EXPERIMENTAL RESEARCHES

QUANTIFICATION OF BACKGROUND EXPRESSION OF INTERFERON BETA IN CELL CULTURE OF SIBERIAN BAT (*MYOTIS SIBIRICUS*)

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RESUME

Background. *The study of the immune response of these mammals to viral infections is necessary to reveal the fundamental mechanisms of the circulation of zoonotic infections in nature. There is a hypothesis about the constantly "on" activity of the interferon pathway proteins, developed evolutionarily in bats to counteract viral infections. We assessed the expression of interferon beta as a marker of the innate immune system in kidney cells of the Siberian bat (Myotis sibiricus, Kastschenko, 1905) MdbK3-14.*
The aim. *Evaluation of the background level of interferon beta (IFN-β) gene expression in bat cells as a marker of the activity of the mammalian innate immune system.*
Materials and methods. *MdbK3-14 cells were grown in 24-well plates. Cell monolayers were detached with trypsin solution and total RNA was isolated. The concentration of mRNA of IFN-β gene transcripts and reference genes beta actin (ACTB) and succinate dehydrogenase subunit A (SDHA) was determined by one-step multiplex RT-qPCR and confirmed by RT-dPCR.*
Results. *Specific primers with a probe for detecting mRNA of the IFN-β gene in bat cells were designed. The detection of SDHA and IFN-β gene transcripts was stable both in RT-qPCR (CV = 0.5 % and CV = 0.2 %, respectively) and in RT-dPCR (CV = 0.8 % and CV = 1.4 %, respectively). In addition, stable detection of ACTB mRNA was achieved using RT-dPCR (CV = 0.8 %), but the average variability value for actin using RT-qPCR exceeded the permissible value (CV = 3.6 % with an acceptable CV ≤ 2 %). The results of quantitative determination in RT-qPCR and RT-dPCR correlated with each other. The expression levels of IFN-β in MdbK3-14 cells averaged 0.97 ± 0.15 relative units in RT-qPCR and 0.13 ± 0.05 relative units in RT-dPCR.*
Conclusions. *In the absence of immune stimulation, background expression of IFN-β occurs in the M. sibiricus kidney cell line.*

Key words: *Myotis sibiricus, cell lines, expression, mRNA, SDHA, ACTB, IFN-β, housekeeping genes, quantitative RT-PCR, digital PCR*

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КОЛИЧЕСТВЕННОЕ ОПРЕДЕЛЕНИЕ ФОНОВОЙ ЭКСПРЕССИИ ИНТЕРФЕРОНА БЕТА В КУЛЬТУРЕ КЛЕТОК СИБИРСКОЙ НОЧНИЦЫ (*MYOTIS SIBIRICUS*)

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РЕЗЮМЕ

Обоснование. *Рукокрылые являются хозяевами и переносчиками широкого спектра зоонозов. Исследование иммунного ответа этих млекопитающих на вирусные инфекции необходимо для раскрытия фундаментальных механизмов циркуляции зоонозных инфекций в природе. Существует гипотеза о постоянно «включенной» активности белков интерферонового пути у рукокрылых для противодействия вирусным инфекциям. В этом исследовании мы оценили уровень активности системы врожденного иммунитета в клеточной линии почки сибирской ночницы (Myotis sibiricus, Kastschenko, 1905) MdbK3-14, взяв в качестве маркера экспрессию интерферона бета.*

Цель исследования. *Оценить фоновый уровень экспрессии гена интерферона бета (IFN-β) в неинфицированных клетках Myotis sibiricus.*

Методы. *Культуру клеток MdbK3-14 выращивали в 24-луночных планшетах. Монослои клеток открепляли раствором трипсина и выделяли суммарную РНК. Концентрацию мРНК транскриптов гена IFN-β и референтных генов бета-актина (ACTB) и субъединицы A сукцинатдегидрогеназного комплекса (SDHA) определяли с помощью одностадийной мультиплексной ОТ-рвПЦР и подтверждали с помощью ОТ-цПЦР.*

Результаты. *Разработаны специфичные праймеры с зондом для детекции мРНК гена IFN-β в клетках рукокрылых. Выявлена стабильная детекция транскриптов генов SDHA и IFN-β как в ОТ-рвПЦР (CV = 0,5 % и CV = 0,2 % соответственно), так и в ОТ-цПЦР (CV = 0,8 % и CV = 1,4 % соответственно). Детекция мРНК ACTB в ОТ-цПЦР также проходила равномерно во всех образцах (CV = 0,8 %), однако в ОТ-рвПЦР выявлена некоторая нестабильность для бета-актина (CV = 3,6 %). Результаты количественного определения в ОТ-рвПЦР и ОТ-цПЦР коррелировали между собой. Установлено, что уровень экспрессии IFN-β в клетках MdbK3-14 сопоставим (в ОТ-рвПЦР в среднем 0,97 ± 0,15 отн.ед.) или несколько ниже (в ОТ-цПЦР в среднем 0,13 ± 0,05 отн.ед.), чем экспрессия белков домашнего хозяйства ACTB и SDHA.*

Заключение. *При отсутствии иммунной стимуляции в клетках почки M. sibiricus наблюдается фоновая экспрессия IFN-β.*

Ключевые слова: *Myotis sibiricus, клеточные линии, экспрессия, мРНК, SDHA, ACTB, IFN-β, гены «домашнего хозяйства», количественная ОТ-ПЦР, цифровая ОТ-ПЦР*

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BACKGROUND

Bats are hosts and vectors of a diverse range of zoonotic infections [1]. Studying the immune response of these mammals to viral infections is essential to uncover the underlying mechanisms of zoonotic pathogen circulation in nature and to assist in understanding the emergence of novel human diseases. The first line of defense in mammals is the innate immune system, composed of various cells and molecules that recognize and eliminate pathogens of viral and bacterial origin, thereby protecting the organism from the development of immunopathological processes.

