IGF-1 AND SOME HOUSEKEEPING GENE CANDIDATES FOR REAL-TIME RT-PCR EXPRESSION STUDIES IN CATTLE

Z. Smolkina, A. Karus

ABSTRACT. *Gene expression analysis is increasingly important in biological research, while real-time reverse transcription PCR (RT-PCR) is becoming the method of choice for high-throughput and accurate expression profiling of selected genes. The present work is meant to contribute to solving problems related to IGF-1 expression measurements in cattle. The first aim of this paper is study of IGF-1 expression in different bovine tissues. The second aim of this study is measuring two housekeeping gene candidates (β2M and G6PDH) expression level for future research as housekeeping gene for IGF-1 relative quantification. To compensate of variations in input RNA amounts and efficiency of reverse transcription, different endogenous housekeeping genes have been quantified. However, there are still no completely satisfactory results. We measured the IGF-1 expression in different cattle tissues with ready-to-use kits from Roche. For the experiments the real-time RT-PCR LightCycler technology was used. The IGF-1 expression determination with SYBR Green I was performed with good linearity (R=0.98) and with average mean squared error (Error=0.319) over three orders of magnitude of target molecules. The highest IGF-1 gene expression was observed in cattle liver. G6PDH is a suitable candidate for finding a good housekeeping gene for IGF-1 in bovine tissues.*

Abbreviations used: *β2M – β2-microglobulin, G6PDH – glucose-6-phosphate dehydrogenase, IGF-1 – insulin like growth factor 1, RT-PCR – reverse transcription polymerase chain reaction*

Introduction

Cytokines are regulatory proteins, which play a central role in the immune system by modulating immune response, including lymphocyte activation, proliferation, differentiation, survival, and apoptosis (Giulietti, 2001). One of cytokines is insulin-like growth factor-1 (IGF-1). IGF-1 is peptide believe to play an important role in the regulation of cellular growth and differentiation. IGF-1 is synthesized and secreted by many tissues. It can act as endocrine hormone that is being transported by the circulation to distant sites of action, but it can also act locally by paracrine or autocrine mechanisms. During postnatal growth, IGF-1 stimulates protein synthesis and improves glucose utilization. The biological activity of IGF-1 is modulated by its binding proteins and receptors (Giulietti, 2001; Pfaffl, 2001). The study of biological regulation for IGF-1 usually involves gene expression assays and frequently requires quantification of RNA.

The present work is meant to contribute to solving problems related to IGF-1 expression measurements in cattle. The first aim of this paper is studying of the IGF-1 expression in different bovine tissues. In many studies, the cytokine (IGF-1) quantification in bovine tissue has been performed with different methods, such as Northern blotting, *in situ* hybridization, RIA (radioimmunoassay) and PCR. Therefore to gain insight into IGF-1 expression our research strategy involves use a real-time reverse transcription polymerase chain reaction (RT-PCR), as a most suitable method for cytokines expression (Giulietti, 2001). For detection and quantification of mRNA (expression of interesting protein), we used only ready to use kits from Roche. RT-PCR has been used with online-detection by LightCycler SYBR Green 1 technology (Rasmussen, 2001).

It is generally recognized, that highest IGF-1 expression rate is observed in liver, followed by kidney and heart, and is lowest in the different skeletal muscles. These results support the understanding, that the liver is the main IGF-1 producing tissue. Although, some authors indicate that skeletal muscle, in particular when taking its absolute mass into account, might considerably contribute to the IGF-1 levels in blood (Pfaffl *et al*., 1998; Pfaffl, 2001; Pfaffl *et al*., 2002; Pfaffl, Mircheva Georgieva *et al*., 2002).

For quantitative analysis of gene expression, RT-PCR often uses housekeeping genes as endogenous controls against which the expression level of a target gene can be normalized (Jung *et al*., 2002; Technical Note, 15/2002):

$$\frac{\text{Amount of target}}{\text{Amount of housekeeping RNA}}$$

Therefore, the second aim of present paper is to find a suitable housekeeping gene candidate for IGF-1 relative quantification. This is already much more complex problem, because the investigators have put effort to find good housekeeping gene candidates for IGF-1. However, there are still no completely satisfactory results.

For this purpose we investigated a useability LightCycler-h-Housekeeping gene selection set particularly two housekeeping genes, β2M and G6PDH for bovine samples.

Keywords: IGF-1, β2M, G6PDH, real-time RT-PCR, LightCycler.

