Capripoxvirus antibodies detection: Relationship between the two methods alpha and beta of virus neutralisation test

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Abstract

Virus neutralisation test (VNT) of capripoxvirus (CaPVs) was studied to assess the post-vaccination (vaccine effectiveness) or post-infection antibodies level using two methods: alpha-VNT and beta-VNT which are generally carried out to measure the Neutralising Index (N.I.) and the serum Antibody titre (T_{Ab}) respectively. The authors have demonstrated that a positive correlation exists between N.I. and T_{Ab} values, this study aimed to add more evidence to this correlation by establishing a graph and its mathematical equations. We found that: N.I. = (1.489 Log T_{Ab}) + 1.331; this serves as a base to calculate N.I. using T_{Ab} values measured by beta-VNT without going through alpha-VNT and vice versa. At the end of this study, we evaluated the equation accuracy by two parameters; the deviation (*d*) and the error percentage, which were *d* = 0.2 and error (%) = 8%, respectively.

Keywords: Capripoxvirus, Embryonic lamb kidney cells, RM65 strain, Vaccine effectiveness, Virus neutralisation.

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Introduction

Sheep pox (SP), goat pox (GP), together with lumpy skin disease (LSD) of cattle are notifiable highly contagious viral diseases caused by viruses that are belonging to Poxviridae family, Chordopaxvirinae sub-family, and Capripoxvirus (CaPVs) genus (Buller *et al*., 2005). These are large (170-260 nm by 300-450 nm), double stranded DNA and enveloped viruses (Tulman, *et al*., 2002), they affect sheep and goat, LSD virus also affects cattle. These diseases are responsible for a significant economic loss in the endemic regions (Bowden *et al*., 2008; OIE, 2017a,b).

Symptoms of CaPVs infections are clinically manifested by hyperthermia, papular eruptions or nodules that can become vesicles (rarely), and secondarily affect the mucous membranes causing internal lesions (Fassi-Fehri *et al*., 1983; Bhanuprakash *et al*., 2006). CaPVs infections morbidity and mortality rates may be as low as 1% and go up to 100% in some outbreaks (Batta *et al*., 1999; Woldemeskel and Ashenafi, 2003).

Sheep pox virus (SPV) and goat pox virus (GPV) transmission is considered to occur primarily via respiratory aerosols (Kitching and Taylor, 1985) and by direct contact with infected animals (OIE, 2017a,b) or contaminated objects, feed and wool (Bhanuprakash, *et al*., 2006). Human infection with CaPVs has never been reported (Bhanuprakash *et al*., 2006; Haller *et al*., 2013; OIE, 2017a).

The diagnosis of CaPVs diseases can be achieved by identifying the specific clinical signs/symptoms and then confirmed in the laboratory by standard virological and/or serological methods. One of the major problems encountered in the CaPVs diagnosis is poor seroconversion, for this raison, the confirmation of the disease is generally based on the detection of capripox virions or antigens through electron microscopy, virus isolation and/or Real-time PCR (OIE, 2017a,b).

Despite the fact that immune response against CaPVs is predominantly cell-mediated, the humoral immunity also plays a role (Kitching and Mellor, 1986), so that, the serum antibodies titration can reflect the protection level of the individual animal, based on that, virus neutralisation test (VNT) is considered as reference and the unique validated serological test available that has been used to evaluate immune status in individual animals or in post-vaccinated populations, it has a strong specificity that can reach 100 % but less sensitivity between 70 % and 96 % for CaPVs, using a standard viral strain and pre-vaccination antibodies control promote the test sensitivity (Bhanuprakash *et al*., 2006).

On the other hand VNT has been assessed as suitable method to farther uses like; prevalence of infection and surveillance, detection of population and individual freedom from infection prior to movement, confirmation of clinical cases, contribute to eradication policies (OIE, 2017a).

All strains of CaPVs so far examined whether derived from cattle, sheep or goats, are antigenically similar (Tulman *et al*., 2001, 2002) and share a major antigen P32 for neutralising antibodies (Chand *et al*., 1994) so

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they are indistinguishable from each other using serology and will cross protect regardless their origin (Kitching and Taylor, 1985; Kitching *et al*., 1987; OIE, 2017a,b). Based on this similarity, several strains derived from sheep and goats were used to produce effective vaccines against LSD (Capstick, 1961; Davies, 1991).