A key component in the activation of the innate immune response is interferon beta (IFN-β). This cytokine is part of the type I interferon family, which is induced directly in response to viral infection [2]. The expression and antiviral activity of *IFN*-β have been described in kidney cells of the Chinese bat, *Myotis davidii*, after infection with Sendai virus, a member of the *Paramyxoviridae* family [3]. It has been established that virus-induced *IFN*-β gene expression is higher in bat cells than in cells from murid rodents [4]. Type I interferons (IFN-α/β) are thought to play a role in the high tolerance of bats to zoonotic viruses [5]. There is a hypothesis that bats have a constitutively active interferon system [6]. It is possible that novel viruses entering the body of bats do not cause immunopathological responses due to the evolutionary development of the constant activity of interferon pathway proteins. This feature may be characteristic of all bat species. However, this area of research remains under-investigated. For instance, foreign researchers have demonstrated that IFN-α is expressed constitutively in all organs of healthy individuals from the Australian black flying fox (*Pteropus alecto*) and the Malayan short-nosed fruit bat (*Cynopterus brachyotis*). IFN-β, on the other hand, was found to be barely detectable in these species [7]. However, both of these bat species are members of the *Pteropodidae* family, suborder Megachiroptera (flying foxes). In contrast, other researchers have identified basal levels of IFN-β and type I IFN subtypes in kidney cells of *Myotis daubentonii*, a species belonging to the suborder Microchiroptera (vesper bats) [5]. Furthermore, in contrast to the findings from fruit bats, the researchers did not identify any specific IFN-α subtypes or unusually high levels of basal IFN expression in *Myotis daubentonii* cells. This suggests a clear distinction between the interferon systems of bats (Microchiroptera) and flying foxes (Megachiroptera). This finding clearly warrants further research into each group of bats.

Previously, we established a continuous cell line, MdbK3-14, derived from the kidney of the Siberian bat (*Myotis sibiricus*, Kastschenko, 1905). This species is widespread and abundant in nature. We found that the replication of flaviviruses, particularly tick-borne encephalitis virus (TBEV), in this cell line, is reduced compared to other cell lines derived from reservoir (Korean field mouse) and laboratory (pig) hosts of the virus [8]. This was demonstrated by impaired virus entry into MdbK3-14 cells, decreased efficiency of RNA replication and production of infectious viruses, delayed viral replication kinetics, and lower final titers of infectious viruses [9]. Additionally, compared to cells derived from laboratory and reservoir hosts, the MdbK3-14 cell culture was less susceptible to TBEV infection. The virus did not cause a cytopathic effect and cell death was not observed as a result of TBEV infection [10]. To investigate the mechanisms of cellular resistance to viral infection, we assessed the basal level of the innate immune response in uninfected MdbK3-14 cells.

This study aimed to evaluate the basal expression level of the *IFN*-β gene, encoding interferon beta, in Siberian bat cells as a marker of innate immune system activity.

The primary method for the quantitative analysis of gene expression profiles was reverse transcription quantitative polymerase chain reaction (RT-qPCR). The genes encoding beta-actin (*ACTB*) and succinate dehydrogenase complex subunit A (*SDHA*) were selected as references. Beta-actin is a crucial protein for cell motility, structure, and integrity in eukaryotic cells [11], while succinate dehydrogenase complex subunit A is an integral part of the protein complex located in the mitochondrial membrane and involved in the Krebs cycle and respiratory chain [12]. The selection of these genes was based on their critical role in maintaining cell viability, which ensures the stability of their expression under various external conditions. The methodology for assessing the expression of *ACTB* and *SDHA* has been previously validated in rodent and artiodactyl cell cultures [13], suggesting that it can be applied to bat cells as well. In addition to the previously described methods, we utilized reverse transcription digital polymerase chain reaction (RT-dPCR) as a further method for quantifying the mRNA concentrations of both the target and reference genes. While RT-dPCR allows for absolute quantification of the target fragment concentration in each sample, it is also advisable to normalize the results using reference genes [14], since the samples may be affected by other factors, such as the reverse transcription process.

MATERIALS AND METHODS

Primer design

A primer-probe set was designed for the detection of bat interferon beta (*IFN-β*) mRNA from *Myotis* species based on sequence data published in the GenBank database under accession numbers XM_005853023 and XM_036329120. Sequence alignment was performed using BioEdit 7.0.5.3 software, incorporating the integrated CLUSTAL W algorithm. Manual adjustment was then performed. The OligoCalc web-based tool was also used for oligonucleotide sequence design. The specificity of the primers and probes was verified through BLAST searches and by PCR, with amplicons visualized on an agarose gel. The design, structure, and validation

of primers and probes for reference genes *ACTB* and *SDHA* have been published previously [13].