Materials and Methods

Total mRNA extraction and cDNA synthesis

The mRNA extraction was performed in bovine (*Bos taurus*) liver, blood and skeletal muscle. Tissue samples of 2 cows and 1 bull were taken immediately after slaughter (blood samples were taken from live animal before slaughtering), instant frozen in dry ice and stored (about 2 hours) at –20 °C until use. Samples were disrupted and homogenized using mortar and pestle. For mRNA isolation, 74–76 mg of homogenized sample was taken. mRNA was purified using the mRNA isolation kit (Roche Diagnostics Corporation, USA), based on a principle of purifying polyadenylated RNA species from sample homogenate non-polyadenylated RNA (rRNA and tRNA) by poly(A)tails. Synthesis of first strand complementary DNA (cDNA) was performed with First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics). The mRNA template (10 μl of the mRNA sample or 10 μl of RNase-free water for negative control) was denatured for 10 min at 65 ºC in a termostated waterbath. During RNA denaturation cDNA master mix (total volume 30.0 μl) was prepared as follows: 6.4 μl H_2O PCR grade, 4.0 μl 10* RT-Buffer, 1.6 μl AMV Reverse Transcriptase, 4.0 μl deoxynucleotide mix, 8.0 μl 25 mM $MgCl_2$, 4.0 μl random primer p(dN)$_6$, 2.0 μl Rnase Inhibitor. The mix of cDNA and denatured sample was treated according to the kit instructions. In order to quantify the produced cDNA, optical density of the cDNA work solution was determined at 260 nm. The stock solution was diluted into a work solution at the 1/100. Final concentration of reverse transcribed total cDNA was about 1.6 μg/ml.

Additionally, optical density of the OD_{260} nm/OD_{280} nm (nucleic acid/protein) absorption ratio was measured. Ratio OD_{260}/OD_{280} in water ranges from 1.4 to 1.8.

Oligonucleotide primers

The primers used for the production of recombinant IGF-1 RNA and for quantitative LightCycler RT-PCR were derived from the bovine IGF-1 sequence (EMBL Ac.no.X15726). They were designed to produce a 240 bp amplification product spanning two RNA splicing sites in a highly conserved region (exon 3–4) of the IGF-1 sequence coding for the mature IGF-1 protein. The suitable genetic sequences were represented in (Pfaffl, 2001; Pfaffl *et al.*, 2002; Pfaffl, Mircheva Georgieva *et al.*, 2002). According to literature data, the IGF-1 primers, for this experiment, were synthesed in TIB MOLBIOL (www.tib-molbiol.com). Primer information and the TIB reference number are listed in Table 1.

Table 1. Sequence of PCR primers, position of the primers (f = forward; r = reverse), position of RT-PCR product, G/C content, Tm, and TIB reference no. of the used published nucleic acid sequences
Tabel 1. Kasutatud praimerid: praimerite järjestused, praimerite ja RT-PCR produkti asukohad, G/C sisaldus, Tm ja TIB referentsnumber

	Position	GC (%)	Tm (ºC)	TIB no.
TCG CAT CTC TTC TAT CTG GCC CTG T f	bt 88-327	52.0	68.9	003120463
GCA GTA CAT CTC CAG CCT CCT CAG A r		56.0	69.0	

For β2M and G6PDH we used primers and hybridization probes from LightCycler-h-Housekeeping Gene Selection Set. The PCR primers are designed to produce DNA fragments of 147 bp for β2M and 123 bp for G6PDH (LightCycler-h-housekeeping gene selection set, 2002).

Quantification by real-time PCR

In this study there were made three PCR series: two for IGF-1 expression and the other one for housekeeping genes amplification. All series of polymerase chain reaction were performed with reversely transcribed cDNA (1.64 μg/ml).

LightCycler PCR for IGF-1

Real time PCR for IGF-1 was conducted by using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics Inc.) in LightCycler Instrument (Roche Diagnostics Inc.). The procedure described by Pfaffl (Pfaffl *et al.*, 2002) has been taken for a basis. In our experiment we used kits from Roche with some changes in the protocol. The master mix of the following reaction components was prepared: 11.2 μl of water, 2.4 μl of

$MgCl_2$ (4 mM), 0.8 µl of forward primer (0.4 µM), 0.8 µl of reverse primer (0.4 µM), 0.8 µl bovine serum albumin (1 µg/ml) and 2 µl of LightCycler FastStart DNA Master SYBR Green I (Roche). An aliquot of 18 µl of LightCycler master mix was added to the LightCycler glass capillaries and 2 µl volume of PCR template was added. The capillaries were closed, centrifuged 5 s 700 g and placed into the LightCycler rotor. Subsequently, the LightCycler PCR reactions were performed by series of the target cDNA as follows:

- For all blood samples the 1/10 dilutions were prepared
- For all liver and skeletal muscle samples the undiluted, 1/10 and 1/100 templates were used
- Negative control

Hot-start PCR protocol was based on three articles (Pfaffl, 2001; Pfaffl *et al*., 2002; Pfaffl, Mircheva Georgieva *et al*., 2002). The cycling conditions of the IGF-1 were similar to Pfaffl (Pfaffl *et al*., 2002), except we used higher temperature T=85 °C, (Pfaffl, 2001) in fluorescence acquisition step instead of 80 °C. The melting curve analysis conditions were taken from (Pfaffl, 2001; Pfaffl, Mircheva Georgieva *et al*., 2001). For details see Table 2.

