creases in comparison to the first hours of life can be observed in the absence of relevant bacterial infection. Careful studies with regard to the age-dependent reference intervals of serum procalcitonin in infants should be conducted to maximize the use of this parameter in the diagnosis of neonatal sepsis.

References

Quantitative Analysis of the Human Epidermal Growth Factor Receptor Messenger RNA Using Reverse Transcription-PCR: A Methodological Study of Imprecision, Vibeke Bech Thogersen,1* Peter Bross,3 Niels Gregersen,2 and Ebba Nexø1 (1 Department of Clinical Biochemistry, KH, Aarhus University Hospital, Nørrebrogate 44, DK-8000 Aarhus C, Denmark, and 2 Research Unit of Molecular Medicine, SKS, Aarhus University Hospital, 8200 Aarhus N, Denmark; * author for correspondence: fax 45 8949 3060, e-mail domain31.ak14 s.akklkvt1@aaa.dk)

Reverse transcription-PCR (RT-PCR), combined with an endogenous and exogenous standard, is used extensively to quantify specific mRNAs, but few studies have evaluated the reproducibility of these assays (1–7). Increased expression of the epidermal growth factor receptor (EGFr) has been observed in several human cancers and has been suggested to predict tumor progression (8–11). Quantitative analysis for the EGFr mRNA using competitive RT-PCR has been described (12–14), but the analytical imprecision has not yet been documented systematically.

A primer pair was designed to flank the first intron of the human EGFr gene (15, 16). The 350-bp cDNA fragment was amplified as described below, using the following primers: primer I (5′-GAC CCT CCG GGA CGG-3′), spanning positions 191–205; and primer II (5′-GGC ATG ATT TTC GTA GTA CAT AT-3′), spanning positions 515–540 and cloned into a pCR II vector (TA Cloning System, Invitrogen). The “EGFr” plasmid was cleaved as above. The PCR products of the EGFr and 3060, e-mail domain31.ak14 s.akklkvt1@aaa.dk) product was cloned into a pCR II vector, purified, and cleaved as above. The PCR products of the EGFr and standard were sequenced on both strands, confirming their identity and the absence of PCR errors (data not shown).

To synthesize the RNA standard, 5 μg of the linear plasmid (containing the 69-bp deletion) was incubated with 100 units of SP6 RNA polymerase according to the manufacturer’s protocol (Promega Corp.). Fifty microliters of 25 mmol/L MgCl2 was added to the mixture before treatment with 20 U of RNase-free DNase I (Promega) for 30 min at 37 °C. The RNA standard was extracted using 1 volume of phenol:chloroform and centrifuged at 13 000g for 15 min at 4 °C. The aqueous phase was mixed with 1 volume of chloroform:isoamyl alcohol (49:1, by volume) and centrifuged as above. One volume of ethanol and 0.5 volume of 7.5 mol/L ammonium acetate were added to the aqueous phase. Precipitation was performed for 30 min at −80 °C, followed by centrifugation at 13 000g for 15 min at 4 °C. The pellet was resuspended in 40 μL of diethyl pyrocarbonate-treated double-distilled water (DEPC ddH2O). PCR was performed before and after the reverse transcriptase step. RNA concentration was determined by using an ultraviolet spectrophotometer (with absorbance at 260 nm of 1 corresponding to RNA = 40 mg/L) (17). RNA standards (3.7 × 10−15 and 3.7 × 10−12 mol/L) were stored in small aliquots at −150 °C and thawed a maximum of four times before disposal.

Human biopsies were obtained immediately after delivery from 12 healthy placenta in accordance with the Helsinki Declaration. From each placenta, four biopsies were removed, frozen immediately in liquid nitrogen, and stored at −150 °C.

EGFr protein was determined using an ELISA technique (18). Protein analysis was performed using a microtiter assay (BCA protein assay reagent, Pierce).

Total RNA was extracted from 20 mg of placenta by adding 800 μL of a denaturing solution (solution D: 4 mol/L guanidine thiocyanate, 25 mmol/L sodium citrate, pH 7, 5 g/L sarcosyl, and 0.1 mmol/L 2-mercaptoethanol) and homogenized with a “turbo mixer” (Heidolph Dixa 600) (19). The RNA pellet was resuspended in 25–40 μL DEPC ddH2O and stored at −150 °C. RNA (1:50 dilution in DEPC ddH2O) was quantified with use of an ultraviolet spectrophotometer (as above). For the imprecision experiments, RNAs from each placenta biopsy were pooled, and 20 aliquots were stored at −150 °C.

The reverse transcriptase reaction was performed as described in the manufacturer’s protocol (Clontech 1st strandTM cDNA synthesis kit). Total RNA (0.5 μg) was converted to cDNA by priming with random hexamer primer (1 μmol/L) and MMLV reverse transcriptase (200 U/μg RNA) in a total volume of 20 μL. A 1:10 dilution was performed, and 10 μL cDNA was added to the PCR buffer containing 1× reaction buffer [10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L MgCl2, and 0.01 g/L gelatin], 100 μmol/L deoxynucleotide triphosphates, 248 pmol primer I, 12.8 pmol primer II, and 1.0 U Taq DNA polymerase in a final volume of 50 μL (Pharmacia Biotech).
A temperature cycling profile was designed. Each cycle consisted of denaturation at 94 °C for 30 s, annealing for 15 s, and extension at 74 °C for 1.5 min. Stepwise, the annealing temperature was decreased as follows: 2 cycles at 61 °C, 4 cycles at 60 °C, 6 cycles at 59 °C, and 28 cycles at 58 °C. The total cycle number was 40.

