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EXPERIMENTAL WORKS

Development of PCR Test System for Detecting Primate Betaherpesvirinae

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Abstract—A PCR system was developed for detecting primate Betaherpesvirinae viruses. Using the alignment of the complete genomes of human, chimpanzee, and macaque rhesus cytomegaloviruses, conserve regions of viral genes were found. Oligonucleotide primers were developed for consensus conserve regions of the CMV UL56 gene. The PCR conditions were optimized and the primer specificity for cytomegaloviruses of different primate species was confirmed.

Key words: cytomegalovirus, primates, polymerase chain reaction, sequencing

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Cytomegalovirus (CMV), a representative of subfamily Betaherpesvirinae, family Herpesviridae, is widespread in the primate population, as well as in other mammals (pigs, guinea pigs, rats, etc.) [3, 10]. This virus belongs to a group of pathogens of the TORCH complex (from Latin Toxoplasmosis, Others, rubella, chlamydiosis, herpes) and is a real danger to a fetus in the event of intrauterine infection [4, 9]. In 5–15% of children infected with CMV, various disturbances are detected later, mainly in connection with disturbances to the brain and hearing [1, 2]. CMV also causes the development of various diseases in adults. Thus, CMV infection is a frequent cause of complications and death in patients after organ transplantation and AIDS patients [3, 5, 7]. Presently, the data indicates that CMV plays a significant role in the induction of some oncological diseases, particularly large and straight intestine cancer [8].

A study of CMV infection in monkeys (epidemiology, specificities of molecular-biological structure, and phylogenesis of CMV in primate order) will allow us to suggest it as an adequate model for studying human CMV infections and, later to approach the problem of prevention and, possibly, the development of specific treatments [10]. Presently, molecular-biological specificities of CMV of separate primate species are poorly studied.

The goal of the present work is to develop a universal test system for detecting viruses from the subfamily Betaherpesvirinae in primates. To solve this goal, the following approaches were applied: the search for and selection of conservative regions of genes on the whole-genome consensus in the nucleotide sequence of Betaherpesvirinae of various primate species, the development of primers for the chosen conservative consensus nucleotide sequence, and the selection of PCR conditions.

MATERIALS AND METHODS

The study was conducted based on monkeys at the Adler Nursery, Institute of Medical Primatology, Russian Academy of Medical Sciences. Blood samples and homogenates of salivary glands tissues of the primates species, including 60 macaque rhesus (*Macaca mulatta*), 50 long-tailed macaques (*Macaca fascicularis*), 35 hamadryas baboons (*Papio hamadryas*), 15 anubis baboons (*Papio Anubis*), 24 green monkeys (*Cercopitecus aetiops*), 6 pig-tailed macaques (*Macaca nemestrina*), as well as blood samples from 120 clinically healthy people (*Homo sapiens*) served as a material for the study. All monkeys, both males and females, were of aged 1–10 years and were kept in aviaries.

A comparative analysis and the alignment of nucleotide sequences were conducted using BioEdit Sequence Alignment Editor (version 7.0.5.2 Copyright 1997–2005), GeneStudio (TM) Professional Edition (version 1.03.72 1999–2004 GeneStudio Inc.) software. Oligonucleotides-primers were chosen using the Primer Premier program (version 5.00).

DNA extraction. DNA extraction from the materials was conducted by the guanidinethiocionate (GuSN) method. The material (100 μl of blood, solitary gland tissue) was transferred to a tube with 300 μl of 5M GuSCN, 1% trithon X-100 (v/v), 20mM EDTA, 50 mM tris-HCl (pH 6.4), and 10μl SiO_2; incubated for 30 min; centrifugated; and washed with precipitate once by 500 ml of irrigating solution containing 5M GuSCN and 50 mM tris-HCl (pH 6.4) and twice by 500 ml of irrigating solution that contains

Table 1. DNA sequences of CMV used to search for conservative gene regions

GeneBank accession number	Detection	Virus
AF480884	*Chimpanzee cytomegalovirus*	*Pongine herpesvirus 4 (Po HV-4)*
AY186194	*Cercopithecine herpesvirus 8*	*Cercopithecine herpesvirus 8 (CeHV-8)*
DQ120516	*Cercopithecine herpesvirus 8 isolate CMV 180.92*	*Macacine herpesvirus 3 (RhCMV)*
AC146904	*Human Herpesvirus 5 PH-BAC isolate*	*Human Herpesvirus 5 (HCMV)*
AC146851	*Human Herpesvirus 5 Towne-BAC isolate*	*Human Herpesvirus 5 (HCMV)*
X17403	*Human cytomegalovirus strain AD 169*	*Human Herpesvirus 5 (HCMV)*
AC146905	*Human Herpesvirus 5 Toledo-BAC isolate*	*Human Herpesvirus 5 (HCMV)*
FJ616285	*Human Herpesvirus 5 strain Towne*	*Human Herpesvirus 5 (HCMV)*