Table 2. Cycling conditions of insulin like growth factor 1 (IGF-1) in a four segment LightCycler real-time RT-PCR. The amplification and quantification programme was repeated 50 times with a single fluorescence acquisition point at an elevated temperature
Tabel 2. Reaalaja RT-PCR nelja segmendiga protokoll IGF-1 mRNA määramiseks. Amplifitseerimise ja kvantifit-seerimise programmi korrati 50 korda ühekordse fluorestsensmõõtmisega kõrgendatud temperatuuril

Segment nr	Target temperature *Temperatuur* (ºC)	Incubation time *Toimeaeg* (s)	Temperature transition rate *Temperatuuri muutmise kiirus* (ºC/s)	Second temperature *Teisene temperatuur* (ºC)	Step size *Astme suurus* (ºC)	Acquisition mode *Mõõte-tingimused*
Denaturation/*Denaturatsioon*, 1 cycle						
1	95	600	20	0	0	None/*Puudub*
Quantification/*Kvantifitseerimine*, 50 cycles						
1 (denat.)	95	15	20	0	0	None/*Puudub*
2 (anneal.)	63	10	20	0	0	None/*Puudub*
3 (elong.)	72	20	20	0	0	None/*Puudub*
4 (quant.)	85	3	20	0	0	Single/*Ühekordne*
Melting Curve Analysis / *Sulamiskõvera analüüs*						
1	95	10	20	0	0	None/*Puudub*
2	60	10	20	0	0	None/*Puudub*
3	95	0	0.1	0	0	Cont./*Pidev*
Cooling/*Jahutamine*						
1	40	30	20	0	0	None/*Puudub*

Selection of housekeeping genes

Real-time PCR for β2M and G6PDH was performed with LightCycler Protocol using LightCycler FastStart DNA Master Hybridization Probes from LightCycler-h-Housekeeping gene selection set and the LightCycler Instrument. In general, a total volume of 20 µl was 5 µl cDNA as a template. 15 templates were prepared for each housekeeping gene analyse due to limited quantify of detection mixes:

- For all skeletal muscle samples the undiluted, 1/10 and 1/100 templates were used
- For all liver samples the 1/10 was prepared and the 1/100 dilutions were made from only two samples (one cow and one bull)
- Positive control

The closed capillaries were centrifuged and placed into the LightCycler rotor. The hot-start PCR amplification and quantification program has 45 cycles and the reaction conditions were taken from user protocol. In addition to the usual protocol the melting curve analysis also has been lead. The following melting curve program was used: 65–95 °C with heating rate of 0.1 °C/s, a 15s incubation time on second segment and continuous fluorescence measurements (Lutz, 2003).

Calculations and statistics

IGF-1 mRNA expression was evaluated by amplification curve analysis of the LightCycler real-time RT-PCR. After incorporation into double stranded DNA (dsDNA), SYBR Green I (DNA binding dye) shows fluorescence emission and increases according to target amplification with cycle number. The exponential phase

of the PCR becomes detectable when the fluorescence signal from accumulated PCR product is greater than the background fluorescence. LightCycler software version 3.5 was used to estimate the reproducibility of the calibration curves (slopes and intercepts). Crossing points were estimated using the "*Fit Points option*" (Rasmussen, 2001).

Expression data of the IGF-1 in different tissues was calculated according to the equation: amount (copies/μl) = (RNA total concentration x 6.023 x 10^{23} molecules)/(MW), where the MW is the molecular weight of IGF-1 (Pfaffl, Mircheva Georgieva *et al*., 2002; Technical Note, 11/2000).

Results

Quantification of IGF-1

Generally in real-time RT-PCR there are two different quantification strategies. The level of expressed genes may be measured by absolute or relative quantitative real-time RT-PCR. Absolute quantification relates the PCR signal to input copy number using a calibration curve, while relative quantification measures the relative change in mRNA expression levels. The reliability of an absolute real-time RT-PCR assay depends on the condition of identical amplification efficiencies for both the native target and the calibration curve in RT reaction and in following kinetic PCR (Bustin, 2000; Rasmussen, 2001; Technical Note, 11/2000). The results of quantification of IGF-1 mRNA are given on Figure 1, where the total fluorescence was measured at the end of each cycle at 85 °C. The negative control contains no input cDNA.