Cell culture

The continuous adherent cell line, MdbK3-14, was derived from the kidney tissue of *Myotis sibiricus* (Kastschenko, 1905) and was established in the Laboratory of Transmissible Infections at the Scientific Centre for Family Health and Human Reproduction Problems [9]. A cell stock of passage 64 was used for this study. The cell culture was maintained in RPMI1640 medium, supplemented with antibiotics and 10 % fetal bovine serum (FBS) from HyClone (Thermo Scientific, UK).

The study was approved by the Biomedical Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problems (Protocol No. 2 dated February 18, 2020).

Cell preparation

A cell monolayer with a confluence of 90–100 % was detached from a flask using trypsin supplemented with 0.5 mM EDTA (T/E). Cells were then resuspended in growth medium (RPMI1640 supplemented with L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL), and 10 % FBS), and the cell concentration was determined using a Goryaev hemocytometer. The resulting cell suspension was adjusted to a concentration of 1×10^5 cells/mL and seeded into 24-well plates, with 1 mL of suspension per well. The plates with cells were incubated for 16–18 hours at 37 °C under 5 % CO_2. Subsequently, the growth medium was removed and replaced with 1 mL of maintenance medium (RPMI1640 with L-glutamine, antibiotics, and 2 % FBS) per well. After 24 hours of incubation, the maintenance medium was removed and the cells were washed three times with serum-free medium. They were then detached using 0.25 mL of T/E. Finally, 0.25 mL of maintenance medium was added to each well to resuspend the cells, which were transferred to 1.5 mL Eppendorf tubes. Cells were pelleted by centrifugation using an Eppendorf MiniSpin centrifuge at 13,400 rpm for 5 min at +4 °C. The cells were then resuspended in 1 mL of sterile phosphate-buffered saline (PBS, pH 7.4). The cell concentration was determined and aliquots containing 5×10^4 cells per sample were transferred to cryovials. The cells were again pelleted using centrifugation at +4 °C, and the supernatant was removed, leaving only the cell pellet. This pellet was then stored at -70 degrees C and used for RNA isolation immediately. All steps involving cell sample collection took place on ice to ensure optimal conditions.

RNA isolation

To assess the expression of the *SDHA, ACTB*, and *IFN*-β genes in MdbK3-14 cells, RNA samples were isolated using the HiPure Total RNA Kit (Magen Biotechnology, Guangzhou) and the RNAse Free DNAse I Set (Magen Biotechnology, Guangzhou) according to the manufacturer's protocol. The purified RNA sample volume was 100 µL. The quality of RNA purification from genomic DNA was assessed by performing an additional PCR without RT reaction using primers specific for the target genes. The RNA preparations that did not show specific fluorescence in RT-quantitative PCR (RT-qPCR) and did not contain the target fragment after standard PCR were considered suitable for further analysis. Negative control RNA samples were included in each experiment.

One-step RT-PCR

One-step multiplex RT-qPCR and RT-dPCR assays were performed using the Luna Universal Probe One-Step RT-qPCR reagent kit (New England Biolabs, USA), following the manufacturer's protocol. The reaction volume was 30 µL. The reaction mix contained two specific primers at a final concentration of 400 nM each, a corresponding probe at 200 nM, and 3 µL of RNA template. The thermal cycling conditions for both RT-qPCR and RT-dPCR were as follows: reverse transcription at 55 °C for 10 min, reverse transcriptase inactivation at 95 °C for 1 min, followed by 45 cycles of PCR. Each cycle consisted of 10 s at 95°C, annealing at a gene-specific temperature (Ta°C) for 1 s; and 20 s at 60 °C. Fluorescence acquisition was performed during the 60 °C step of each cycle using the FAM, ROX, and Cy5 channels. The quantification cycle (Cq) for RT-qPCR was defined as the first cycle in which the fluorescence signal intensity exceeded ten standard deviations above the background fluorescence. Fluorescence from cycles 1 to 10 was considered background. Data analysis for RT-qPCR was performed using Bio-Rad CFX Manager v3.1 software (Bio-Rad Laboratories Inc., USA).

RT-dPCR was performed using the SCI Digital S500 automated digital PCR system (TurtleBiotech, China) according to the manufacturer's protocol. This platform partitions the sample into more than 20,000 independent, uniformly sized microdroplets within the microcavities (wells) of a digital PCR chip using an automated system device. Following reaction completion, the fluorescent signal from each microdroplet is detected and enumerated. The concentration of specific RNA molecules in the original sample was determined based on the Poisson distribution principle using SCI Digital v1.0.0.0P1.7 software (TurtleBiotech, China).

Assessment of PCR linearity and efficiency

For each RNA sample, a series of three 10-fold serial dilutions (10^{-2} to 10^{-5}) was prepared in RNase-free water.

For the RT-qPCR assay, the mean Cq value and standard deviation were calculated for each dilution. A standard curve was generated using the mean Cq values. RT-qPCR efficiency (*E*) was calculated using the formula {10^(-1/k) – 1} × 100, where k is the slope of the standard curve, and expressed as a percentage. Reaction efficiency was considered acceptable within the range of 90 % ≤ *E* ≤ 110 %.