The previous serological evidences indicate that CaPVs strains cross-react immunologically (Davies and Otema, 1981; Kitching and Taylor, 1985; Kitching and Mellor, 1986); hence, the ex-vivo serological tests can reveal the level of animal protection regardless the CaPVs strain. A Neutralising Index of ≥1.5 means the animal is protected but under that doesn't mean the animal is not (Kitching *et al*., 1986; OIE, 2017a,b).

The principle of this study is to prepare nine references sera (SR1 to SR9) by double fold dilutions from one reference serum (antiserum-LSDV) then determine their antibody titres (T_{Ab}) by beta-VNT and measure their Neutralisation Index (N.I.) by alpha-VNT. Among these two methods, beta method is commonly used and is more accurate whereas alpha method requires a large amount of serum sample.

The main objective of this study was to establish a linear trendline graph by Excel 2007; using the found values of T_{Ab} and their corresponding N.I. of the nine sera, therefore, determine their mathematical equations, based on these equations we can deduce the values of N.I. by T_{Ab} values previously determined without performing the alpha-VNT, by the same we can conclude the values of T_{Ab} via N.I values previously determined without performing the beta-VNT. Furthermore, we aimed to estimate the minimum threshold of serum T_{Ab} that theoretically protects the immunized individual animal.

To assess the accuracy of the found graph and the mathematical equations on real conditions, ten sera samples from immunized sheep were used in order to find the deviation values and the error percentage between the theoretical (by using the found equations) and the experimental results.

Materials and Methods

Cell cultures

The Algerian law prohibits the slaughter of pregnant females including species of sheep, cattle, goat, horse and camel, except in some strict conditions under the decision of the veterinarian duly authorized by the concerned authority, more details are in the Executive Decree Nº. 91-514 of 22 December 1991 on animals prohibited for slaughter (Décret exécutif n°91-514 relatif aux animaux interdits à l'abattage, 1991).

In this case, slaughtered ewes matrices were carried out randomly from the slaughterhouse (in Algiers) to laboratory to check out the presence of embryonic lambs which serve for the isolation and expansion of primary embryonic lamb kidney cells (ELK cells).

The Primary ELK cells were grown from trypsinized fetal lamb kidney cortical tissue according to the method described in FAO, Animal Production and Health paper 118 (1994) with slight modifications (Fassi-Fehri *et al*., 1983; Rweyemamu *et al*., 1994; Mirakabadi and Moradhaseli, 2013).

The ELK cells were cryopreserved for further uses following the method described in ATCC (ANIMAL CELL CULTURE GUIDE) (Ian Freshney, 2016; ATCC®, 2017). All the experiments of this study were carried out by ELK cells between the 1st and the 3rd subcultures (Fig. 1.a).

Virus strain and Antiserum

During this study, we have used the attenuated vaccine strain of sheepoxvirus RM65 (Ramyar and Hessami, 1968), previously supplied by the reference laboratory FAO for Africa of Senegalese Institute for Agricultural Research (I.S.R.A.) to Pasteur Institute of Algeria in 1995.

The Master Seed Lots inoculums (MSL) were prepared on ELK cells subcultures and stored at -70°C for further uses (Fig. 1.b, c, d, e and f). The inoculums were titrated using Spearman-Karber's method (Karber, 1931) as described by Villegas and Purchase (1989).

We used the reference positive antiserum-LSDV raised experimentally on infected cattle at the Institute for Animal Health (IAH), Pirbright Laboratory, UK, in 2011, the CaPVs Reference Laboratory of IAH provided the laboratory of production and development of viral veterinary vaccines at the Pasteur Institute of Algeria by this antiserum named: LSD+ cattle serum 2006, VN84, 37 DPI.

