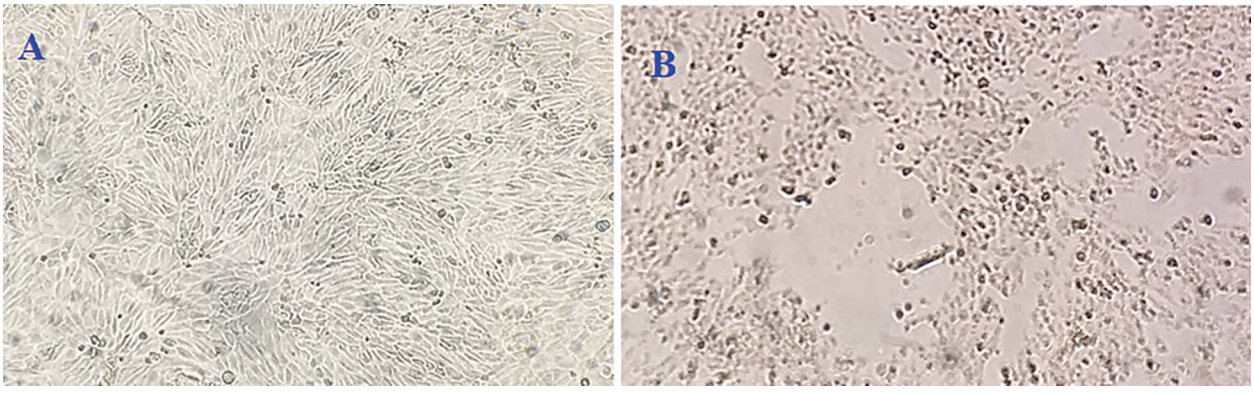

Figure 4. Microscopic view in live (A) and toxin-treated (B) MDCK cell culture

Table1. Comparison of TNT and MDCK neutralization test results

Test	Positive Samples		Pearson correlation coefficient (r)	Negative Samples	
	Number of samples (n)	Average Result (İÜ/ml)		Number of samples (n)	Result
TNT	21	7.36	0.952b	10	(-)
MDCK	21	8.40a		10	(-)

[a] p>0.05 [b] p<0.01

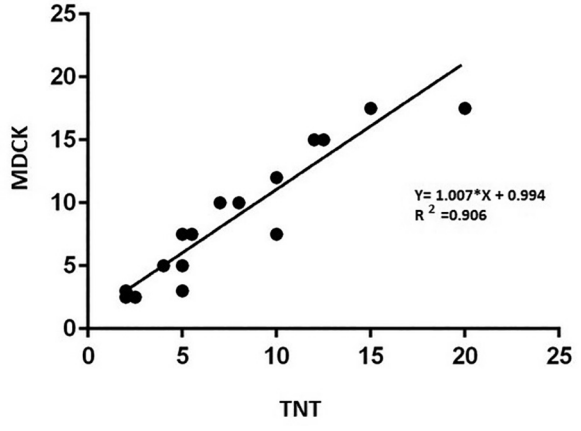

Figure 5. Linear regression curve between TNT and MDCK cytotoxicity assays

Discussion

In accordance with the provisions of the "European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes"; it is expected that a minimum number of animals or none should be used in testing and procedures that are less painful or do not cause permanent damage should be adopted (EDQM, 2022b). For these purposes, it is essential to promote the use of alternative test methods. The sensitivity on this issue is constantly increasing with collaborative studies and revisions in international directives (Lang ve ark, 2018, EDQM, 2022c). Clostridial vaccines have an important place in current studies. The widespread use of these vaccines in husbandry, their production in multiple combinations, their standard tests on experimental animals and the use of many animals for each antigen cause these vaccines to be among the priority targets. However, an important problem in developed serological-based tests is the difficulty in obtaining standardized antigens, antibodies or reference substances along with the extraction, identification, characterization, purification or standardization of these substances. These deficiencies often affect the precision of the analyses (Kulpa-Eddy et al., 2011; Stokes et al., 2011; Romberg et al., 2012). In this respect, procedures using a specific cell line instead of an experimental animal have significant advantages over other in vitro methods in terms of using the same standards and not requiring further purification, extraction and elution procedures (Bormann et al. 2006).