To assess RT-dPCR linearity, the expected RNA concentrations of the dilutions were compared with the observed values. The RNA concentration in the 1×10^{-2} dilution was considered the starting concentration. Expected RNA concentrations for the 1×10^{-3} and 1×10^{-4} dilutions were calculated by dividing the starting concentration by 10 and 100, respectively. For each dilution, the mean concentration obtained by RT-dPCR was calculated and used as the observed concentration, along with its standard deviation.

Assessment of RT-qPCR and RT-dPCR repeatability

Intra-assay repeatability was determined as the coefficient of variation (CV, %) and expressed as $\log_{10}$ concentration. For RT-qPCR, the CV was calculated from three independent replicates of each RNA dilution performed within a single assay on the same day using the same instrument. For RT-dPCR, the CV was determined from two independent replicates of each RNA dilution.

Statistical analysis

RT-PCR linearity was assessed using regression analysis of the calibration curves. The relationship between Cq and the sample dilution factor for RT-qPCR, and between the observed and expected RNA concentrations for RT-dPCR, were considered linear when the coefficient of determination (R^2) was > 0.8. To assess data variability, the standard deviation of the mean values was determined. Outlier Cq values for RNA were excluded using the quartile method [15]. The Pearson correlation coefficient (*r*) was used to identify correlations between RNA concentrations obtained by RT-dPCR and Cq values obtained by RT-qPCR. Relative *IFN-β* expression from RT-qPCR results was calculated using the ΔΔCq method and normalized to the *SDHA* and *ACTB* reference genes. Normalized *IFN-β* expression from RT-dPCR results was calculated as the ratio of *IFN-β* mRNA concentration to the geometric mean of the reference gene concentrations. Calculations were performed using MS Office Excel 2003, MaxStat Lite v.3.06, and CFX Manager software.

RESULTS AND DISCUSSION

PCR specificity

The selected primer pairs for the *ACTB, SDHA*, and *IFN*-β genes demonstrated high specificity, producing amplicons of the expected size and no additional or non-specific bands at an annealing temperature of 55 °C (Fig. 1). The identical PCR conditions for all genes allowed for multiplexing of the reaction, thereby enhancing the comparability of the data obtained.

RT-qPCR analysis revealed a clearly defined sigmoidal shape of the amplification curves for the target mRNAs of the studied genes, with negligible background fluorescence. In all plots, the fluorescence curves for the 10-fold serial RNA dilutions ranging from 10^{-2} to 10^{-4} crossed the threshold line at approximately 3-cycle intervals, corresponding to a 10-fold dilution of the samples. Samples with a 10^{-5} dilution for the *ACTB* and *SDHA* genes were identified as outliers based on the quartile test and were excluded from further analysis. No increase in fluorescence signal was observed in the negative control samples, indicating the absence of DNA contamination and confirming the specificity of *ACTB, SDHA*, and *IFN*-β mRNA detection in bat cells. The overall dynamic range of the assay was 10,000:1.

PCR linearity and efficiency

The coefficient of determination (R^2) for linearity in RT-qPCR exceeded 0.99 for all calibration curves (Fig. 2). This indicates that across all assay variants, the relationship between Cq and target RNA concentration conforms to a linear regression model and provides a good fit to the observed data. The mean RT-qPCR efficiency values fell within the optimal range of 90 % to 110 % inclusive [16, 17] and were 105 % (*ACTB*), 92 % (*SDHA*), and 101 % (*IFN*-β), with calibration curve slopes of 3.22, 3.53, and 3.31, respectively.

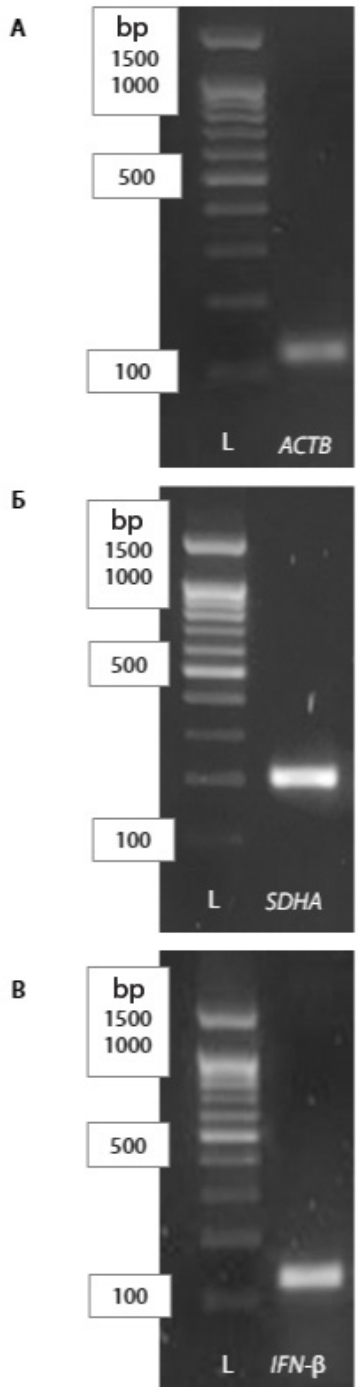

FIG. 1.
Specificity of PCR for fragments of ACTB, SDHA and IFN-β mRNA transcripts of bats at an annealing temperature of 55°C. A – ACTB gene, expected fragment length 120 bp; Б – SDHA gene, expected fragment length 209 bp; B – IFN-β gene, expected fragment length 119 bp. L – DNA size ladder, the bands of 100, 500, 1000 and 1500 base pairs are labeled aside of the gel

