Sensitivity of *Pipistrellus Nathusii* Kidney Diploid Cell Strain to Viruses of Rhabdoviridae, Reoviridae, Bunyaviridae and Paramixoviridae Families

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Abstract

The discovery of a significant number of viral pathogens in bat tissue samples and excrement point to a potential prominent role of chiropters in the maintenance and spread of human and animal diseases. It also indicates the potential sensitivity of bat cells to a broad spectrum of viruses. The migratory pipistrelle bat, *Pipistrellus nathusii*, inhabits northeastern Europe and typically migrates to the southwest. Our study revealed the sensitivity (susceptibility) of a diploid cell line, derived from the kidney of *P. nathusii* to several transmissible animal disease causative agents such as Epizootic hemorrhagic disease, Akabane disease, Vesicular stomatitis virus and Peste des petit ruminants. High sensitivity of the *P. nathusii* kidney diploid cell line to viruses from various taxonomic groups allows them to be recommend for extensive use in virological studies.

Introduction

Currently, members of the order Chiroptera are considered to be one of the most important reservoirs and carriers of causative agents of particularly dangerous human and animal diseases. [1–3]. Genomes of viruses from different taxonomic groups have been detected in tissue and excrement samples taken sampled from bats [4]. Identification of these pathogens in bats has been carried out mainly using molecular genetics. Studying the biological properties of virus isolates which transmit from Chiropterans to target animal species is hampered by the absence of vivaria for bats, as well as the particular immune response of this mammalian species [5]. Furthermore, the most frequently used (“traditional”) cell cultures are not effective enough for the isolation of viruses persisting in bats.

Therefore, obtaining cell cultures from tissues and organs of different subspecies of bats is relevant and solves problems regarding primary isolation and subsequent investigation into the biological properties of viruses. Additionally, it helps the assessment of the possible role of tissue-donor bats in the formation of natural foci of infection.

Studies on the susceptibility of bat cells have only been carried out on a few viral pathogens; most relating to causative agents of human disease. I. Eckerle et al. immortalized trachea cells lines from *Carollia perspicillata* (Seba’s short-tailed bat) and *Eidolon helvum* (Straw-colored fruit bat) of the suborders Yangochiroptera and Yinpterochiroptera, respectively. These cell lines were characterized by susceptibility to viral infection with Rift-Valley fever virus and Vesicular stomatitis virus (Indiana strain) [6]. G. Vrameri et al. obtained cell lines from twenty different organs of Black flying foxes (*Pteropus alecto*) which were sensitive to Hendra and Nipah viruses [7]. A new bat cell line (NIV-BtEPC), taken/harvested from the embryo of a *Plocamopherus ceylonicus*, developed by D.T. Mourya and colleagues was susceptible to Chandipura (CHPV) and novel adenovirus (BtAdv-RLM) [8].

Earlier, our group developed a stable epithelial cell line from the kidneys of *P. nathusii* (PKDCL) [9]. In another study, this diploid cell line demonstrated the ability to maintain reproductions of Bluetongue virus, Rift Valley fever, Lumpy skin disease virus and Myxoma virus [10].

This study aimed at further exploring the range of susceptibility of diploid cell lines, derived from bat tissues to viruses from different taxonomic groups. Including those posing a risk of being introduced to, and spreading in, European countries.

Results And Discussion

Morphologically, PKDCL is characterized by epithelial-like polygonal cells containing oviform or ellipsoidal karyons with 1–3 (sometimes more) rounded nucleoli varying in size. The nuclear matrix was uniform (Fig. 1).

Virological tests carried out between passages 14–43 showed commonality between, and active proliferation of, the cells in the culture.

In this period, with the replanting coefficient 1:2 – 1:3, the confluent monolayer developed within 48 hours and persisted without changing the medium for 45 days (the period of observation).

Earlier, we demonstrated the sensitivity (permissivity) of this diploid cell strain to bluetongue virus, lumpy skin disease virus and myxoma virus [10]. In this study, we assessed its sensitivity to RNA-viruses belonging to different families: Rhabdoviridae, Bunyaviridae, Reoviridae and Paramixoviridae. For this study, viruses causing transmissible animal infections were selected, including the Peste des petits ruminants virus, which is currently posing a risk of being introduced to, and spreading in Russia.