It can be seen that a lot of templates show fluorescence what differs in broad range in Figure 1. Therefore some samples have relatively flat curve.
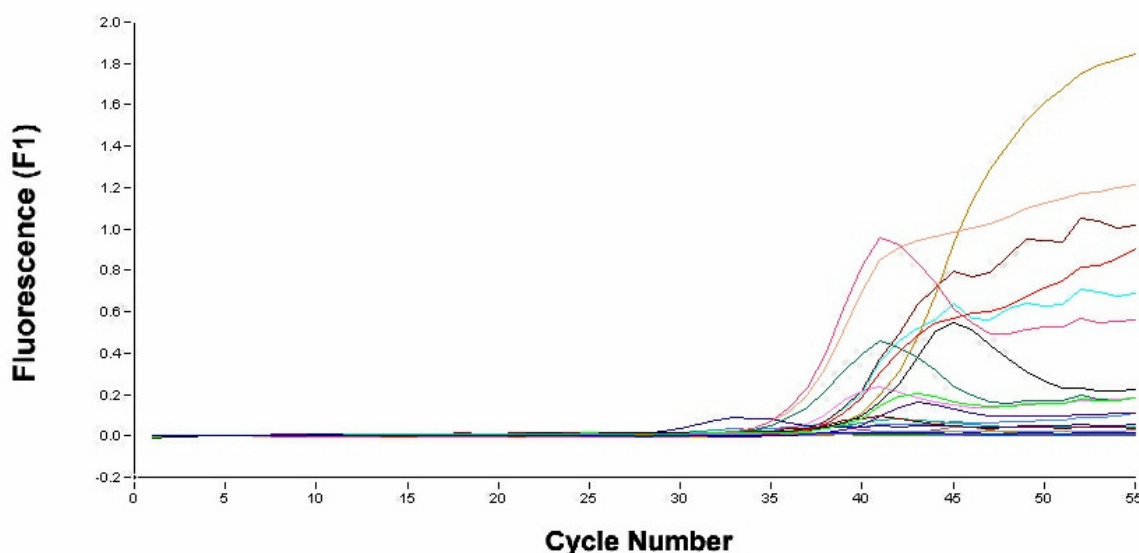

Figure 1. Fluorescence *vs*. the number of cycles for differing initial amounts of template copies. Data from an experiment by using a LightCycler to quantify copy number of IGF-1 in bovine samples
Joonis 1. *Veise IGF-1 kvantifitseerimine LightCycleril. Fluorestsentsi sõltuvus tsükli numbrist erinevatel matriitsi lähtekontsentratsioonidel*

On the other hand, Figure 2 presents the melting curve analyses where the melting peaks are determined by plotting the negative derivate of fluorescence emitted by each sample during the increase of temperature by which PCR products were slowly denaturated. Fluorescence was measured continuously. The DNA product can be identified as a peak at above Tm 90 °C. The primer-dimers melting temperature Tm is lower than 82 °C. The negative control and some templates had primer-dimer Tm at about 80 °C.

IGF-1 is relatively low expressed in cattle tissues, therefore we observed many samples with high primer-dimer level. The primer-dimers often had the higher peak in 1/100 diluted samples.
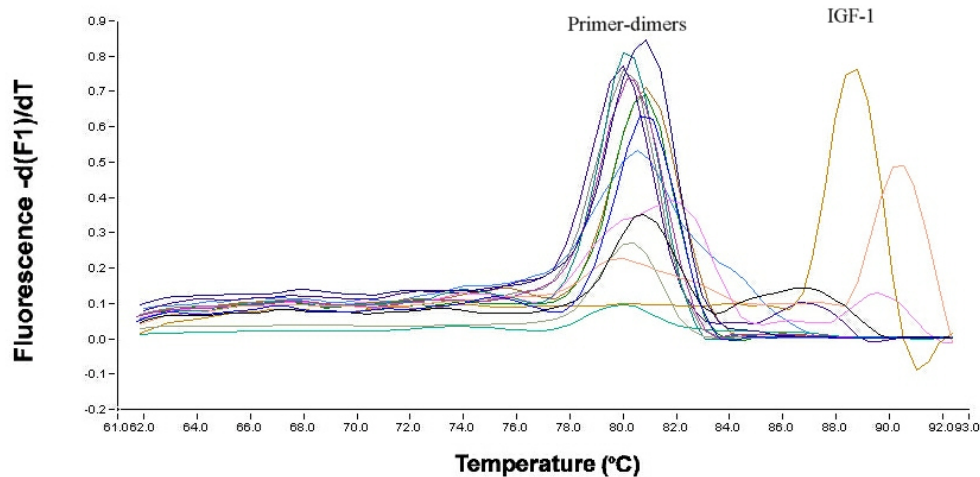
Figure 2. Amplification of IGF-1 dilution series of different bovine tissues: melting curve analysis of IGF-1 amplification product demonstrates the gradual reduction in fluorescence as temperature increases
Joonis 2. *IGF-1 amplifitseerimine veiste kudede lahjenduste seeriatest. Sulamiskõvera analüüs iseloomustab IGF-1 amplifitseerimiseprodukti fluorestsentsi vähenemist temperatuuri tõstmisel*

Confirmation of primer and PCR-product specificity

Specificity of the desired products in different bovine tissues was documented with melting curve analysis. The system uses a fluorescent dye (SYBR Green I) which binds specifically to double strand DNA in order to detect the accumulation of PCR products so eliminates the need for electrophoresis for the detection of the specific amplicon. With the LightCycler, the accumulation of amplicon can be visualised real time, and the specific product can be determined by its characteristic melting temperature (T_m).