In RT-dPCR, at high template loads (1:100 dilution), the distribution of positive wells across the chip surface was uneven, with an evident depletion towards the right edge of the image. Visually, this artifact occupied approximately 10–20 % of the chip capacity (Fig. 3, row "1 × 10^{-2}"). As a similar pattern was observed in the second RT-dPCR replicate (image available upon request), it is unlikely that this phenomenon is due to a defect in a single chip. Further investigation and optimization of the SCI Digital S500 system are required to elucidate the nature and potential consequences of this uneven distribution of positive signals when processing high (up to saturating) mRNA concentrations. At RNA template dilutions of 1:1000 and 1:10,000, the distribution of positive wells on the chip was random and uniform (Fig. 3, rows "1 × 10^{-3}" and "1 × 10^{-4}"), allowing for further analysis.

In a series of 10-fold dilutions of total RNA, it is theoretically expected that the concentration of the target mRNA should also change 10-fold with each dilution. To assess the reliability of RT-dPCR quantification using the SCI Digital S500 system, expected mRNA concentrations were calculated based on the 1:100 dilution as the reference point. A regression analysis was then performed to evaluate the agreement between the observed and expected mRNA concentrations. The observed data for all target genes demonstrated a strong linear correlation with the expected values ($R^2 > 0.98$, Fig. 4), indicating a good fit to the linear model. These findings suggest that, despite the uneven signal distribution observed at high template concentrations, the data obtained accurately reflects the true mRNA concentration and is suitable for evaluating *IFN*-β expression.

Repeatability of RT-qPCR and RT-dPCR assays

The coefficients of variation for intra-assay reproducibility of *SDHA, ACTB*, and *IFN*-β quantification are presented in Table 1.

In Siberian bat MdbK3-14 cells, *IFN*-β mRNA detection was more stable with RT-qPCR than with RT-dPCR (CV = 0.2 % vs. CV = 1.4 %), although both CV values fell within the optimal range (CV ≤ 2 %). RT-qPCR demonstrated higher sensitivity for *IFN*-β mRNA detection, as it consistently detected mRNA at a 10^{-5} dilution (Cq = 39.9 and CV = 0.3 %). In contrast, RT-qPCR consistently yielded negative results under identical conditions (Table 1). Due to the fact that both the reaction mixtures and RNA preparations were identical in the two assays, it is apparent that optimization of the experimental conditions is necessary, taking into consideration the specific characteristics of the dPCR platform.

The stability of *SDHA* gene transcript detection between RT-dPCR and RT-qPCR was comparable (CV = 0.8 % and CV = 0.5 %, respectively), falling within the acceptable range for intra-assay variability.

ACTB mRNA detection by RT-dPCR was uniform across all samples (CV = 0.8 %), with little deviation from the mean quantitative value. In contrast,

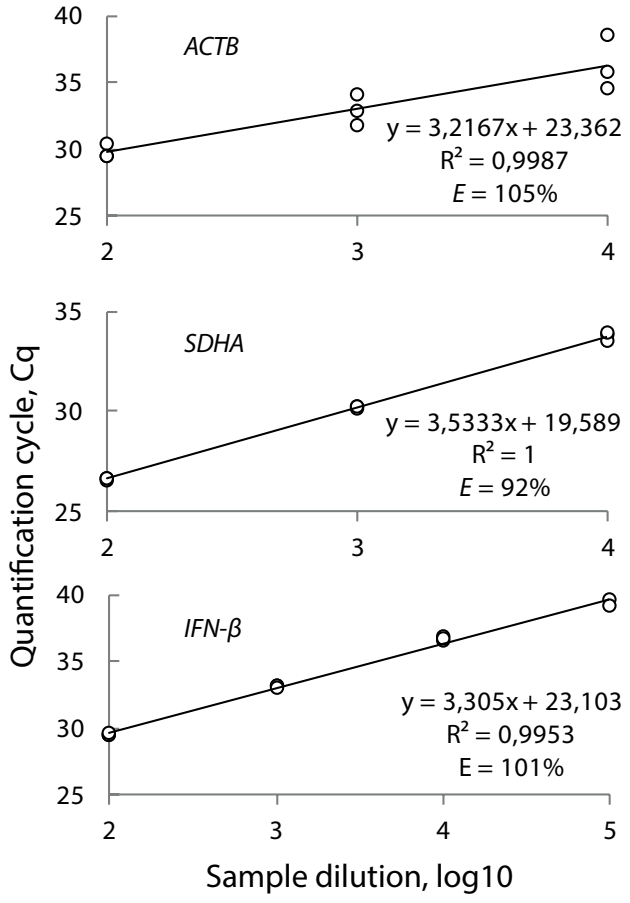

FIG. 2.
Evaluation of the linearity (R^2) and efficiency (E) of RT-qPCR targeting bat genes for beta-actin (ACTB), succinate dehydrogenase subunit A (SDHA) and interferon beta (IFN-β). Error bars indicate standard deviations of three replicates of RT-qPCR

