Study of Cultural Characteristics and Interference of Peste Des Petites Ruminants Virus and Sheep Pox Virus in Co-Culture

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Abstract: The paper presents cultural characterization of Peste Des Petites Ruminants (PPR) virus and sheep pox virus in co-culture. During the study we selected optimal cell cultures such as lamb kidney cells (LK), lamb testicles cells (LT) and goat gonad cell line (CG-91) allowing to obtain virus suspension with high infective activity. The optimum period of cultivation is 7 days and the infecting dose of PPR and sheep pox viruses is 0,01 $TCID_{50}$/cell. The interference phenomenon absence between the two viruses is confirmed by electron microscopic studies, as well as the cytopathic effect of these viruses in cell culture. Subject to the above parameters highly active virus-containing suspension can be obtained for PPR and sheep pox associated vaccine production.
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1. Introduction

PPR is a viral disease of sheep and goats, characterized by high fever, necrotizing stomatitis, hemorrhagic gastroenterocolitis, bronchopneumonia and mucosal and lymphoid tissuesyndrome. The disease agent is an RNA virus belonging to the genus Morbillivirus, family Paramyxoviridae (Barrett, 1987, Dhinkar et al., 1992, Lefevre, 1990, Taylor, 1979).

Sheep pox virus is a DNA virus belonging to the family Poxviridae, genus Capripoxvirus. It is acute contagious disease with papule and pustule lesions of the skin and mucous membranes. The virus is mainly localized in pocks. The virus has no antigenic variants. After recovering animals have long-lasting immunity (at least two years) and the formation of antibodies that are passed to offspring through colostrum and milk (Taylor et al, 2007, Lefevre 1982).

Given the epizootic situation in the various countries which showes wide circulation of the above-mentioned diseases, there is a need for new associated vaccines aimed at specific prevention, reduction of morbidity and elimination of the pathogens.

Rogozin I.I. considers that the use of associated live vaccines can cause phenomenon of interference, but it is not clearly manifested and optimal combination of the vaccine strains leads to successful immunization (Chifney et al, 1973, FAO-WHO-OIE 1992-2001).

Co-culturing of the above viruses reduces the time of virus-containing suspension accumulation, as well as material and labor costs.

Using associated vaccines, as well as single injection with several vaccines has long been practiced in human and veterinary medicine. This can reduce the timing and number of injections to decrease stress for animals and to reduce material and labor costs. Development of the associated vaccines is usually conducted on the basis of existing monovaccines.

In this regard, we have conducted a study on the cultural characteristics of PPR and sheep pox viruses in co-culture, as well as the study of the interference phenomenon.

2. Materials And Methods

Virus strains

- PPR attenuated strain “G45- MK”;
- Sheep pox attenuated strain “NISKHI”.

Cell culture

- Goat gonad cell line (CG-91);
- Lamb kidney cell line (LK);
- Sheep kidney cell line (SK);
- Bovine kidney cell line (BK);
- Lamb testicles cell line (LT).

Preparation of a primary cell culture

To obtain the primary cell culture we used Roger Adams method (R.Adams 1983).

Cultivation of continuous cell culture

For producing of continuous cell culture a vial with frozen cell culture was removed from the deep freezer and immediately immersed for 1-2 minutes in a water bath at (37 ± 1)ºC. The contents of the vial was aseptically pipetted into the vessel, with a growth

medium and viable cells were counted according to the accepted method. The resulting cell suspension was poured into 1,5-liter dishes with the inoculum concentration of 20 million cells and cultured at a temperature (37 ± 1)ºC. In the following days the growth medium was renewed when it was necessary. And further a complete monolayer was formed.

Infection of cell culture

When infecting a cell culture we poured out the growth medium and added PPR and sheep pox viruses at a dose of 0,01 $TCID_{50}$/cell in the amount of 150-170 ml per a vessel. The infected cell cultures were incubated at (37 ± 0,5)ºC. The infected cell culture was examined daily with the use of microscope. When70-80% of a monolayer was affected by cytopathic effect of the virus, vessels were frozen at -20ºC for 12-18 hrs. Thawing was performed at a room temperature. The resulting virus-containing suspension was used for further research or the next passage.