In order to compare the performance of positive antiserum-LSDV, a beta-VNT was embarked on an inter-laboratory exercise, it was performed at the IAH and compared with that of another reference laboratory ARC-OVI (Agricultural Research Council's-Onderstepoort Veterinary Institute), South Africa, the comparison was performed by their producers for quality control purposes (OIE, 2011).

Our results are quoted below in the results section in order to compare them with the two reference laboratories results.

Virus Neutralisation Tests (VNTs)
Beta-VNT

The beta-VNT (varying-serum/constant-virus) was performed in order to determine the T_{Ab} of the reference antiserum-LSDV, this T_{Ab} value was compared to those found by ARC-OVI and IAH (OIE, 2011) and also allows us to prepare nine sera RS1 to RS9 by two-fold dilutions of the reference antiserum-LSDV, ranging from 1/2 to 1/512 respectively, so we could calculate each serum T_{Ab} of the nine prepared sera ($\text{Log } T_{Ab\,RS2} = \text{Log } T_{Ab\,RS1} - 0.3$).

To realize the beta-VNT, the reference serum (antiserum-LSDV) was heat inactivated at 56^0C for 30

min, then two-fold serial serum dilutions were prepared (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512), the antiserum-LSDV was tested in duplicate wells (50 μl/well).

A volume of 50μl/well of the standard virus strain RM65 at a titre of 100 $TCID_{50}$ was maintained similar in the well plate, then incubated at 37°C for 90 min to promote virus-antibody adsorption, after that 100μl of cell suspension (10^4 cells/well) was added to all the 96-well plate (OIE, 2017a).

Alpha-VNT

We performed the alpha-VNT (constant-serum/varying-virus) in order to calculate the Neutralisation Index (N.I.) for the nine references sera RS1 to RS9 prepared previously (with known T_{Ab} values) according to the method described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2017a).

To realize the alpha-VNT, a constant dilution 1/5th (without FBS) was done for each serum RS1 to RS9, and also for a negative control serum that serves for comparison to calculate N.I., a 50 μl of each test serum was added to the wells of columns 1 to 9 covering the rows A to H in the 96-well plate, the inoculum of RM65 virus strain was used to prepare a serial ten-fold dilution from 10^0 (the stock inoculum) to 10^{-8} $TCID_{50}$/ml, 50 μl of each dilution was added to columns 1 to 9 respectively, eight repetitions for each virus dilution (rows A to H), the wells of columns 10, 11 and 12 represented negative cell control, FSB toxicity control and test serum toxicity control respectively, the plate was incubated at 37°C for 90 min to enhance the virus-antibody adsorption, and then 100μl of cell suspension of 10^4cell/well was added to all the 96-well plate.

The final reading of both VNT methods was under microscopic examination for Cytopathic Effect (CPE) detection according to Spearman-Karber's calculating method (Karber, 1931) after the 9th day of incubation at 37°C, 5% CO_2 and 90% of humidity.

For alpha-VNT, The logarithm antibody titre (Log T_{Ab}) represents the reciprocal value of logarithm serum dilution that protects 50% of the repetitions (N.D.$_{50\%}$), (Log10 T_{Ab} = - Log dilution).

For beta-VNT, the N.I. represents the Log_{10} titre difference between the titre of the virus in the negative control serum and in the test serum [N.I. =Log_{10} (negative control serum viral titre) -Log_{10} (test serum viral titre)] (OIE, 2017a). The virus titre was determined by the endpoint dilution that presents CPE at 50% of repetitions according to Karber method (Karber, 1931).

The N.I. and Log T_{Ab} values obtained by alpha-VNT and beta-VNT of all references sera from RS1 to RS9 were used to establish the linear graphic and find the linear equation:

N.I. = (p Log T_{Ab}) + b (Where p is the slope and b is a constant).

To assess the equation accuracy, ten sera samples from immunized sheep were used in this study to determine the deviation and the error percentage of the resulting equation.

Results

The beta-VNT performed on the antiserum-LSDV on ELK cells gave an average T_{Ab} value of 2.1 N.D.$_{50\%}$ (equivalent of the endpoint dilution 1/128). Consequently, the T_{Ab} values of the nine sera RS1 to RS9 prepared from the antiserum-LSDV by two-fold dilution and their corresponding N.I. are shown on Table 1.