10mM tris-HCl (pH 7.3), 50 mM NaCl, and 50% ethanol. DNA was eluted in 50 μl of 0.1 M tris-EDTA (TE) buffer (pH 8.3) [6]. For PCR amplification 5 μl were used, the rest was kept under −20°C.

PCR-analysis. Amplification was conducted in 25 μl of solution that contains 50 mM KCl, 10 mM tris-HCl (pH 8.4), 3 mM $MgCl_2$, 0.01% of gelatin, 100 ng of each primer, 0.2 mM of each dNTP, and 2.5 U of Tag DNA-polymerase. Primers were synthesized in Sintol (Moscow). As a positive control, DNA was used that was extracted from cultural medium of human fibroblast cells that was infected by laboratory strain AD169 human CMV. PCR was conducted on a Tercik instrument (DNA Technology, Moscow).

PCR products were analyzed electrophoretically in 2% agarose gel with 0.5 μg/ml of ethidium bromide. Electrophoresis was conducted for 15 min at 50 A, 10 V/sm. Amplification products were visualized on a transilluminator at a UV wavelength of 330 nm. The positive reaction was judged by an amplicon band that corresponded to a size of 200 bp. Nucleotide sequences were sequenced on an ABI Prism 3100 automatic sequenator (Applied Biosystems, United States) at Postgenome and Nanotechnology Innovations (Moscow).

RESULTS AND DISCUSSION

Using GeneBank database, we conducted a search for the known whole-genome nucleotide sequences of CMV in humans and monkeys of various species. The sequences presented in Table 1 were chosen for analysis. We proceeded based on the assumption that a comparative analysis of nucleotide sequences will allow us to identify conservative regions of DNA sequences that are specific to the entire subfamily Betaherpervirinae. The analysis was conducted in two stages. At the first stage, we compared whole-genome sequences of human CMV and obtained a consensus sequence (HCMV consensus). At the second stage, we conducted a comparative alignment of HCMV consensus with whole-genome sequences of chimpanzee CMV (PoHV-4) and macaque rhesus (RhCMV and CeHV-8). The percentages of homology are given in Table 2.

As shown in Table 2, the percentage of homology among CMV of various species is rather small, which indicates the probable ancient origin of the virus and early evolutionary divergence of CMV in primate order; it also explains the species specificity of the virus. The alignment of whole-genome sequences of CMV of chimpanzee (AF480884) and macaque rhesus (AY186194 and DQ120516) with consensus sequence HCMV consensus allowed us to select maximally homologous regions of nucleotide sequences. As a result of the search of conservative regions by all lengths of the consensus sequence, several regions with 100% homology were revealed.

The analysis indicated that the UL56 gene fragment, which encodes DNA-binding protein with a key role in viral DNA wrapping, is a suitable choice of primer [11]. The given gene fragment contains the longest homologous regions and is the most conservative, not only in subfamily Alphaherpesvirinae [5], but also in subfamily Betaherpesvirinae. The given fact allowed to assume that the mentioned region must be conservative for all primate CMVs, including those not yet studied. The results of the conducted comparative alignment of UL56 gene region of primates CMV are presented in Fig. 1.

Using the Primer Premier program (version 5.00), primers were selected for the given region. The search

Table 2. Homology of whole-genome sequences of CMV of various species of primates

Virus	Homology			
	HHV-5	PoHV-4	CeHV-8	RhCMV
HCMV consensus (HHV-5)	–	60.2	45.6	45.9
Pongine herpesvirus 4 (PoHV-4)	60.2	–	44.6	45.2
Cercopithecine herpesvirus 8 (CeHV-8)	45.6	44.6	–	95.3
Rhesus cytomegalovirus (RhCMV)	45.9	45.2	95.3	–

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                91610     91620     91630     91640     91650     91660     91670     91680     91690     91700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
HCMV consensus  TTGAACACCGGGTTGTCCTCGAAAGCTTGAATGCTGAGGGATGTGATGAGGTCGATGATCCTGTTGGGGGCGGCAAAGACCGACCCCACGAACATGCGCT
PoHV-4          ........G..A..........G..C.............C..A.....A..........C.................G..............
CeHV-8          .....A.....................................A..T.............C.....................A.GA....
RhCMV           .....A.....................................A..T.............C.....................A.GA....
Cconsensus      .....M..S..R..........R..Y.............Y..R..K..R..........Y.................S........M.KR....