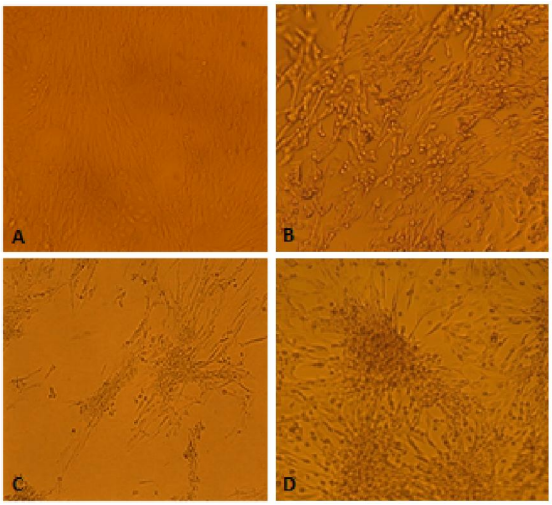
Determination of virus infectivity in Cell Culture

PPR and sheep pox virus infectious activity was determined by titration in LK cell culture grown in vitro by stationary method. 10-fold dilutions of virus-containing material were prepared with a supporting medium and 1,0 ml of each dilution was added to the vials with cell culture. Four vials were infected with each dilution. Vials with cell culture inoculated with supportive medium were the controls. Infected and control cell cultures were incubated at (37 ± 0,5)ºC under stationary conditions. A medium containing 600 ug/ml glutamine, antibiotics, and 2% bovine serum was used as a supportive. Titration results were evaluated on 12-14th days after inoculation by the appearance of specific cytopathic changes in the infected cell cultures and in the absence of cytopathic effect (CPE) in control vials. The greatest dilution causing CPE in cell cultures was considered as the virus titer, which was calculated by the Reed I.J., and Muench H.A. method (1938).

3. Results

Sensitivity of cell cultures to PPR and sheep pox viruses was determined by the appearance of CPE and the level of virus accumulation. At a separate cultivation CPE of PPR virus was observed as stellate cells with refractory appendices separating from the glass and forming gaps in the monolayer. Sheep pox virus CPE was observed as foci of photorefractive rounded cells and slow spread of CPE on the cell monolayer, after which there was a separation of the affected cells from the glass.

During co-cultivation we observed mixed CPE with foci of PPR and sheep pox virus. Mixed CPE was expressed with the formation of stellate cells with refractory apendices, which occurred on a background of light-refracting rounded cells. Further cultivation leaded to focal separation of cells from the glass and gaps formation (Figure 1).



A – control cell culture, B – sheep pox virus, C – PPR virus, D – PPR and sheep pox co-culturing

Figure 1 – PPR and sheep pox viruses CPE in LK cell culture. Magnification x 400

Infectivity of virus-containing suspension was determined by titration in lamb kidney cell culture. The virus presence in the resulting suspension was further confirmed by electron microscopy. Results of studies to determine cell cultures sensitivity and the virus biological activity are shown in Table 1.

Table 1 – Sensitivity of different cell cultures to PPR and sheep pox viruses n=3

Cell culture	Viruses	Incubation period, days	Infectious activity, lg $TCID_{50}$/ml
LK	PPR	7-10	4,00 ÷ 4,75
	Sheep pox	5-6	5,50 ÷ 5,75
LT	PPR	6-9	4,00 ÷ 4,50
	Sheep pox	4-5	5,25 ÷ 5,75
CK	PPR	8-9	4,00 ÷ 4,25
	Sheep pox	8	3,75 ÷ 4,00
SK	PPR	7-8	3,00 ÷ 3,50
	Sheep pox	7-8	3,75 ÷ 4,00
CG-91	PPR	5-7	4,00 ÷ 4,75
	Sheep pox	4-6	5,25 ÷ 5,50

The results of the studies (Table 1) show that the most active PPR and sheep pox virus-containing material was obtained in such cell cultures as LK, LT, and CG -91. Bovine kidney cell culture and sheep kidney cell culture proved to be less sensitive to these viruses.

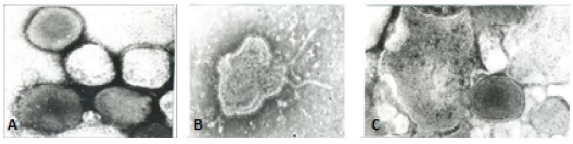
Infectivity of the mixed PPR and sheep pox virus suspensions was determined by titration in cell culture using specific sera to the viruses. The results of this study are presented in Table 2.

Table 2 - Infectious activity of PPR and sheep-pox virus at separate cultivation and co-cultured in LK cell culture n=3

Biological material	Virus	Incubation period, day	Infectious activity, lgTCID$_{50}$/ml
PPR virus-containing suspension	PPR	8-9	5,16 ± 0,08
Sheep-pox virus – containing suspension	Sheep-pox	6-7	6,08 ± 0,08
Mixed PPR and sheep-pox virus-containing suspension 1:1	PPR	7	4,91 ± 0,08
	Sheep-pox		5,66 ± 0,04

The data in Table 2 show that the level of PPR virus infectivity was 4,91 lg TCID$_{50}$/ml and sheep pox virus infectious activity was 5,66 lg TCID$_{50}$/ml while simultaneous co-introducing PPR and sheep-pox viruses in proportion 1:1 into the cell culture. The incubation period was 7 days.

