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Comparative characterization of *Leporipoxvirus* members' reproduction in continuous cell culture

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SUMMARY

Examination of the virus-cell interactions is of both scientific and practical importance. Our study was aimed at comparative characterization of rabbit myxoma virus and Shope fibroma virus biological properties that manifested during the virus reproduction in RK-13/2-03 clonal continuous rabbit kidney cell culture. It was demonstrated that the viruses varied in infection development and cytopathic effect duration in RK-13/2-03 cell culture. Apparent lesions in cell monolayers infected by myxoma virus and fibroma virus at similar multiplicity of infection and cultivation temperature were observed on day 2 and day 3 of cultivation, respectively, as well as maximum cell lesions with evident degeneration were observed on day 3 and day 6 of cultivation, respectively. Myxoma virus was accumulated at titre of 6.25–6.50 lg $TCID_{50}$/0,2 cm^3, and Shope fibroma virusa was accumulated at titre of 5.50–5.75 lg $TCID_{50}$/0.2 cm^3. Shope fibroma virus demonstrated such infectivity during three passages and myxoma virus demonstrated such infectivity during twenty passages. Prepared cultures were identified as myxoma virus and Shope fibroma virus with molecular genetic analysis. Tests of the viruses for their antigenic relatedness showed that antibodies against myxoma virus were able to neutralize Shope fibroma virus also. NT titres of antibodies against both viruses were similar (1:8). RK-13/2-03 cell culture was found to be highly permissive to Shope fibroma virus that had been isolated from the diseased rabbit and not been an attenuated variant.

Keywords: continuous cell culture, myxoma virus, Shope fibroma virus, virus-specific cytopathic effect

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Сравнительная характеристика репродукции вирусов рода *Leporipoxvirus* в перевиваемой культуре клеток

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РЕЗЮМЕ

Изучение характера взаимодействия вируса с клеткой имеет как научное, так и практическое значение. Целью настоящих исследований явилась сравнительная оценка проявления ряда биологических свойств вирусов миксомы и фибромы Шоупа кроликов при репродукции в клональной перевиваемой линии клеток почки кролика RK-13/2-03. Показано, что развитие инфекции и длительность проявления цитопатического действия в культуре клеток RK-13/2-03 изучаемых вирусов различны. Видимые поражения клеточного монослоя при одинаковой множественности заражения и температуре

культивирования для вируса миксомы наблюдали на 2-е сут, для вируса фибромы – на 3-и сут, максимальное поражение клеток с выраженной дегенерацией отмечали на 3-и и 6-е сут культивирования соответственно. Вирус миксомы накапливался в титре 6,25–6,50 lg $ТЦД_{50}$/0,2 $см^3$, а вирус фибромы Шоупа – в титре 5,50–5,75 lg $ТЦД_{50}$/0,2 $см^3$. Данную инфекционную активность регистрировали у вируса фибромы Шоупа на протяжении трех, а вируса миксомы – двадцати пассажных уровней. Молекулярно-генетический анализ подтвердил, что полученные культуральные материалы идентифицируются как вирусы миксомы кроликов и фибромы Шоупа. При определении антигенного родства было установлено, что антитела, полученные к вирусу миксомы, способны нейтрализовать и вирус фибромы Шоупа. Титр антител в реакции нейтрализации обоих вирусов был идентичен и составил 1:8. Установлен высокий уровень пермиссивности культуры клеток RK-13/2-03 к вирусу фибромы Шоупа, изолированному от больного кролика и не являющемуся аттенуированным вариантом вируса.

Ключевые слова: перевиваемая культура клеток, вирус миксомы, вирус фибромы Шоупа, цитопатическое действие вируса

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INTRODUCTION

Continuous cell lines used as a cell substrate for virus and antigen accumulation for antiviral vaccine development have significantly contributed to biotechnological process effectiveness and stability. Use of continuous cell lines has contributed to significant decrease in risks of endogenous contamination that could be associated with used primary cell culture and to solving of some ethical problems associated with donor tissue collection from mammals and in general enhancement of target product biosafety.

Possibility of continuous cell line use for cultivation depends mainly on permissiveness of the selected cell system for the virus.

Consequently, examination of virus-cell interactions and subsequent optimization of cultivation conditions and parameters are of both scientific and practical significance.