                91710     91720     91730     91740     91750     91760     91770     91780     91790     91800
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
HCMV consensus  CCTCCCCGTCCAACGCCTTTTCCCCGAGCACGAAGATGTCCTCCACGTCCTCCCCGTACAGATGGCGACTGATGCCGTTCATGAGCGCCCGGCACAGCTG
PoHV-4          ........G...G.A.............G.....................G.............GT..........A....A.....G..
CeHV-8          G...G.G.GAA.G.AG.....................A.....................G.AC.TG....G.A..........GA...T......G..
RhCMV           G...G.GAGAA.G.AG.....................A.....................G.AC.TG....G.A..........GA...T......G..
Cconsensus      S...S.SRKMM.R.RS.............R....R.....................R.RS.KR....K.VY.........SR...KR.....S..

                91810     91820     91830     91840     91850     91860     91870     91880     91890     91900
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
HCMV consensus  GTGATACACATTTAGCTGCTGGATGGTGATGCCCACCCGCTTGACGATAACCTCCGAGGTACGGGACCAGTAGGTAAAATCCGACAAGGAATATATTCGT
PoHV-4          ...G..G.....................CC.............T...........A.....................................
CeHV-8          ......G.T.............C...C.T..C.............T.....................................................
RhCMV           ......G...............C...C.T..C.............T.....................................................
Cconsensus      ...R..S.Y.............S...S.K.YS.............K...........R.....................................

                91910     91920     91930     91940     91950     91960     91970     91980     91690     9200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
HCMV consensus  TCCGGTATATCCGTAAACAGGTTGTACTCCCTCAGCGCCTCCTCCGCCTCCTGGATGTAGCTGTGGTAGGCCGATGAAGAAGAGAATAGGCTTTTGAGGG
PoHV-4          ..........................................................................A..G.............
CeHV-8          G.........................................................A.....A....A.....C...T.........
RhCMV           G.........................................................A.....A....A.....C...T.........
Cconsensus      K.........................................................R.....R....W.....R..B...Y.........
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Fig. 1. Comparative alignment of nucleotide sequences of *UL56* gene fragment of CMV of primates. Primers locations are underlined.

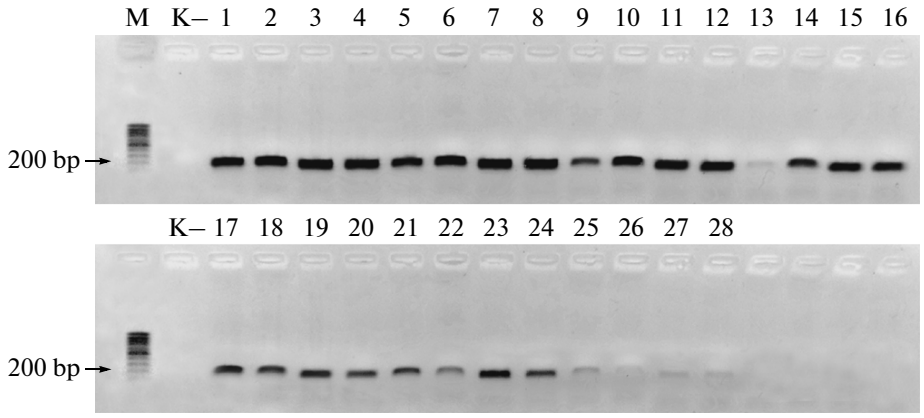

Fig. 2. Electrophoregramm of PCR products using positive control: *M*, marker of molecular mass of phage λ DNA/ HindII; *K*, negative control; 1–28. positive control; annealing temperature: (1–4) 61°C; (5–8) 62°C; (9–12) 63°C; (13–16) 64°C; (17–20) 65°C; (21–24) 66°C; (25–28) 67°C; concentration of Mg ions: (1, 5, 9, 13, 17, 21, 21) 2 mM; (2, 6, 10, 14, 18, 22, 26) 2.5 mM; (3, 7, 11, 15, 19, 27) 3 mM; (4, 8, 12, 16, 20, 24, 28) 3.5 mM.