To determine the possibility of PPR and sheep-pox virus co-cultivation the virus-containing suspension saccumulated both separately and co-cultured were investigated by electron microscopy to detect defective interfering particles. The results of electron microscopy of purified PPR and sheep-pox viruses are presented in Figure 2.



A - sheep pox virus, B – PPR virus, C – PPR and sheep pox viruses
Figure 2 - negative staining, magnification x100 000

Thus, the results obtained during culturing and the results of electron microscopy (Figure 2) indicate the absence of interference phenomena between PPR and sheep pox viruses in cell culture, i.e. mutual suppression of reproductive characteristics, suggesting the possibility of PPR and sheep-pox viruses co-cultivation in one biosystem.

To determine the optimal cell culture infective dose lamb kidney cell culture was infected simultaneously with PPR and sheep pox viruses at the doses of 0,001, 0,01, 0,1 and 1,0 TCID$_{50}$/cell in 4 tubes for each dose of the virus. The results of this study are presented in Figure 3.

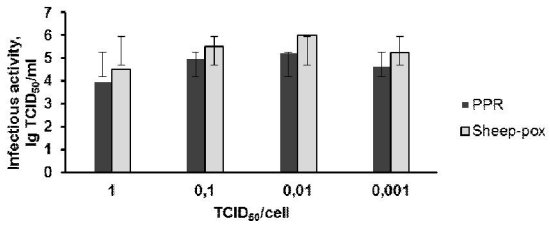


Figure 3 - Determination of the minimum infective dose for PPR and sheep-pox viruses co-cultivation in lamb kidney cell culture

From the data presented in Figure 3 we can conclude that at a multiplicity of infection dose 0,01 TCID$_{50}$/cell the PPR virus accumulates with in 5,25 lg TCID$_{50}$/ml. The use of a lower dose leads to the virus accumulation to maximum 4,00 lg TCID$_{50}$/ml.

The results of sheep pox virus infectious dose determination showed that at a multiplicity of infectious dose of 0,01 TCID$_{50}$/cell the virus activity reached 6,00 lg TCID$_{50}$/ml. The increase of infection dose up to 1,0 TCID$_{50}$/cell leaded to more rapid destructive changes in the cell culture monolayer, but the virus titer decreased to 5,25 lg TCID$_{50}$/ml ($P < 0.05$). This can be explained by the fact that a large dose of virus infection contributes to the formation and accumulation of defective interfering particles, which ensure the persistence of sheep pox virus in cell culture. Application of 0,001; 0,1 and 1,0 TCID$_{50}$/cell infectious dose decreased the virus titer down to 4,50 ÷ 5,50 lg TCID$_{50}$/ml.

The study of PPR and sheep pox virus incubation period during co-cultivation was performed in lamb kidney cell culture within 1-10 days at a temperature (37 ± 0,5)ºC. The infectious activity in virus-containing suspension obtained at different terms of cultivation was determined by titration in cell culture. The results are presented in Figure 4.

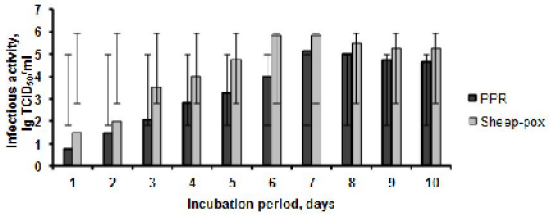


Figure 4 - The incubation period of associated PPR and sheep pox virus suspension

The studies have shown (Figure 4) that the optimal period for PPR and sheep pox viruses co-cultivation is 7 days. The level of virus titer was 5,16 lg $TCID_{50}$/ml and 5,83 lg $TCID_{50}$/ml respectively. Further cultivation reduced the infectivity titer of PPR virus to 4,66 lg $TCID_{50}$/ml (P> 0,1), sheep pox virus to 5,25 lg $TCID_{50}$/ml (P> 0,1).

4. Discussion

Existing methods of specific prophylaxis are not always reliable. In case of a serious epizootic situation, whenthe circumstances require the immunity to multiple infections, monovalent vaccines do not give the desired result. The associated immunization of animals can be of help in this case. The advantage of such immunization is that vaccinated animals acquireimmunity to two or more infections simultaneously. Application of associated vaccines opens a new stage in the field of practical immunology (Kovalenko, 1966).

KovalenkoYa.R. notices that it is important to find new means of specific prophylaxis of infectious diseases. In this regard, production of live, chemical and associated vaccines as well as reduction of the multiplicity of injections becomes more and more important (Kovalenko, 1966).