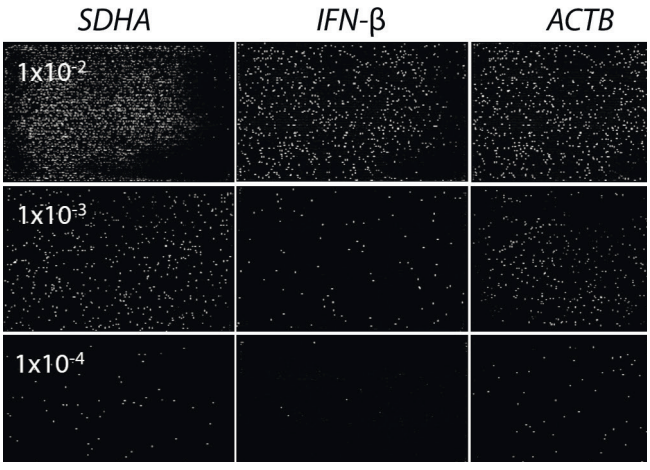

FIG. 3.
Positive droplets distribution in the chips «1x10^{-2}», «1x10^{-3}» and «1x10^{-4}» with triplex RT-dPCR reactions in the FAM (for detection of SDHA transcripts), ROX (IFN-β), and Cy5 (ACTB) fluorescence channels. The RNA template dilution in each chip was 1:100, 1:1000, and 1:10000, respectively. White dots indicate a positive PCR-amplification signal in the corresponding channel

some instability in beta-actin detection was observed with RT-qPCR (CV = 3.6 %). It is generally accepted that for accurate quantitative PCR results, intra-assay reproducibility should correspond to CV values of ≤ 2 % [17]. A CV value exceeding this acceptable threshold indicates higher data variability within the studied sample set. In the case of *ACTB*, this may be attributed to technical in the RT-qPCR process. This suggests the need to optimize the RT-qPCR conditions in order to improve the stability of *ACTB* mRNA detection throughout the experiment, by automating as many manual procedures as possible, such as the preparation of RNA sample dilutions and the pipetting of samples into the PCR mixture. This is expected to reduce the variability in the quantitative data obtained.

Agreement analysis of RT-qPCR and RT-dPCR results

Pearson correlation analysis revealed a strong negative correlation between Cq values obtained from RT-qPCR and mRNA concentrations measured by RT-dPCR for all genes under study (Fig. 5). Therefore, the quantitative mRNA concentrations derived from RT-qPCR and RT-dPCR assays are consistent with each other, and an inverse linear relationship exists between them, such that an increase in mRNA molecule concentration measured by RT-dPCR corresponds to a decrease in Cq value calculated by RT-qPCR.

Assessment of basal IFN-β expression in the MdbK3-14 cell line

The results obtained suggest that there is a continuous basal expression of interferon beta in the MdbK3-14 cell line (Fig. 6). When measured by RT-dPCR, the expression level was approximately 10-fold lower than that of housekeeping genes (mean 0.13 ± 0.05 relative units). However, when measured by RT-qPCR, the *IFN*-β expression was comparable to the expression of reference genes (0.97 ± 0.15 relative units).

The observed differences in the relative levels of *IFN*-β expression can be attributed to two factors. Firstly, the values obtained for *IFN*-β expression by RT-dPCR may be slightly underestimated due to technical limitations. Specifically, the uneven distribution of positive wells on the chip (Fig. 3) can significantly affect the accuracy of mRNA quantification. Secondly, the high variability in *IFN*-β expression levels measured by RT-qPCR results in a wide range of values, ranging from 0.4 to 1.6 relative units (Fig. 6). Considering both factors, it can be hypothesized that the actual basal level of *IFN*-β expression lies within the range of 0.2–1 relative units.

TABLE 1

REPEATABILITY OF RT-qPCR AND RT-dPCR ASSAYS FOR mRNA OF *ACTB*, *SDHA* AND *IFN*-β GENES IN MdbK3-14 CELL LINE

Gene	RNA dilution	RT-dPCR, mRNA transcripts, log10 copies/μL			RT-qPCR, Cq		
		log10 copies/μL	SD	CV, %	Mean*Cq	SD	CV, %
IFN-β	10^{-2}	2.8	1.5	0.8	30.2	0.1	0.1
	10^{-3}	1.6	0.8	1.2	34.1	0.1	0.1
	10^{-4}	0.5	0	1.5	37.1	0.6	0.3
	10^{-5}	0	0	0	39.9	0.6	0.3
	Mean intra-assay reproducibility for *IFN-β* detection, %: 1.4				Mean intra-assay reproducibility for *IFN-β* detection, %: 0.2		
SDHA	10^{-2}	3.5	1.8	0.3	26.7	0.02	0.1
	10^{-3}	2.6	1.5	0.8	30.2	0.05	0.2
	10^{-4}	1.6	0.8	1.3	33.7	0.23	0.7
	Mean intra-assay reproducibility for *SDHA* detection, %: 0.8				Mean intra-assay reproducibility for *SDHA* detection, %: 0.5		
ACTB	10^{-2}	3.4	2.4	1	29.9	0.51	1.7
	10^{-3}	2.6	1.1	0.5	32.9	1.11	3.4
	10^{-4}	1.6	0.7	1	36.3	2.03	5.6
	Mean intra-assay reproducibility for *ACTB* detection, %: 0.8				Mean intra-assay reproducibility for *ACTB* detection, %: 3.6		

Note. * – Mean Cq (*n* = 4 intra-assay replicates).