The linear positive correlation between N.I. and T_{Ab} (Table 1) is displayed on the graph (Fig. 2.), this graph has a linear equation Y = a X + b hence IN = (p Log T_{Ab}) + b and Log T_{Ab} = (IN - b) / p (where p is the slope and b is a constant).

After drawing the graph by Excel 2007 the equation values were calculated automatically giving N.I. = (1.489 Log T_{Ab}) + 1.331 while the coefficient of determination R^2 = 0.987, thereby Log T_{Ab} = (N.I. − 1.331)/1.489, based on this equation we calculated the minimum threshold of serum T_{Ab} that theoretically protects the immunized individual animal against CaPVs diseases.

Furthermore, we can derive its value directly on the graph (Fig. 2) by projecting the point of N.I. taking into consideration that the animal is protected when the N.I. ≥1.5 (OIE, 2017a), so that, at N.I. = 1.5, Log T_{Ab} = 0.11 N.D.$_{50\%}$ which corresponds approximately to the endpoint dilution (1/1).

We assessed the equation accuracy by two parameters; the deviation (d) and the error percentage, through the T_{Ab} results using beta-VNT performed on ten immunsera samples from immunized sheep, the values obtained for antibody titres were used on the equation (N.I. = (1.489 Log T_{Ab}) + 1.331) as a tool to calculate the N.I. which represents the theoretical calculated N.I. (N.I.cal), these values were compared with the experimental N.I. values (N.I.exp) obtained with alpha-VNT.

The absolute deviation (d) was determined by d = |N.I.exp - N.I.cal| and the error percentages: error (%) = d / N.I.cal (Table 2).