It is particularly difficult to fight against infectious diseases in large farms where there are many animals on a limited area. And those animals are all under threat of several simultaneous diseases. In this case associated immunization of the animals can be very valuable (Kovalenko, 1966).

Using associated immunization reduces labor efforts and facilitates practical veterinary workers, as associated immunization creates immunity to several infections in a short time. And in some cases it improves the immunogenic properties of the applied antigens (Kovalenko, 1966).

An integrated approach is necessary to study the possibility of PPR and sheep pox viruses' co-cultivation for the development of prophylactic means. As it is known selection of optimal biosystems and cultivation parameters work-out is an integral part of the antiviral vaccines manufacturing technology development.

The success of viruses cultivation depends especially on the successful selection of the cellular substrate. The main criteria for this are the high yield of virus and viral antigen, the relative simplicity of the culture conditions and the vaccine safety. Some viruses replicate well in cell cultures of different origin, others - prefer the natural host cells or differentiated cells, or require specific cultivation conditions (temperature reduction, treatment with trypsin, particular infection dose), and some viruses can not be reproduced outside a susceptible organism (Guo, 2003). According to domestic and foreign authors PPR and sheep pox viruses can be cultivated in lamb kidney (LK) and lamb testicles (LT) cell cultures and in such continuous cell lines as goat gonads (CG -91), African green monkey kidney (Vero) and sheep kidney (SK) (Kutumbetov, 1990, Durojaiye et al, 1985, Gilbert et al, 1962, Laurent, 1968, Rossister et al, 1985, Adams, 1983, Asim et al, 2009, Koreba, 1984).

Our results are consistent with the references containing the data that the most active viral material can be obtained by PPR and sheep pox virus culturing in LK, LT and CG-91 cell cultures. Thus, we have chosen lamb kidney cell culture for further studies of the interference phenomena and determination of the cultivation parameters.

Interference phenomenon is widespread among plant, bacterial and animal viruses and manifested in different animal species, chicken embryos and tissue cultures. Interference of animal viruses was first reproduced by Hoskins in monkeys which were consistently infected with neurothropic and then panthropic strains of yellow fever virus. The phenomenon of interference can occur between different strains of the same virus (homologous interference) between immunologically close viruses, and between viruses which are quite different immunologically (heterologous interference). The possibility of simultaneous cell infection with two, three or even five viruses with different biological tropism was proved experimentally (Syurin, 1966). After PPR virus attachment to cell receptors the viral envelope and the cell membrane surface are merged(at neutral pH). PPR virus replication occurs in the cell cytoplasm and does not depend on the function of the nucleus. Sheep pox virus replication also occurs in the cytoplasm. But despite the fact that replication occurs in the cytoplasm, there is evidence that participation of nuclear proteins in the post-replicative transcription is needed (Vasilyev, 2004). Perhaps this plays an important part for the absence of interference between PPR and sheep pox viruses during co-cultivation in cell culture, which is also confirmed by electron microscopy analysis of virus-containing suspension.

Virus accumulation in the cell culture also depends on an infection dose that mainly affects the length of the virus accumulation, and not on the harvest. The dose of 0,001-0,1 $TCID_{50}$ per cell is usually used to infect monolayer cultures in order to accumulate the virus. The virus accumulation is the sum result of multi cycle reproduction. In case of dealing with cell cultures the viability of which decreases rapidly, and viruses which do not have a short cycle of reproduction, multiplicity of infection

is very important (Sergeev, 1976). Having conducted the studies to determine the optimal infective dose we found out that the PPR virus infectious dose was 0,01 $TCID_{50}$/cell and infectious activity titer was within 5,25 lg $TCID_{50}$/ml. Sheep pox virus infecting dose was 0,01 $TCID_{50}$/cell and the virus infectious activity was 6,00 lg $TCID_{50}$/ml.

The study of PPR and sheep pox viruses' incubation period in co-cultivation has shown that the optimal incubation period is 7 days, where the level of infectious activity was 5,16 lg $TCID_{50}$/ml and 5,83 lg $TCID_{50}$/ml respectively. Further culturing reduced the infectious activity of the PPR virus to 4,66 lg $TCID_{50}$/ml, sheep pox virus infectious activity was down to 5,25 lg $TCID_{50}$/ml.

Conclusion

As a result of the implemented studies the optimal cell culture line was selected for PPR and sheep pox viruses co-cultivation. Optimal cultivation conditions were worked out. The absence of interference between PPR and sheep pox viruses in co-culture was established. This was confirmed by the results of electron microscopy and the presence of characteristic cytopathic changes in cell culture. The study results will be used for the development of associated vaccines.

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