Viruses of *Poxviridae* family infect many mammal species, propagate in target cell cytoplasm affecting both skin and internal organs and can be responsible for epidemics causing significant economic losses [1, 2]. The family is divided into two subfamilies: *Chordopoxvirinae* and *Entomopoxvirinae* [3]. The first subfamily comprises 11 virus genera that cause disease in animals (vaccinia, monkeypox [4], buffalo pox [5], sheep and goat pox [6], lumpy skin disease [7], fowl pox [8], rabbit myxomatosis and fibromatosis [9, 10], squirrel pox [11], etc.), as well as in humans (smallpox). The second subfamily comprises 3 virus genera circulating in insects. There are genera in *Chordopoxvirinae* that have limited host range including viruses of *Leporipoxvirus* genus causing disease only in rabbits, hares and squirrels.

Rabbit fibromatosis (Shope fibroma) is a viral disease of domestic and wild rabbits characterized with confined nodules and diffuse cirrhosis (fibromas) under skin and mucosa, the main pathognomonic feature of disease.

Rabbit myxomatosis is an infectious acute highly contagious viral disease characterized with serous-purulent conjunctivitis, edematous-tremelloid cell infiltration on head and perineum, skin tumors.

Both diseases are caused by DNA viruses. These viruses do not significantly distinct from vaccinia virus in their morphological features. Mature myxoma virus is 250–370 nm in size, and mature Shope fibroma virus is 125–240 nm in size. Myxoma virus virions are of brick-like form with rounded corners, have villous outer surface, dumb bell-shaped nucleotide and double-stranded DNA [12].

Mature Shope fibroma virus virion is of brick-like form with rounded edges. Fibroma and myxoma viruses are related in antigenic and immunogenic properties and therefore rabbits convalescent from fibromatosis are resistant to myxomatosis agent [13].

Both viruses replicate in chicken embryonated eggs but cause different lesions. Myxoma virus cultivation on chorioallantoic membrane results in formation of characteristic foci (pustules) and subsequent embryo death, whereas fibroma viruses induces mild changes and embryo remains alive. Myxoma virus causes apparent cytopathic effect characterized with cell rounding and cell layer degeneration in primary and continuous newborn rabbit kidney cell cultures. Fibroma virus reproduction in primary and continuous cell culture induces mild cytopathic effect [12].

Poxvirus reproduction in cell culture is characterized by its replication in the cytoplasm and release of the mature virions from the cell with its destruction or without cell membrane disruption (exocytosis). The induced cytopathic effect is characterized with pyknosis, cell shrinkage and destruction. However, members of the said virus

family have their own specific manifestations during their *in vitro* interactions with cells. Hinze and Walker detected mild cellucidal effect caused by Shope fibroma virus cultivated in rabbit kidney cells [14].

There are limited data on cultivation of *Leporipoxvirus* family viruses in Russian literature.

Considering the abovementioned, our study was aimed at comparative characterization of myxoma and Shope fibroma virus biological properties manifested during their reproduction in RK-13/2-03 clonal continuous rabbit kidney cell culture as a basis for improvement of the technology for production of vaccines against the diseases caused by these pathogens.

MATERIALS AND METHODS

A clone of continuous RK-13/2-03 rabbit kidney cell subline (Cat. No. 36.2) obtained from the FRCVM Cell Culture Collection [15]; rabbit-pathogenic Shope fibroma virus isolated from pathological materials (infectivity at 5th passage in rabbits – 4.11 lg ID_{50}/ml); rabbit myxoma virus (B-82 vaccine strain) with infectivity of 6.5 lg $TCID_{50}$/cm^3 were used. The virus strains were obtained from the Governmental Collection of Microorganisms Causing Dangerous, Highly Dangerous Including Zooanthroponic and Foreign Animal Diseases (CKP Reg. No. 441429, http://ckp-rf.ru/ckp/441429/).

Specific rabbit serum against myxoma virus (NT titre – 1:8) was used.

The following nutrient media were used: liquid Eagle's DMEM medium supplemented with alanyl-glutamine and glucose, 4.5 g/l, and Dulbecco solution (NPP PanEco, Russia); Eagle's MEM (Sigma, USA); fetal bovine serum (Biological Ind., Israel).

RK-13/2-03 rabbit kidney cell culture was cultivated in culture flasks (25 cm^2 growth area) and 96-well plates (Corning, USA).