By contemplating the N.I.exp and N.I.cal illustrated on Table 2, we can see that the maximum absolute deviation achieved was 0.37 with a maximum error percentage of 14.85%, the mathematical equation was estimated with an average absolute deviation d = 0.2, in addition, the average error percentage was equal to 8%. So that, the new mathematical equation: N.I. = p Log T_{Ab} + b ± d (where p is the slope, b is a constant and d is the average deviation).
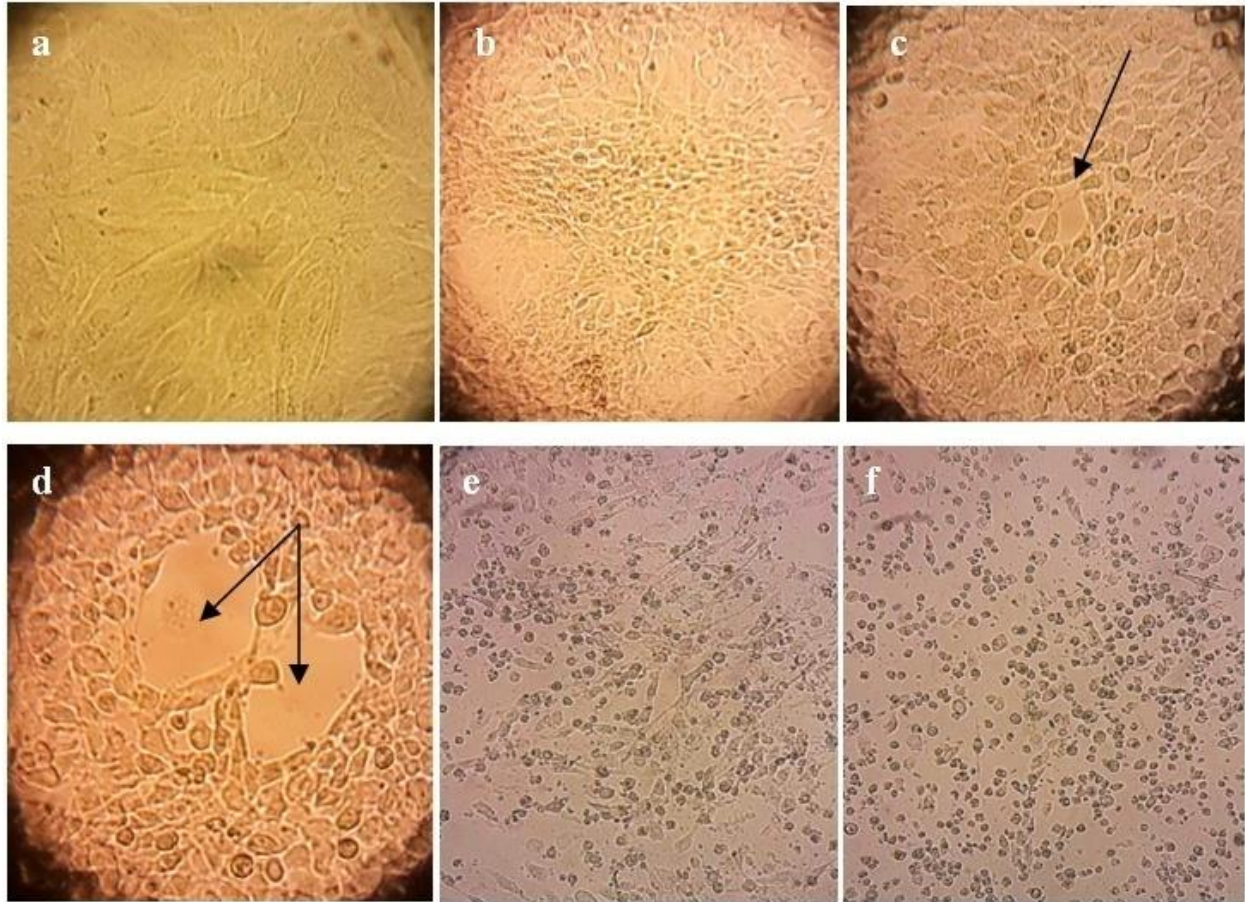
Fig. 1. Microscopic observation of uninfected and infected ELK Cells monolayer with the RM65 vaccine sheep pox virus strain. **(a)** Uninfected ELK Cells culture (100x). **(b)** Two to three days post-infected ELK Cells showing the start of cytopathic effect by cytoplasm granulation (100x). **(c and d)** Three to four days post-infected ELK Cells, the cytopathogenic effects (CPE) appeared as small islands of cell lysis (thin arrows) (100x). **(e and f)** Four to six days post-infected ELK Cells the CPE propagate gradually and cells lysis generalized on the entire cellular layer (50x).
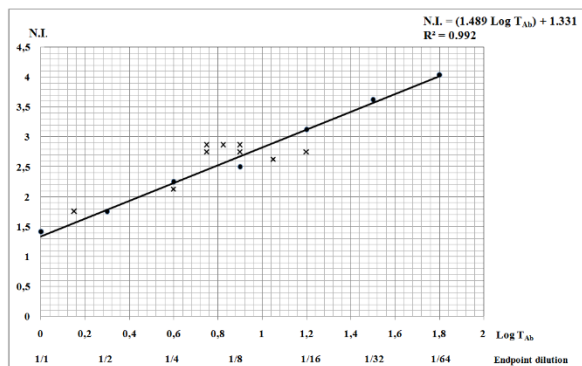
Fig. 2. The linear trendline of the Neutralisation Index (N.I.) and Log Ab Titre (T_{Ab}). N.I. = (1.489 Log T_{Ab}) + 1.331: is the linear equation of the graph. R^2 = 0.992: is the determination coefficient of the equation. (•) Point of the reference sera used to draw up the linear trendline and to establish the equation. (X) Point of the immunsera used for the equation accuracy determination.

Table 1. Log Antibody titre (T_{Ab}) for the nine references sera (RS) and their corresponding Neutralisation Index (N.I.).

Reference sera (RS)	Log T_{Ab}	Endpoint dilution	N.I.
Antiserum-LSDV	2.1	1/128	lack of RS
RS1	1.8	1/64	4.041
RS2	1.5	1/32	3.625
RS3	1.2	1/16	3.125
RS4	0.9	1/8	2.5
RS5	0.6	1/4	2.25
RS6	0.3	1/2	1.75
RS7	0	0	1.4125
RS8	0	0	1.25
RS9	0	0	0.75

Table 2. The absolute deviation (*d*) and the error percentage values (error (%)).