For competitive RT-PCR, a titration series of the RNA standard was added to a constant amount of total sample RNA. The RT-PCR was performed as described above. PCR products were analyzed by gel electrophoresis (Fig. 1B). Band density of the PCR products was determined using a computerized analyzing system (Qgel, Quantigel Corp.). Because of the presence of identical sequences in the EGFr cDNA and standard cDNA, competitive amplifications of these two templates led to hetero-duplex formation, as shown by denaturation/renaturation of a mixture of EGFr and standard cDNAs. After densitometry, the target and standard bands were corrected for hetero-duplex and size differences. The concentration of the RNA standard and the ratio between standard and EGFr band density was linear (Fig. 1C); therefore, the EGFr concentration in the sample corresponded to the standard concentration at the equivalence point (ratio = 1).

Amplification kinetics of target and standard was evaluated by adding equal amounts of EGFr and standard linear plasmid (1 ng) to a single PCR mixture. After 25, 27, 30, 35, and 40 cycles, small portions of the PCR mixture were removed and analyzed by gel electrophoresis as above. The mean ratio between EGFr and the RNA standard was 1.03 ± 0.16 (mean ± SD, n = 5; data not shown), indicating that the amplification kinetics for the two fragments are fairly similar throughout the PCR.

To determine the imprecision of competitive RT-PCR for EGFr mRNA, competitive RT-PCR of pooled RNA was performed in duplicates on 19 separate days. Total and within-run CVs were 28% and 20% higher than the 10% CV reported for the EGFr protein assay.

The imprecision of the detection system was estimated on the basis of PCR products frozen in small portions. Gel electrophoresis and scanning were performed on 7 different days, analyzing five samples each day. Total and within-run CVs were 8% and 6%.

Fig. 1. Construction of standard and quantification of the EGFr mRNA by using competitive RT-PCR.

(A) An EGFr cDNA fragment was cloned into pCR™ vector II. A 69-bp RNA standard was obtained by PCR using primers II and III; the linear EGFr plasmid was used as template, followed by cloning and in vitro transcription using SP6 RNA polymerase. A titration series of the RNA standard (0–365 × 10⁻²¹ mol/µg RNA in a 2:3 dilution scheme) were added to a constant amount of total RNA (0.5 µg), followed by RT-PCR. (B) PCR products were separated in a 3% agarose/ethidium bromide gel. STD, RNA standard; H, hetero-duplex. Lane M, molecular weight marker (14.174 RF DNA/HaeIII digest); lanes 1–5, serial dilution of standard and a constant amount of total RNA; lane 6, control reaction without a template. (C) The gel was subjected to digital scanning densitometry (Eagle Eye™, Stratagene) and analyzed using a computerized analyzing system. The target and standard were corrected by adding one-half of the hetero-duplex band to each band. Because of size differences between standard (281 bp) and target (350 bp), the standard was multiplied with 350/281. The RNA standard concentration per µg RNA was plotted against the ratio of band densities of standard and target. The equivalence point was determined by using linear regression and is indicated by the arrow. Biopsies a (●) and b (■) from the same placenta are shown.
When analyzing biopsies, it is important to be aware of variation due to tissue inhomogeneity. The CV duplicates (23) for the mRNA concentrations determined in two biopsies from the same placenta (n = 12) was 38%. In two placentae, the variations between two sets of data (178 and 478 × 10⁻¹¹ mol/μg RNA and 273 and 396 × 10⁻¹¹ mol/μg RNA) were considerable. When these data were excluded, the CV was reduced to 20%. Based on our results, we recommend examining at least two biopsies from the same tissue and accepting data only if the difference between the samples meets predefined criteria for acceptance.

Only a few groups have reported on the imprecision of mRNA analysis using competitive PCR (1–9, 11, 14). Becker-André and Hahlbrock (25) were among the first to describe a competitive PCR assay by adding a standard (competitor) after the reverse transcriptase reaction, allowing mRNA quantification by referring to a housekeeping enzyme mRNA. Two reports using this assay examined three different mRNAs and reported an imprecision between 14% and 27% (1, 24). Because the standard was added after the PCR, this variation reflects the PCR only. In competitive RT-PCR, the standard is added before the reverse transcriptase reaction (25). Ferre (24) developed a competitive RT-PCR analysis for HIV-I with a within-run analytical imprecision of 19%, which is comparable with the within-run imprecision we describe for the EGFr mRNA.

The amount of EGFr mRNA in the same samples was determined to be 170 (80–300) × 10⁻¹¹ mol/μg RNA (mean and range, n = 10). The EGFr protein concentration in the placenta samples was 10.6 (7.4–14.4) × 10⁻¹² mol/mg membrane protein (mean and range, n = 12). We did not find any significant correlation between EGFr mRNA and EGFr protein concentration in the placenta (Pearson r = 0.30, 2p = 0.40, n = 10) (data not shown). The lack of correlation may be explained by a combination of the narrow range covered for the EGFr, the high analytical imprecision for the mRNA analysis, or posttranscriptional regulation.

We thank Anna Lisa Christensen for excellent technical assistance. This work was supported by the Danish Cancer Society, Danish Research Council, Danish Research Academy, and Aarhus University Hospitals Research Initiative.

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Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population. CF has a