RK-13 cell subline was cloned to increase genetic homogeneity level using end-point dilution method in 96-well plastic plates [16]. Prepared clones were cultivated in Eagle's MEM containing 20% of fetal bovine sera and 50% of conditioned medium with pefloxacin (antibiotic) (20 μg/ml) for 10–14 days. The most promising RK-13/2-03 clone was selected for further cultivation out of 42 line clones based on their growth and virus-reproducing property assessment results. Karyologic analysis at 18th passage showed that cells with 56 chromosomes corresponded to the modal number that was 22%. Cytomorphological examinations showed that clonal culture monolayer consisted of epithelial-like cells of polygonal shape. The monolayer formed within 48–72 hours at the seeding density of 100 ths cells/ml. The prepared clonal RK-13/2-03 cell subline retained its original growth properties and morphological characteristics through 50 passages (observation period).

Shope fibroma and myxoma (B-82 strain) viruses were cultivated in 2-day-old RK-13/2-03 cell culture grown in DMEM supplemented with 10% fetal bovine serum. Before inoculation the growth medium was removed from the flasks, the cell monolayer was washed twice with Dulbecco solution for removing remained serum and then the viruses were added at the multiplicity of infection of 0.002–0.003 $TCID_{50}$/cell. The virus was left for adsorption at (33.0 ± 0.5) °C for 60 min. Then, maintenance Eagle's DMEM supplemented with 2% fetal bovine serum was added to the flasks. Inoculated cell culture was incubated at (33.0 ± 0.5) °C up to the evident virus-induced cytopathic effect (CPE). The cell monolayer was observed for the virus-induced lesions by examination of the culture flasks under Olympus inverted microscope (Olympus, Japan).

Shope fibroma and myxoma virus infectivity was determined by conventional method of titration in RK-13/2-03 cell culture. For this purpose, RK-13/2-03 cells were seeded in 96-well plates at concentration of 150 ths cells/cm^3. Two days later, the cells were inoculated with 10-fold dilutions (10^{-1}–10^{-7}) of Shope fibroma and myxoma viruses upon confluent monolayer formation and then were incubated at temperature of (33.0 ± 0.5) °C in 5% CO_2 atmosphere and at 95%-relative humidity. The observation period was 10 days. Titration results were assessed based on the virus-induced CPE, the titre was calculated using Reed and Muench method and expressed as lg $TCID_{50}$/0.2 cm^3.

Virus DNAs were extracted with QIAamp DNA Mini kit (Qiagen, Germany) according the manufacturer instruction.

Primers proposed by Y. Li et al. [17] and S. Albini et al. [18] were used for detection of Shope fibroma and myxoma virus genomes.

Polymerase chain reaction (PCR) products were purified with Cleanup Standard kit (ZAO Evrogen, Russia) with subsequent Sanger direct sequencing using Applied Biosystem 3130 XL DNA Analyser (Applied Biosystems, USA) and reagents of BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA).

Conventional neutralization test was used for demonstration antigenic relatedness between myxoma and Shope fibroma virus. RK-13/02-03 cell suspension (at concentration of 100 ths cells/cm^3) was added to 96-well plates and the plates were incubated at temperature of (37.0 ± 0.5) °C in 5% CO_2 atmosphere and at 95%-relative humidity for 48 hours. Normal and myxoma virus-specific rabbit sera were titrated in duplicate (1:2–1:64) in two plates; then, 1,000 doses of myxoma virus were added to the first plate and 1,000 doses of Shope fibroma virus were added to the second plate. Serum-virus mixture was kept at temperature of (33.0 ± 0.5) °C in 5% CO_2 atmosphere and at 95% relative humidity and transferred to the plates with cell culture and then incubated under the abovementioned conditions for 10 days. Results were recoded starting with day 3.

RESULTS AND DISCUSSION

Apparent manifestations of the viruses' reproduction were observed on day 2 (Fig. A) and day 3 (Fig. B) of incubation of myxoma virus (B-82 strain) and Shope fibroma virus, respectively, in RK-13/2-03 cell culture. Lesions in the culture caused by viruses at the beginning of the infection were characterized with rounded cell accumulation and small bundle cluster formation. Characteristic small disruptions of the cell layer were observed in the culture infected with myxoma virus. Changes in the infected monolayer: cell layer destruction with the formation of a "grid" of elongated cellular elements surrounded by rounded cells with sharply contoured thick walls, became more apparent on day 3 of this virus cultivation (Fig. C).