was conducted using the following algorithm: primers length 20 ± 2, amplicon length 200–500 bp. As a result of the search, in the sequence of the UL56 gene, several nucleotide sequences corresponded to the aforementioned characteristics. However, a further analysis of oligonucleotides on the formation of hairpins, dimmers, cross dimmers, and false annealing revealed the most stable pair of primers of the following sequence: left primer, 5'-ctc cac gtc ctc ccc gta-3'; right primer, 5'-agg cgc tga ggg ata caa c-3', which amplificated a region with the length 200 bp. Checking the primer sequences for specificity using BLAST indicated 100% homology in only the primate UL56 gene. The localization of primers is given in Fig. 1.

PCR optimization was conducted by the following parameters: annealing temperature was changed from 61 to 67°C with a step of 1°C and the concentration of Mg ions was changed from 2 to 3.5 mM with a step of 0.5 mM. Electrophoregramms of PCR products are given in Fig. 2. In the process of PCR optimization, it was indicated that the system operates in a wide range of temperature and concentration of Mg^{2+} ions.

To decrease the capability of unspecific binding of primers with DNA target, the following conditions of

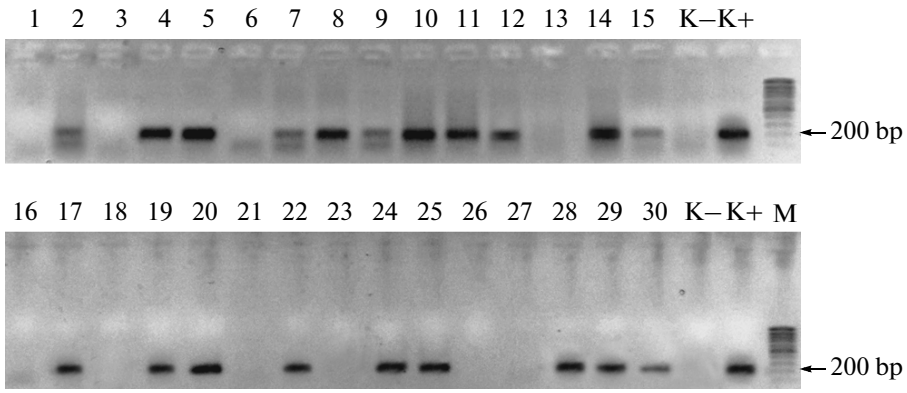

Fig. 3. Electrophoregram of random screening on CMV DNA samples extracted from various species of primates. *M*, marker of molecular mass of phage λ DNA/ HindII; *K*, negative control, K^+, positive control; No. 1–3. *M. nemestrina*; No. 4–8. *C. aetiops*; No. 9–13. *P. hamadryas*; No. 14–17. *M. fascicularis*; No. 18–22. *M. mulatta*; No. 23–25. *P. anubis*; No. 26–30. *H. sapiens.*

PCR were chosen: Mg^{2+} concentration 3 mM, temperature regime 95°C for 2 min; five cycles at 95°C for 50 s, 64°C for 50 s, 72°C for 50 s; and 40 cycles at 95°C for 40 s, 62°C for 35 s, 72°C for 35 s, and storage at 10°C.

To choose the abilities of the given test system PCR to reveal CMV of various species of primates, 190 DNA samples were selected that were extracted from the blood and salivary gland of primates of the following species: *M. mulatta, M. fascicularis*, *P. hamadryas*, *P. Anubis*, *C. aetiops*, and *M. nemestrina*, as well as 120 DNA samples extracted from human blood. The results of the amplification of separate DNA samples of various species of primates are presented in Fig. 3.

As can be seen from Fig. 3, positive samples on CMV are revealed in various species of primates, including *M. nemestrina* (No. 2), *C. aetiops* (No. 4, 5, 7, 8), *P. hamadryas* (No. 9, 10, 11, 12), *M. fascicularis* (No. 14, 15, 17), *M. mulatta* (No. 19, 20, 22), *P. Anubis* (No. 24, 25), and *H. sapiens* (No. 28, 29, 30).

To confirm the positive PCR results, the obtained amplicons were sequenced. An analysis of sequences revealed 98% homology with UL56 gene fragments of Betaherpesvirinae in various species of primates. Thus, the developed test system enabled us to reveal CMV of various species of primates and can be recommended for the further use.

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