There are also a lot of reasons (Missel *et al.*, 2002; Technical Note, 9/2000; Technical Note 11/2000) why Figure 2 show that some templates had only primer-dimers and no amplification product. In our study the first possible cause of the increase of primer-dimers might be connected with changing of the structure of a master mix with unsuccessful attempt to amplification the IGF-1. The water volume was decreased, the primer amount was increased and the bovine serum albumin was added as a detergent. $MgCl_2$ and LightCycler FastStart DNA Master SYBR Green I quantities were unchanged. Our preliminary experiment shows, that twice lower primer amount and without usage of BSA only a few templates had the IGF-1 amplification product (Figure 3). The second reason might have been the insufficient low concentration of the primers what can result in inadequate annealing.
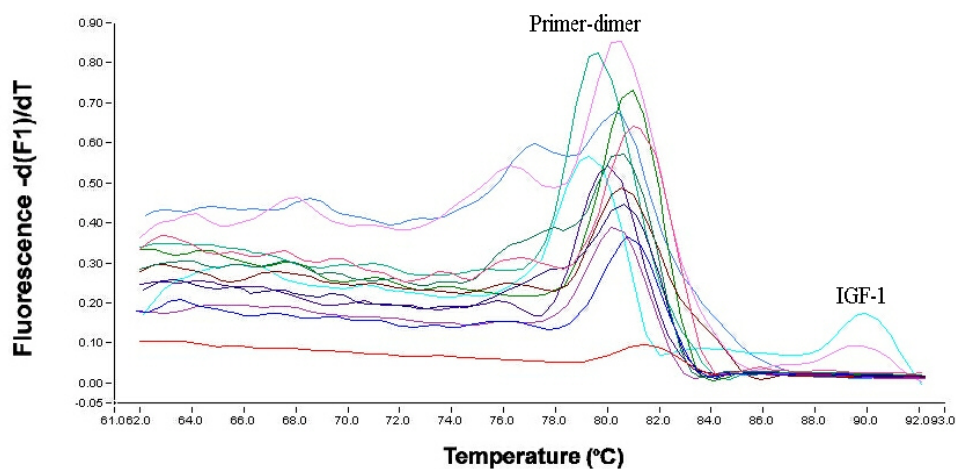

Figure 3. Melting point analysis of preliminary experiment
Joonis 3. *Eeleksperimendi sulamispunkti analüüs*

Distribution of mRNA expression

The highest gene expression was observed in cattle liver. The standard curve was made by using *Fit Points option*. The standard curve is the linear regression line through the data points on a plot of crossing point (threshold cycle) versus logarithm of sample concentration. The value shown at the bottom of the standard curve on Figure 4 is derived from the calculated regression line:

Slope=−3.22 (=−1/logE), where E=2.0, efficiency of the reaction.

Y-Intercept=35.50 (=logNcp/logE), Ncp=5.96x10^9, amount of PCR product at Cp

In theory the slope should be around −3.3. Slopes lower than −4 are indicative of low PCR efficiency.