Fig. Dynamics and nature of CPE caused by myxoma viruses and Shope fibroma virus in RK-13/2-03 cell culture (magnification 100×). Myxoma virus manifestations on day 2 (A), Shope fibroma virus manifestations on day 3 (B), myxoma virus-associated lesions on day 3 (C), fibroma virus-associated lesions on day 4 (D), fibroma virus-associated lesions on day 5 (E), intact RK-13/2-03 cell (F)

Progressive destructive changes were observed in cell monolayer infected with fibroma virus on day 4 of incubation (Fig. D); maximum cell lesions with evident degeneration were observed on day 5–6 of cultivation (Fig. E). No such changes were observed in intact RK-13/2-03 cell culture (control) (Fig. F).

Thus, dynamics of myxoma virus and Shope fibroma virus –induced CPE was different in RK-13/2-03 cell culture and the CPE became apparent on day 3 and 6, respectively. The nature of cell monolayer lesions was identical throughout the whole cultivation cycle.

Some differences were found when viruses' accumulation during cultivation. Myxoma virus infectivity reached 6.25–6.50 lg $TCID_{50}$/0.2 cm^3 during cultivation period (3 days), whereas Shope fibroma virus titre was 5.50–5.75 lg $TCID_{50}$/0.2 cm^3 on day 6 of cultivation. Shope fibroma virus and myxoma virus demonstrated such infectivity levels during three and twenty passages, respectively.

Poxvirus amplicons were produced with PCR and nucleotide sequencing was carried for their identification. Resulting sequences were compared with gene sequences deposited in GenBank database using Blast software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular genetic analysis confirmed that the resulting virus-containing materials were myxoma and Shope fibroma viruses.

Neutralization tests of the viruses for their antigenic relatedness showed that antibodies against myxoma virus were also able to neutralize Shope fibroma virus. Antibody titres against myxoma and Shope fibroma viruses were identical and equaled to 1:8. It was indicative of antigenic relationship between myxoma and Shope fibroma viruses.

Significant differences in dynamics of CPE caused by Shope fibroma and myxoma viruses in RK-13/2-03 cell culture were found. Apparent cell monolayer lesions induced by myxoma virus and Shope fibroma virus at similar multiplicity of infection and cultivation temperature were observed on day 2 and day 3, respectively, that was consistent to the findings obtained during reproduction of these viruses in primary cell cultures [12].

Cytomorphological examinations showed that cell culture lesions induced by the studied viruses were characteristic of *Poxviridae* family members; rounded cells and their agglomerations in the form of pox plaques were the main CPE manifestations [19].

The above-mentioned CPE differed from the CPE induced by Mix-98 strain of myxoma virus characterized by rounded cell pulling to the lesion centre with spider-like bundle formation [20].

These two antigenically related viruses cultured under similar conditions differed in their infectivity. Myxoma virus accumulated at higher titres since its B-82 vaccine strain had been adapted to the said cell culture. However, Shope fibroma virus isolated from organ lesions without preliminary adaptation to RK-13/2-03 cells also actively propagated in the cells as compared to other viruses of *Poxviridae* family. For example, lumpy skin disease virus required longer period of adaptation to continuous cell cultures. Characteristic lesions in RK-13/2-03 cell monolayer were reported starting with the 3rd passage, no visible changes were observed in 1st-passage cell culture, minor changes were observed in 2nd-passage cell culture [21].

Studies of these two viruses showed no distinct cultivation peculiarities and confirmed their antigenic relatedness. Sequencing of culture virus-containing materials showed their relevance to the taxonomic status.

CONCLUSION

Comparative characterization of myxoma virus and Shope fibroma virus reproduction in continuous rabbit kidney cell line revealed differences in cytopathic effect dynamics. The viruses induced identical lesions in RK-13/2-03 cell culture characterized by rounding of epithelium-like monolayer cells followed by their blotch-like agglomeration. Increasing destructive processes result in cell layer destruction, formation of grid-like structures surrounded by rounded cells.

It was found that myxoma virus being a culture vaccine strain (B-82) had a higher infectivity.

Neutralization test demonstrated that myxoma virus and Shope fibroma virus were antigenically related and belonged to *Leporipoxvirus* genus.

RK-13/2-03 cell culture was found to be highly permissive to virulent Shope fibroma virus isolate recovered from the diseased rabbit.

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