Overall, the basal level of IFN-β expression is comparable to or slightly lower than that of housekeeping genes, such as beta-actin and the succinate dehydrogenase complex subunit A. This finding may indicate a potential role for interferon beta in maintaining the viability of *M. sibiricus* kidney cells, or it could suggest continuous activation of the innate immune response in the Siberian bat.

Therefore, the linearity, sensitivity, and reproducibility parameters of RT-qPCR and RT-dPCR suggest that a reliable assessment of the innate immune response necessitates enhancing the sensitivity of RT-dPCR for *IFN*-β and improving the reproducibility of RT-qPCR for *ACTB*. The quantification of *SDHA* mRNA is consistent across both RT-PCR methods and does not require optimization.

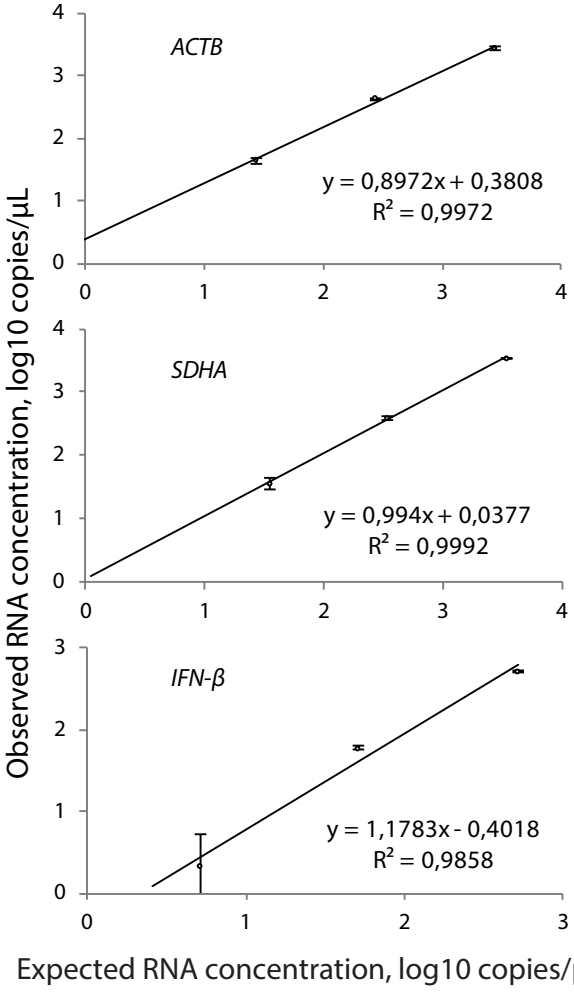

FIG. 4.
The linearity (R^2) of the RT-dPCR was evaluated using linear regression model of observed changes in mRNA concentrations in comparison to the changes in estimated expected concentrations. RNA concentrations were expressed in log10. Error bars represent the standard deviation of two RT-dPCR replicates