It is known that the studied canine MDCK cell line is very sensitive to epsilon toxin (Petit et al., 1997; Borrmann et al., 2001; Soler Javer et al., 2004). Interestingly, kidney cell lines (MDBK) of species naturally affected by ETX, such as sheep, goats, cattle, and kidney lines of rodents and primates are resistant to the toxin. Similarly, it is known that many cell lines, including Vero, are not sensitive to ETX (Uzal

et al., 2014; Navarro et al., 2018). In this respect, the MDCK cell line constitutes a good model for studies on *C.perfringens* epsilon toxin. It is seen that the MDCK-ATCC/CCL-34 cell line selected in this study is used in various studies on ETX (Borrmann et al., 2006, Salvarini et al., 2013). Supporting the current literature (Soler Jover et al., 2004; Stiles et al., 2013; Uzal et al., 2014), in the microscopic observations of MDCK cells exposed to the toxin, depending on the exposure level and duration, the cells lose their spindle shape and their regular adherent structures, the nuclei becomes swollen and rounded, their nuclei becomes dark and pycnotic, and the monolayer structure due to cell lysis is disrupted and intercellular spaces emerge.

The results of TNT and MDCK toxicity analysis in this study indicate that the mean of 21 positive serum samples in terms of epsilon antitoxin titre did not differ statistically between the groups and there was a strong correlation between the tests. Although limited in number, these results seem comparable to the results of different studies on epsilon antitoxin levels. Borrmann et al. (2006) in their study that compared the antibody titres in MDCK cells of 113 rabbit sera from two different vaccines with the results of the Paul Ehrlich Institute, showed a good correlation ($r=0.60$ and $r=0.72$ for vaccines A and B, respectively), although slightly lower than our results and stated that this correlation was statistically significant ($p<0.01$). In another study (Salvarini et al., 2013) with a smaller number of samples (in 7 rabbits, one of which was negative), the correlation between the results obtained with mouse and MDCK serum neutralization tests is seen to be much higher ($r=0.997$, $p<0.05$). In addition to ETX, various studies on different clostridial toxins (such as *C. septicum* alpha toxin, *C. novyi* type B alpha toxin) and different cell lines also yielded results indicating a similarly strong correlation (Borrmann et al., 2006; Redhead et al., 2011; Salvarini et al., 2010; Sinitskaya et al., 2015). All these data show that cell culture neutralization tests can be used successfully to evaluate the potency of different clostridial antigens. In addition, the data obtained from this study shows that the accuracy of the results obtained with the MDCK cell culture method is also comparable to the antibody-based ELISA methods. In a study conducted by Arslan et al. (2016) on different clostridial antigens, finding of a high correlation between TNT and ELISA methods for *C.perfringens* epsilon toxoid ($r=0.95$, $p<0.01$, $n=20$) indicates that the results of both alternative methods display parallelism among themselves.

Titration of sera containing varying concentrations of *C. perfringens* epsilon antitoxin in MDCK cell culture shows linear results to standard method of TNT. A similar relationship has been reported by various researchers between TNT and Vero cell culture seroneutralization tests for *C.septicum* alpha antitoxin (Salvarini et al., 2010; Redhead et al., 2011; Sinitskaya et al., 2015). The linear range studied (2-20 IU/ml) overlaps with antitoxin levels in clostridial vaccines containing epsilon toxoid.

It is known that various non-specific reactions can develop in ELISA analysis, independent or dependent on antigens. In addition to false positive reactions that may arise from the binding of different immunoglobulins to solid surfaces in the sample, interactions between different proteins, reactions caused by buffer components or reactions from secondary antibodies may change the selectivity of the method. Advanced purification of antigens or antibodies may be required to remove non-specific bindings, but it is still possible to obtain results that affect specificity/sensitivity (Waritani et al., 2017). Since toxin seroneutralization tests in cell culture are based on the principle that the toxin is denatured in the presence of antitoxin and does not have a harmful effect, as in in vivo toxin neutralization tests, one of the crucial advantages of these methods is that there are no concerns about non-specific reactions as in serological-based tests. In the present study, the fact that no false positive reactions were obtained in negative serum samples supports these claims. Similar results were also expressed by other researchers (Salvarini et al., 2010, 2013). The risk that may occur in this type of test is the shedding of cells due to contamination of the cell culture, low OD due to the plate not taking up the dye, and an impact on sensitivity. To prevent these contaminations, penicillin-streptomycin is added to the cell culture growth medium (EMEM). In addition, by creating toxin-antitoxin control, live cell control (only EMEM) and toxin control wells in each plate, possible factors that can change the specificity can be monitored.

Considering the toxicity of Epsilon toxin in mouse and MDCK cell cultures, the findings of this study conducted for the determination of antitoxin levels in serum samples indicate that the seroneutralization procedure performed in MDCK cell culture gives appropriate results. In addition to giving accurate and reliable results, this alternative method has significant advantages such as reducing the number of experimental animals and improving animal welfare, fast and easy application,

testing more than one serum sample on the same plate and working with similar reference standards. However, in terms of standardization of the results, it is evident that further studies should be carried out in laboratory conditions on the validation of the methods.

Ethics committee for the use of experimental animals and other ethics committee decisions and permissions: Bornova Veterinary Control Institute Experimental Animals Ethics Committee Report / 29.03.2017

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