Sera	Log T_{Ab}	N.I.exp	N.I.cal	$d = $ |N.I.exp- N.I.cal|	error(%) = d/N.I.cal
S1	0.15	1.75	1.687	0.062	3.682
S2	0.6	2.125	2.299	0.174	7.584
S3	0.9	2.75	2.707	0.043	1.584
S4	1.2	2.75	3.115	0.365	11.711
S5	0.9	2.875	2.707	0.168	6.202
S6	0.9	2.875	2.707	0.168	6.202
S7	0.825	2.875	2.605	0.270	10.357
S8	0.75	2.75	2.503	0.247	9.857
S9	1.05	2.625	2.911	0.286	9.823
S10	0.75	2.875	2.503	0.372	14.850
The average deviation					0.2
The average error percentage					8 %

Discussion

The T_{Ab} result of the antiserum-LSDV found in this study (T_{Ab} = 2.1 N.D.$_{50\%}$, equivalent of endpoint dilution 1/128) using ELK cells is in good agreement, almost in similar levels of T_{Ab} that were detected by both institutions IAH (Log T_{Ab} = 2.2 N.D.$_{50\%}$, equivalent of endpoint dilution 1/160) on lamb testis or bovine dermis cells and OVI (Log T_{Ab} = 2.1 N.D.$_{50\%}$, equivalent of endpoint dilution 1/128) on Madin Darby Bovine Kidney cell lines. In addition, the first serum dilution at IAH was 1/5, while that at OVI was 1/4 (OIE, 2011) but we started in this study at 1/2 dilution, we also noticed that the used cell type did not affect the results of T_{Ab} (OIE, 2011).

We noticed in table 1 (SR 7, 8 and 9) that the Neutralisation Index method (alpha-VNT) is more sensitive because it has detected neutralising antibodies while the antibody titre method (beta-VNT) did not. Also The variable sensitivity of tissue culture to CaPVs and the consequent difficulty of ensuring the use of 100 TCID$_{50}$ make the Neutralisation Index (alpha-VNT) the preferred method, even though, it does require a larger volume of test sera (OIE, 2017a).

The coefficient of determination R² = 0.987 of the found equation N.I. = (1.489 Log T_{Ab}) + 1.331 show that N.I. and T_{Ab} results demonstrated on Table 1 have a strong positive correlation.

N.I. ≥1.5 are considered positive which means the animal is protected (OIE, 2017a,b). Based on the resulting minimum threshold (T_{Ab} = 0.11 N.D.$_{50\%}$, equivalent of endpoint dilution 1/1), we can consider any raw serum sample that protects half of repetitions

using the previously described VNT methods as serum sample of a protected animal, but under these values particularly following vaccination in which the response is necessarily mild, does not imply that the animal is not protected (Kitching *et al.*, 1987; OIE, 2017a). On the other hand, no data was found regarding the T_{Ab} threshold that reflects the animal protection for CaPVs, but two previous studies indicated that sera for a protected animal have an endpoint dilution of 1/16 (Log T_{ab} = 1.2 N.D.$_{50\%}$) (Barmana *et al.*, 2010) and an endpoint dilution of 1/10 (Log T_{ab} = 1 N.D.$_{50\%}$) (Boshra *et al.*, 2015), but these studies did not include if sera T_{Ab} values below 1.2 and 1 N.D.$_{50\%}$ indicate that the animals are not protected and did not determine the minimum threshold of protective sera T_{Ab}.

The deviation $d = 0.2$ does not significantly change the minimum threshold of serum T_{Ab} that theoretically protects the immunized animal, so it does not affect the equation accuracy.

Using alpha-VNT allows to determine the N.I. but not the T_{Ab}, this method based on fixed serum dilution and variable virus dilutions is more labor-intensive, time-consuming and needs more sera volume than the beta-VNT (OIE, 2017a), despite the fact that beta-VNT seems to be advantageous, it gives only the T_{Ab} without revealing the protection level. This study showed that both alpha and beta VNT are reliable for determining antibody responses since one of them can be used to calculate the result of the other using the established equation.