The IGF-1 expression determination with SYBR Green I was performed with linearity (R=0.98) and with mean squared error (Error=0.319) over three orders of magnitude of molecules. IGF-1 expression differences and/or stability in different tissues will be investigated by increased number of samples (animal).
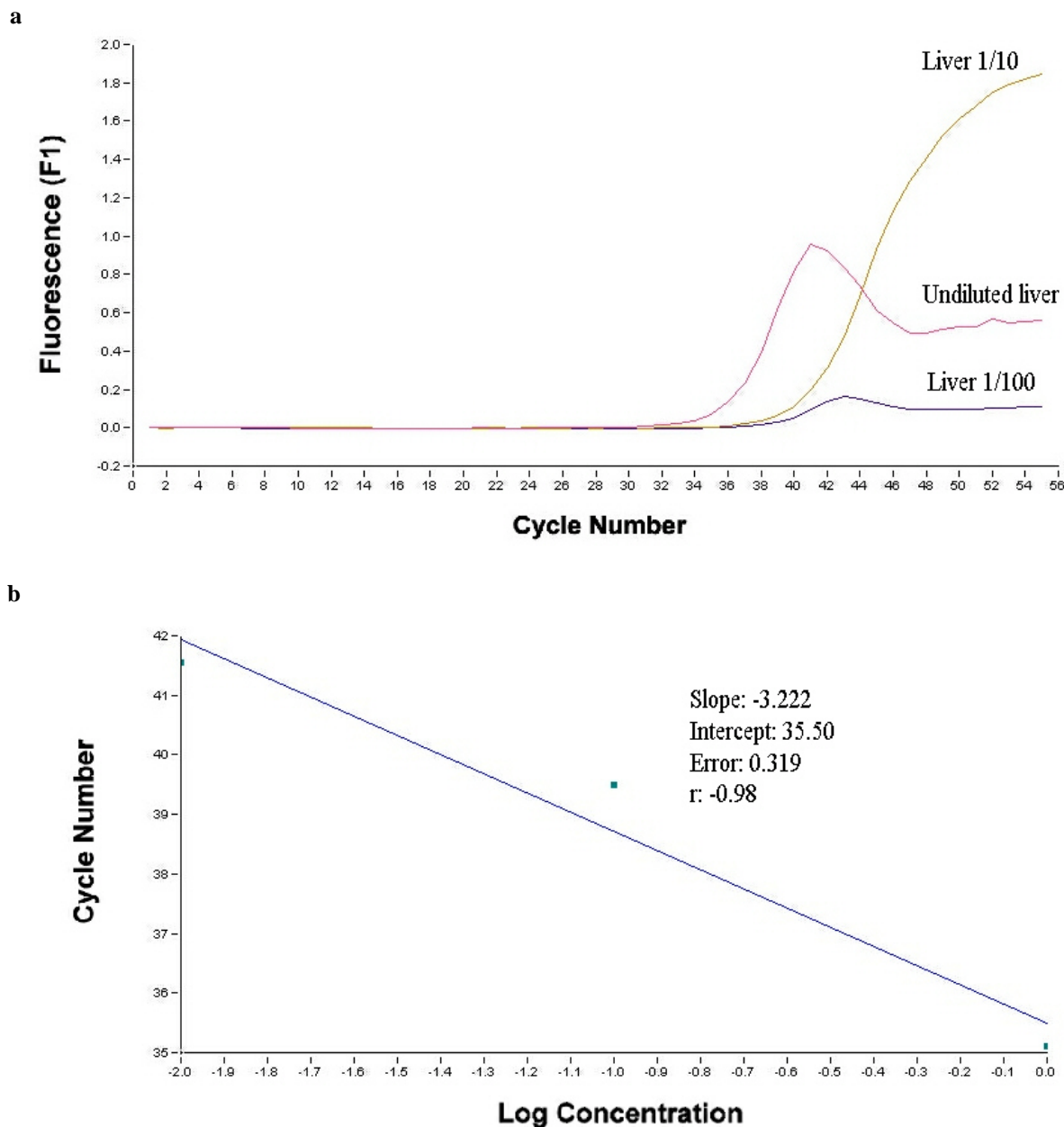
a

b

Figure 4. Expression of IGF-1 mRNA in liver. Figure a shows raw fluorescence data for dilutions of a stock solution of IGF-1 cDNA. Panel b shows a linear standard curve in three magnitudes of copies of IGF-1
Joonis 4. IGF-1 mRNA ekspressioon maksas. Joonis a näitab fluorestsentsi sõltuvust IGF-1 cDNA töölahusetest lahjenduse seerias Joonisel b on toodud lineariseeritud standardkõver kolme magnituudi võrra erinevate IGF-1 cDNA koopiate arvu korral

Quantification of some housekeeping gene candidates

The results of the second part of the experiment are shown in the next figure (Figure 5). For suitable housekeeping gene selection we used the LightCycler-h-Housekeeping Gene Selection Set. This set detects five housekeeping genes, which span a wide medium range of expression levels and also applied for human. Specially developed primer/probe mixes (included in the set) guarantee RNA-specific detection. In this study we used only two housekeeping genes: β2-microglobulin (β2M) and glucose-6-phosphate dehydrogenase (G6PDH). Housekeeping gene candidates quantification was performed using hybridization probes, which allows to detect only the specific product. Both housekeeping gene were analyzed in parallel in different bovine tissues to find for the best candidates. In undiluted and 1/10 diluted samples of skeletal muscle, the G6PDH gave positive results (Figure 5). However it did not give an opportunity the design the standard curve, because the 1/100 diluted sample had no positive expression of G6PDH.
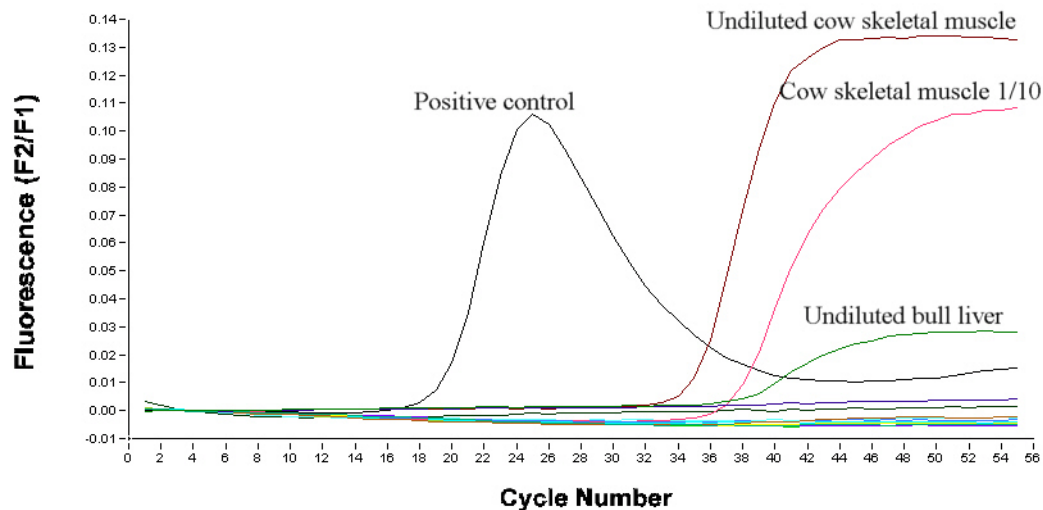