The reproduction of VSV, with a multiplicity of infection (MOI) of 0.1 – 0.01 TCD₅₀/cell in PKDCL, revealed early signs of CPE in the form of slight rounding of cells on the second day post-inoculation. On the third day, the delamination of infected cells from the substrate, followed by lysis and destruction of the monolayer, was observed in the cell culture. The accumulation of the virus in PKDCL reached $7,5 \pm 0,25 \lg \text{TCID}_{50}/\text{cm}^3$. In the control cell culture (L-929), this parameter was $6,75 \pm 0,25 \lg \text{TCID}_{50}/\text{cm}^3$.

Akabane virus (MOI 0,1 – 0,01 TCD₅₀/cell) did not cause CPE. Its titer did not exceed $3,5 \pm 0,25 \lg \text{TCID}_{50}/\text{cm}^3$, corresponding to the amount of initially introduced virus. While the accumulation of AKV in the control cell culture (CV-1) was up to $6.75 \pm 0.25 \lg \text{TCD}_{50} / \text{cm}^3$.

EHDV with MOI of 0,1 – 0,01 TCD₅₀/cell, caused slight CPE on the second day post-infection which was expressed by a partial lysis of the monolayer. Cell destruction was noted, in addition to elongated cells along the substrate forming strands. Infectious activity of EHDV in *P. natussii* kidney cell culture was $5,0 \pm 0,25 \lg \text{TCD}_{50}/\text{cm}^3$. In the control culture CV-1, its activity was $6,25 \pm 0,25 \lg \text{TCD}_{50}/\text{cm}^3$.

Migratory routes of bats cover territories of Russia with a high density of small ruminants. Furthermore, causative agents of disease affecting animals belonging to the subfamily Caprine, including PPRV, may

potentially be circulating in these territories, as well. Consequently, we assessed the sensitivity of PKDCL to this virus.

It was found that in PKDCL, the PPR virus with a MOI rate of 0,1 – 0,01 TCID₅₀/cell caused rounding and detachment of infected cells from the substrate accompanying with lysis and destruction of the monolayer on the fourth day post virus introduction. The virus accumulated in PKDCL cells and reached levels of $7.75 \pm 0.25 \text{ lg TCID}_{50}/\text{cm}^3$. That is much higher than in the control infected sensitive cell culture – GT ($4,25 \pm 0,25 \text{ lg TCID}_{50}/\text{cm}^3$).

In parallel, to compare the dynamics of CEP development, we used cultures sensitive to each virus (CV-1, L-929 or GT). The summarized results of virological studies are presented in Table 1.

Table 1
Sensitivity (susceptibility) of the diploid cell line *P. nathusii* kidney to viruses n = 3

№	Virus, Family, type of genome	Diploid cell line <i>P. nathusii</i> kidney		Sensitive (control) cell culture	
		Time of CPE observation of (dpi)	infectious activity TCID ₅₀ /ml	Cell Line	Infectious activity TCID ₅₀ /ml
1	VSV, <i>Rhabdoviridae</i> , RNA	3	$7,5 \pm 0,25$	L-929	$6,75 \pm 0,25$
2	AKAV, <i>Bunyaviridae</i> , RNA	n/o	$3,5 \pm 0,25$	CV-1	$6,75 \pm 0,25$
3	EHDV, <i>Reoviridae</i> , RNA	3	$5,0 \pm 0,25$	CV-1	$6,25 \pm 0,25$
4	PPRV, <i>Paramixoviridae</i> , RNA	4	$7,75 \pm 0,25$	GT	$4,25 \pm 0,25$
Note: * not detected					

In the experiments testing the susceptibility of DCLPK cells to the viruses, the control (uninfected) DCLPK remained its typical morphology, and no CPE was observed.

The characteristics of CPE caused by the viruses in DCLPK cells is presented in Fig. 2.