Conclusion

This study has demonstrated that one of the two methods of VNT can therefore be used to calculate the values sought by the second method since the deviation was not significant. On the other side, this study can help to resolve the problems linked with the lack of samples volumes and even exploit again some results of old studies where just one method of VNT was used in order to assess the protection level of post-vaccinated individual animals (Kali, 2014) and vaccine effectiveness studies which would certainly improve the fight against CaPVs diseases and help the eradication policy. This study can also expand data for internal references sera preparation that would be useful for further applications.

The VNTs are ex-vivo methods, doing more studies on this subject could help to strengthen the use of these tests as alternative to the in-vivo challenge test that assess the individual protection against the CaPVs diseases and reduce the use of animals for this purpose.

Suppliers list
Institute for Animal Health (IAH), Pirbright Laboratory, UK, in 2011, the Capripoxvirus Reference Laboratory of IAH.
Senegalese Institute for Agricultural Research (I.S.R.A.), the reference laboratory F.A.O. for Africa.
Conflict of interest
The authors declare that there is no conflict of interest.

References

ATCC®. 2017. ANIMAL CELL CULTURE GUIDE; tips and techniques for continuous cell lines. Accessed 08 January, 2017, from www.atcc.org: https://www.atcc.org/~/media/PDFs/Culture%20G uides/AnimCellCulture_Guide.ashx

Barmana, D., Chatterjeea, A., Guhaa, C., Biswas, U., Sarkar, J., Roy, T.K., Roy, B. and Baidya, S., 2010. Estimation of post-vaccination antibody titre against goat pox and determination of protective antibody titre. Small Rum. Res. 93, 76-78.

Batta, M., Katoch, R., Mandeep, S., Joshi, V. and Nagal, K. 1999. Epidemiological observation on goatpox in Himachal Pradesh. Indian Vet. J. 76(8), 683-684.

Bhanuprakash, V., Indrani, B., Hosamani, M. and Singh, R. 2006. The current status of sheep pox disease. Comp. Immunol. Microbiol. Infect. Dis. 29, 27-60.

Boshra, H., Truong, T., Nfon, C., Bowden, T.R., Gerdts, V., Tikoo, S., Babiuk, L.A., Kara, P., Mather, A., Wallace, D.B. and Babiuk, S. 2015. A lumpy skin disease virus deficient of an IL-10 gene homologue provides protective immunity against virulent capripoxvirus challenge in sheep and goats. Antiviral Res. 123, 39-49.

Bowden, T.R., Babiuk S.L., Parkyn, G.R., Copps, J.S. and Boyle, D.B. 2008. Capripoxvirus tissue tropism and shedding: A quantitative study in experimentally infected sheep and goats. Virology. 371, 380-393.

Buller, R.M., Arif, B.M., Black, D.N., Dumbell, K.R., Esposito, J.J., Lefkowitz, E.J., McFadden, G., Moss, B., Mercer, A.A., Moyer, R.W., Skinner, M.A. and Tripathy, D.N. 2005. Family poxviridae. In: Fauquet, M.A., Mayo, J., Maniloff, U., Desselberger, L.A. and Ball, L. (Eds.), Virus Taxonomy: Classification and Nomenclature of Viruses. 8th Report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, San Diego, California, pp: 117-133.

Capstick, P. 1961. Annual Report. Kenya Veterinary Department, Kenya, pp: 45-47.

Chand, P., Kitching, R. and Black, D. 1994. Western blot analysis of virus-specific antibody responses for capripox and contagious pustular dermatitis viral infections in sheep. Epidemiol. Infect. 113(2), 377-385.

Davies, F.G. 1991. Lumpy skin disease of cattle: A growing problem in Africa and the Near East. Accessed on 08 September, 2017: http://www.fao.org/docrep/u4900t/u4900T0d.htm# lumpy skin disease of cattle: a growing problem in africa and the near east.

Davies, F. and Otema, C. 1981. Relationships of capripox viruses found in Kenya with two Middle Eastern strains and some orthopox viruses. Res. Vet. Sci. 31, 253-255.