Figure 5. Amplification of G6PDH in undiluted and diluted samples from bovine tissues
Joonis 5. *G6PDH amplifitseerimine veise kudede lahjendamata ja lahjendatud proovides*

At first sight the idea to use the housekeeping genes from human seems absurd. On the other hand, IGF-1 primers were designed to produce an amplification product which spanned at least two exons in the highly conserved coding region (CDS) of the appropriate coding sequence of multiple species: homology >94% in IGF-1 primers between cattle, sheep, pig, water buffalo, primates and human (Pfaffl, 2001; Pfaffl *et al*., 2002; Pfaffl, Mircheva Georgieva *et al*., 2002). Therefore we assumed that similarly to IGF-1, whose primers can be used either for bovine, ovine, human or mouse sequences, the positive results can be achieved using the primers from human housekeeping set (see Figure 5). But we also did not exclude probability to get the negative results because there is no information about G6PDH and β2M sequences primers homology in human and cattle. As mentioned above, we succeeded to amplify and measure the quantify of G6PDH mRNA in bovine tissues using primers provided with human set.

We failed to detect the β2M expression in cattle tissues using human primers.

Discussion

In this study we investigated the expression of the IGF-1 and some housekeeping gene candidates using real-time RT-PCR by LightCycler technology.

The first PCR series were for IGF-1 expression measurement. We performed two experiments to solve the problems raised by contradictory information from different reaction protocols. For example, differences in temperature in fluorescence acquisition step, melting curve analysis conditions, mastermix components and their final concentrations. The procedure described by Pfaffl (Pfaffl *et al*., 2002) was been taken for a basis and hot-start PCR protocol was based on three articles (Pfaffl, 2001; Pfaffl *et al*., 2002; Pfaffl, Mircheva Georgieva *et al*., 2002). The first experiment shows, that at twice lower primer amount and without bovine serum albumine only few templates had the IGF-1 amplification product. The second attempt to amplify the IGF-1 at improved conditions was more successful, whereas the quantification and melting curve show decrease in primer-dimers. The small optimization procedure gives us the opportunity to establish the standard curve (E=2.0). Results of our experiment confirm the results of earlier investigations (Pfaffl *et al*., 1998; Pfaffl, 2001; Pfaffl *et al*., 2002;

Pfaffl, Mircheva Georgieva *et al.*, 2002) that IGF-1 is highly expressed in cattle liver. In contrast, the IGF-1 expression in muscle and blood cells was lower in our study. At recent time there is also available an improved procedure of Neuvians (Neuvians, 2003), whose scientific paper wass published later.

Following PCR series were made for finding the best housekeeping gene candidate for IGF-1 relative quantification. The idea of using the LightCycler-human-Housekeeping gene selection set for this purpose was born from the opportunity of IGF-1 multi-species primers being widely used.

Among two investigated genes the G6PDH gives positive results in bovine tissues. Unfortunately human β2M primers are absolutely not suitable for bovine β2M amplification. In other words received results gave a matter for further studies the G6PDH as a suitable housekeeping gene in bovine tissues.

In conclusion, our results affirm the simple introduction and high performance of the IGF-1 and G6PDH as a housekeeping gene RT-PCR for expression studies in cattle, if the necessary optimization steps in PCR protocols are implemented.