Analysis of the results of the virological studies has shown that the subcultures of these cells are sensitive to VSV, EHDV and PPRV, and can be considered as a promising cell substrate for primary isolation and further study of these viral pathogens.

With respect to the high infectious activities of VSV and PPRV in DCLPK, exceeding their activities in the control sensitive cell cultures, we carried out a control of their authenticity using molecular methods.

For this purpose, amplicons of VSV and PPR viruses were produced, and nucleotide sequencing was carried out to determine which branch they belong to. Obtained sequences were compared with sequences from the GenBank database by using "Blast" software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). It was confirmed that the obtained culture substrates contain VSV and PPRV.

Additionally, the authenticity of CPE caused by PPRV was confirmed by two serological methods: neutralization test (NT) and ELISA. To perform the NT we used 2-day cell monolayer on the 40th passage grown in 96-wells templates.

The reaction was evaluated based on the dynamics/manifestation of CPE, and by comparing the values of the Reed-Muench virus titrations between normal and specific sera. The calculations revealed a neutralization index (NI) of $10^{3,25}$ or 3,25 lg.

Antibodies against PPRV were detected in serum samples from sheep immunized by PPRV and infected cultural material (see material and methods).

Thus, the results of NT and ELISA tests confirm the authenticity of the PPR virus cultivated in the diploid *P. nathusii* kidney cell line.

Conclusions

The results of the evaluation of the sensitivity (susceptibility, permissivity) of the diploid cell line derived from the kidney of *P. nathusii*, demonstrated a high level of its permissivity to causative agents of Vesicular stomatitis, Epizootic hemorrhagic disease and Peste des petits ruminants.

Reproduction of the above-mentioned viral agents in the cell culture was accompanied by expressed cytopathic effect beginning from the very first passage without preliminary adaptation of the viruses to cells.

P. nathusii inhabits and migrates throughout the area from southern and middle Europe to the middle east. Considering the sensitivity of the cell culture derived from the kidney of this species to BTV, RVFV, LSDV and MV, demonstrated earlier [10], and to VSV, EHDV and PPRV, described in this paper, we can conclude that the diploid cell line derived from *P. nathusii* kidney has a broad spectrum of susceptibility to viruses from different taxonomic groups. It allows us to recommend using them for primary virus isolation and other virological studies, as well as to consider this species as a possible source, carrier (transmitter) and reservoir for the transmissible viral diseases mentioned above.

Material And Methods

Cell cultures

In this study, the diploid cell line *P. nathusii* (*Pipistrellus nathusii* Keyserling & Blasius, 1839; Chiroptera: Microchiroptera: Vespertilionidae) kidney cell strain (PKDCL) developed earlier [10] and cryopreserved in liquid nitrogen at different passage levels was used.

To assess the reproduction and dynamics of the development of the cytopathogenic effect (CPE), we used the following susceptible cell cultures as controls for each virus:

- CV-1 line (African Green Monkey Kidney Fibroblast continuous cell line);
- L-929 (clone strain L cell culture from subcutaneous tissue of mouse),
- GT (primary goat kid testicle cell subculture)

obtained from The Cell Cultures Collection of the Federal Research Center for Virology and Microbiology [11].

Viruses

Another object of this study are several transmissible viruses belonging to different families,

- Vesicular stomatitis virus (VSV, Rhabdoviridae), Indiana strain with infectivity $6,75 \pm 0,25 \lg$ TCID₅₀/cm³ in cell culture L-929;
- Epizootic hemorrhagic disease virus (EHDV), Alberto strain, infectivity $6,25 \pm 0,25 \lg$ TCID₅₀/cm³, in CV-1 cell culture;
- The 8935 Akabane virus strain (AKAV) at the activity $6,75 \pm 0,25 \lg$ TCID₅₀/cm³ in cell culture CV-1
- Peste des petits ruminants virus (PPRV), «45G 37/35-K» strain, with activity $4,25 \pm 0,25 \lg$ TCID₅₀/cm³ in cell culture from testicles of goatling (GT).