Décret exécutif n°91-514 relatif aux animaux interdits à l'abattage. 1991. JOURNAL OFFICIEL DE LA REPUBLIQUE ALGERIENNE. Retrieved from: http://www.joradp.dz/hfr/.

Fassi-Fehri, M., El Harrak, M. and Bertin, F. 1983. Etude comparée du développement de deux souches vaccinales du virus claveleux sur cellules testiculaires et rénales d'agneau : Résultats préliminaires. Rev. sci. tech. Off. int. Epiz. 2(2), 499-507.

Haller, S.L., Peng, C., McFadden, G. and Rothenburg, S. 2013. Poxviruses and the evolution of host range and virulence. Infect. Genet. Evol. 21, 15-40.

Ian Freshney, R. 2016. Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications. 7th edition, Wiley-Blackwell editions, New Jersey, pp: 728.

Kali, K. 2014. Etude comparative de la cinétique des anticorps anti-claveleux post vaccinaux, évalués par séro-neutralisation sur cultures cellulaires, primaire RM et de lignée continue VERO. Alger: E.N.S.V.-Alger.

Karber, G. 1931. Beitrag zur kollectiven behandling pharmakologischer reihenverche. Archive fur Expperimentelle Pathologie. 162, 480-483.

Kitching, R.P., Hammond, J.M. and Black, D.N. 1986. Studies on the major common precipitating antigen of capripoxvirus. J. Gen. Virol. 67, 139-148.

Kitching, R.P., Hammond, J.M. and Taylor, W.P. 1987. Single vaccine for the control of capripox infection in sheep and goats. Res. Vet. Sci. 42(1), 53-60.

Kitching, R.P. and Mellor, P.S. 1986. Insect transmission of capripoxvirus. Res. Vet. Sci. 40(2), 255-258.

Kitching, R.P. and Taylor, W.P. 1985. Clinical and antigenic relationship between isolates of sheep and goat pox viruses. Trop. Anim. Health Prod. 17(2), 64-74.

Mirakabadi, A.Z. and Moradhaseli, S. 2013. Comparative Cytotoxic Evaluation of Free and Sodium Alginate Nanoparticle-Encapsulated ICD-85 on Primary Lamb Kidney Cells. Iran J. Cancer Prev. 6(3), 151-159.

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OIE. 2011. OIE Reference Laboratory Reports; lumpy skin disease and sheep pox and goat pox. Annual reports of OIE Reference Centres.

OIE. 2017a. Chapter 2.7.13. sheep pox and goat pox. Retrieved 08 July, 2017, from OIE, 2017: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.13_S_POX_G_POX.pdf.

OIE. 2017b. Chapter 2.4.13. Lympy Skin Disease. Retrieved 08 July, 2017, from OIE, 2017: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.13_LSD.pdf.

Ramyar, H. and Hessami, M. 1968. Development of an attenuated live virus vaccine against sheep pox. Arch. Inst. Razi. 20, 77-80.

Rweyemamu, M.M., Sylla, D., Palya, V. and Prandota, J. 1994. Quality control testing of rinderpest cell culture vaccine. Standard operating procedures.

FAO Animal Production and Health Paper (118), pp: 117.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish, G.F. and Rock, D.L. 2001. Genome of Lumpy Skin Disease Virus. J. Virol., 75(15), 7122-7130.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Sur, J.-H., Sandybaev, N.T., Kerembekova, U.Z., Zaitsev, V.L., Kutish, G.F. and Rock, D.L. 2002. The Genomes of Sheeppox and Goatpox Viruses. J. Virol. 76(12), 6054-6061.

Villegas, P. and Purchase, H.G. 1989. A laboratory Manual for the isolation and Identification of Avian Pathogens, 3rd edition, University of Pennsylvania, new Bolton centerkennett, PA, pp: 187-188.

Woldemeskel, M., Ashenafi, H., 2003. Study on skin diseases in sheep from northern Ethiopia. Dtsch Tierarztl Wochenschr. 110(1), 20-22.