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IGF-1 ja mõne *housekeeping geeni* kandidaadi ekspressiooni uurimine veistel reaalaja RT-PCR-ga

Z. Smolkina, A. Karus

Kokkuvõte

Geeni ekspressiooni analüüsil on tähtis roll bioloogilistes uurimustes. Reaalaja pöördtranskribeeriv polümeraasi ahelreaktsioon (RT-PCR) on saanud täpseks ja eelistatud meetodiks geenide ekspressiooni määramisel. Käesolev uurimus oli suunatud veiste IGF-1 mRNA määramisega seotud probleemide lahendamisele. Töö esimene eesmärk oli IGF-1 kvantifitseerimise võimaluse selgitamine erinevates veiste kudedes. Uurimise teine eesmärk on iseloomustada kahte *housekeeping geeni* kandidaati beeta-2-mikroglobuliini ($\beta2M$) ja glükoos-6-fosfaadi dehüdrogenaasi (G6PDH) ning määrata nende ekspressiooni tase. Saadud tulemused võiksid leida kasutamist IGF-1 suhteliseks (relatiivseks) kvantifitseerimiseks nende kui *housekeeping geenide* abiga. Kirjanduses ei leidu informatsiooni ühegi IGF-1 kvantifitseerimiseks vajalikke tingimusi täielikult rahuldava *housekeeping geeni* kohta. Nimetatud kahe eesmärgi saavutamiseks kasutati uurimismaterjalina veiste täisverd, skeletilihast ja maksa. mRNA eraldati kudedest, kasutades *mRNA isolation kit* (Roche Diagnostics Inc, USA). Seejärel sünteesiti mRNAst *First Strand cDNA Synthesis Kit for RT-PCR* (AMV) (Roche Diagnostics Inc) abiga cDNA. Puhtuse kontrollimiseks määrati iga proovi jaoks optiliste tiheduste suhted 260 ja 280 nm. Polümeraasi ahelreaktsiooni matriitsina kasutati cDNA (1,64 µg/ml), mille kontsentratsioon määrati 260 nm juures. cDNA-st lahjenduste seeriad tehti vahetult enne igat PCR-i. IGF-1 amplifitseerimiseks kasutati *LightCycler FastStart DNA Master SYBR Green I* (Roche Diagnostics Inc) LightCycler tehnoloogiat (Roche Diagnostics Inc.). Iga reaktsiooni segu sisaldas 11,2 µl vett, 2,4 µl $MgCl_2$ (4 mM), mõlemat praimerit 0,8 µl (0,4 µM), 0,8 µl veise vereseerumi albumiini (1 µg/ml), 2 µl LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics Inc) ja 2 µl cDNA matriitsi. Praimerid (tabel 1) telliti kirjanduse (Pfaffl, 2001; Pfaffl *et al*., 2002; Pfaffl, Mircheva Georgieva*et al*., 2002) põhjal firmast *TIBMOLBIOL*. PCR protokolli (tabel 2) väljatöötamise lähtealustena kasutati kolme artiklit (Pfaffl, 2001; Pfaffl *et al*., 2002; Pfaffl, Mircheva Georgieva *et al*., 2002).

Kõrgeimat IGF-1 ekspressiooni täheldati veiste maksas. Tulemuste andmetöötlus, kasutades *Fit Points option*'i, võimaldas konstrueerida hea lineaarsusega (R=0,98) ja keskmise ruutveaga (Error=0,319) standardkõvera (joonis 4b). *Housekeeping geenide* uurimisel kasutati polümeraasi ahelreaktsiooni matritsiina sama cDNA (1,64 µg/ml). Reaalaja PCR-l $\beta2M$ ja G6PDH määramiseks kasutati *LightCycler FastStart DNA Master Hybridization Probes* ja *LightCycler-h-Housekeeping gene selection* kitte. Protokoll ja reaktsioonisegu koostis vastasid kiti juhendile. Uurimuses kasutati inimese $\beta2M$ ja G6PDH praimerid. Idee kasutada inimeste $\beta2M$ ja G6PDH praimerid teiste liikide sh veiste uurimiseks on diskuteeritav. Samas, IGF-1 jaoks valitud praimerid olid multiplekssed ning suure liikidevahelise homoloogiaga, mis lubab samu praimereid kasutada inimesel, veisel, seal, lambal ja teistel liikidel. Info G6PDH ja $\beta2M$ praimerite homoloogia kohta veiste ja inimese vahel puudub, mistõttu ei olnud välistatud ka negatiivsed tulemused. Human-$\beta2M$ praimerite kasutamisel veiste kudedest eraldatud mRNA-st transkribeeritud cDNA amplifitseerimist ei täheldatud, seega nimetatud praimerid ei ole kasutatavad veiste geenide määramisel. Töö tulemusena leidis aga kinnitust (joonis 5), et G6PDH on sobiv kandidaat IGF-1 relatiivse kvantifitseerimise referentsgeeni (*housekeeping geeni*) valikul edasisteks ekspressiooniuuringuteks veiste kudedes.

Võtmesõnad: IGF-1, $\beta2M$, G6PDH, reaalaja RT-PCR, LightCycler.