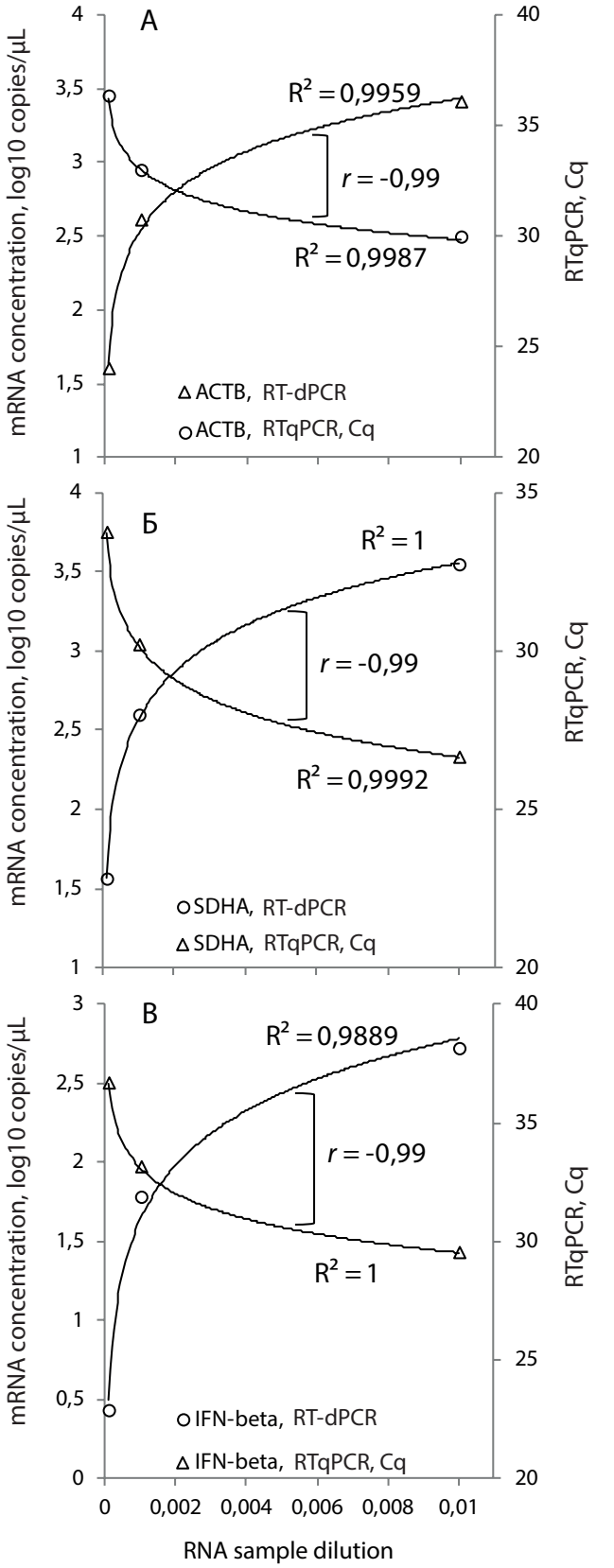

FIG. 5.
There are strong negative correlation between mean Cq values and mRNA transcripts targeting bat genes. A –beta-actin (ACTB), Б – succinate dehydrogenase subunit A (SDHA), B – interferon beta (IFN-β). Solid lines reflect the logarithmic trend model; R^2 reflects the goodness of fit of logarithmic model; r – Pearson`s correlation coefficient between two datasets (brackets)

It has previously been demonstrated that the results obtained through RT-dPCR are comparable to those obtained through RT-qPCR, with dPCR exhibiting higher sensitivity in estimating RNA concentration [18], detecting trace amounts of template in the sample. In this study, RT-dPCR analysis allowed for the quantitative detection of hepatitis B virus (HBV) RNA in serum samples as low as 10^2 copies/mL. Furthermore, it has been shown that RT-dPCR improves the interpretation of Cq values obtained through RT-qPCR in SARS-CoV-2 diagnostics, making it a useful complementary method for enhancing the accuracy and reliability of RNA quantification. This can also help improve the interpretation of RT-PCR results [19]. Our findings align with published data and we believe RT-dPCR to be a valuable tool for gene expression studies. It is particularly promising for investigating small differences in target gene expression levels. However, certain factors such as the high cost of reagents and consumables (e.g. microfluidic chips), the relatively lengthy and labor-intensive process of setting up and running the dPCR, and the need for additional optimization and validation of the RT-PCR protocol for a specific dPCR platform can complicate the analysis of large numbers of samples. It should be noted that establishing a reliable RT-dPCR procedure requires the use of at least three synthetic positive RNA controls with known concentrations, representing high (10,000–10,000,000 copies per reaction), medium (100–10,000 copies), and low (1–100 copies) target fragment loads. RT-dPCR results can only be considered trustworthy if the measured RNA concentrations within each range correspond to the expected values. This complexity becomes particularly significant when multiple targets and reference genes are analyzed, along with a comprehensive set of control assays in subsequent routine RT-dPCR experiments. This implies the inclusion of a negative control sample (without template) in each RT-dPCR run to monitor for contamination during RNA purification, reaction mixture preparation, and chip loading. Additionally, one positive control sample containing a known concentration of each target should be included to confirm reaction efficiency. Furthermore, it is advisable to include an internal RNA control with a known concentration in each reaction to assess RT-PCR efficiency and detect inhibitors in test RNA samples. At the current stage of technology development, RT-qPCR remains the preferred method for large-scale screening studies, while RT-dPCR can be used as a complementary confirmatory technique.

CONCLUSION

The study, which employed two independent methods (quantitative RT-PCR and digital RT-PCR), demonstrated that there is uninduced basal expression of interferon beta in the Siberian bat kidney cell line. The expression levels are comparable to, or slightly lower than, those of housekeeping proteins such as beta-actin and succinate dehydrogenase complex subunit A.

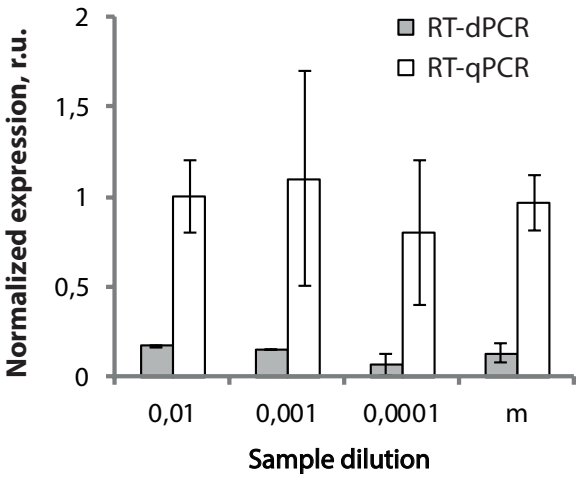

FIG. 6.
Relative normalized expression of IFN-β in the MdbK3-14 cell line. Error bars represent the standard deviation of the mean values; m – the mean expression level of IFN-β in three dilutions of RNA samples. Expression values are normalized to the reference genes ACTB and SDHA and are shown relative to the zero expression level

The developed primer-probe sets are suitable for the detection of *ACTB, SDHA*, and *IFN*-β mRNA in bat cells. Both RT-qPCR and RT-dPCR approaches used in this study demonstrated comparable linearity and yielded similar results statistically. While RT-qPCR requires more optimization to reduce technical errors and variability in mRNA quantification, it has a faster processing time and is more cost-effective than RT-dPCR.

This study was conducted using the equipment of the Core Facility "Center for Development of Advanced Personalized Health Technologies", Scientific Centre for Family Health and Human Reproduction Problems, Irkutsk.

Conflicts of interest

The authors declare no conflicts of interest.

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