The viruses received from The State Collection of Microorganisms Causing Dangerous or Highly Dangerous Diseases, Including Zoonoses, and Exotic Diseases <http://ckp-rf.ru/ckp/441429/>

Medium and solutions

To promote cell growth, we used Eagle's Medium and Dulbecco's Modified Eagle Medium (DMEM, HyClone, USA), fetal bovine serum (FBS, HyClone, USA) and antibiotics (ciprofloxacin 10 µg/ml and amphotericin 5 µg/ml).

When changing the medium, we dispersed the cell monolayer using a mixture of 0.25% trypsin solution and 0.02% Versene solution 1:3 pre-warmed to 37 °C.

The cell cultures were maintained by a conventional method of serial passages at $37 \pm 0,5$ °C in an atmosphere containing elevated gaseous carbon dioxide levels, up to 5%, and a relative humidity of 95%.

Methods

The sensitivity (permissivity) of the *P. nathusii* kidney diploid cell line (PKDCL) was tested by inoculation with a corresponding viral material, with a multiplicity of infection (MOI) of 0.1 – 0.01 TCD₅₀/cell (50% tissue culture infectious dose), into a culture flask containing a confluent cell monolayer. Exposition lasted 60 min. After that, we added a supporting medium containing 2% FBS and incubated the infected culture at 37,0 ± 0,5°C until the CPE of the virus became visible.

The infectious activity of a virus was determined by titration in a virus-sensitive (susceptible) cell culture, using the time of onset of CPE as an index. The virus titer was determined using the Reed and Muench method.

DNA extraction was performed with QIAamp_DNA Mini kit/ QIAamp® Viral RNA kit (QIAGEN, Germany) according to the instruction manual. [13, 14].

The authenticity of PPRV and VSV was confirmed using molecular genetic methods. For the detection of the PPRV genome, we used ID Gene™ Peste des Petits Ruminants Duplex kit (IDvet, France) and primers as described by Kwiatek et al. [12]. To detect the VSV genome, we used primers proposed by Rodriguez et. al [13] followed by sequencing the obtained PCR product.

Purification of PCR products was performed with a Cleanup Standard kit (Evrogen, Russia) followed by Sanger sequencing with BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Austin, TX, U.S.A.) on Applied Biosystem Genetic Analyser 3130 XL (USA).

For additional confirmation of the PPRV authenticity, we performed a neutralization test according to a convenient scheme [15–18] and ELISA test (ID Screen® PPR Competition test kit, France). For this purpose, we tested serum samples taken from three PPRV immunized sheep (“zero-serum”) and specific sera collected from animals on the 14th day post-inoculation of the viral material obtained from a titration of a virus in the *P. nathusii* kidney diploid cell line from wells of 7th dilution after the development of a pronounced CPE.

The animal experimental protocols were approved by the Ethics Committee of the Federal Research Center for virology and Microbiology (Protocol № 1 from February 1, 2021) and were conducted according to Russian legislation and international (ARRIVE) guidelines in animal biosecurity facilities (77.99.03.001.K.000702.0405) of the Federal Research Center for Virology and Microbiology (FRCVM), Volginskiy, Russia.

Virus-neutralizing activity of a specific serum was determined using PPRV (strain 45G35/37-K, MOI of 5,75 lg TCD₅₀/cm³) grown in a green monkey kidney continuous cell line (Vero).

Declarations

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Author contributions

Povolyaeva O. – investigation, visualization, writing – original draft

Chadaeva A. – investigation

Sindryakova I. – investigation

Lapteva O. – investigation

Yurkov S. – conceptualization, methodology, project administration, supervision

Competing Interests Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

Confluent monolayer of PKDCL at the twelfth passage level (magnification×150)

Figure 2

CPE caused by the viruses in DCLPK at different passages (A-D: experimental and E-H: control): A – CPE caused by VSV, 12th passage; B– CPE caused by AKAV, 16th passage; C – CPE caused by EHDV, 25th passage; D – CPE caused by PPRV, 42nd passage; E-H – control (uninfected) cell cultures, 12th, 16th, 25th and 42nd passage, respectively (magnification $\times 150$).