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ORIGINAL PAPER

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Impact of formalin-fixation and paraffin-embedding on the ratio between mRNA copy numbers of differently expressed genes

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Abstract Several studies have shown that specific mRNA sequences can be successfully detected in formalin-fixed, paraffin-embedded tissues using reverse transcriptase-polymerase chain reaction (RT-PCR). Here, we test the hypothesis that gene expression levels can be accurately quantified in formalin-fixed, paraffin-embedded tissues by determining the ratio between the copy number of the mRNA molecule of interest and the mRNA copy number of a so-called housekeeping gene. The mRNA copy numbers of the variably expressed multiple drug resistance gene (MDR)-1 and four housekeeping genes (hypoxanthine phosphoribosyl-transferase-1, glyceral-dehyde-3-phosphate dehydrogenase, beta-actin, and elongation factor-1a) were quantified by real-time-quantitative RT-PCR before and after formalin-fixation and paraffin-embedding of 576 tissue samples (heart, kidney, spleen, liver) from three beagle dogs. The results indicate that fixation and embedding drastically altered the ratios between the different mRNA copy numbers and that the relative expression levels of MDR-1 per any of the housekeeping genes were artificially increased or decreased up to more than tenfold. It would thus appear questionable to normalize quantitative expression data from fixed and embedded tissues by using housekeeping genes as reference. In contrast, tissue autolysis of up to 24 h and long-term storage of embedded tissues of up to 20 years had no additional effects.

Keywords Quantitative gene expression · Real-time reverse transcriptase polymerase chain reaction · Formalin fixation · Paraffin embedding

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Introduction

Formalin-fixation and paraffin-embedding has been the method of choice for the processing and long-term storage of diagnostic tissue samples for nearly a century. This treasure trove for medical research has become even more valuable in the past years with the advent of molecular biological techniques that allow for the precise quantification of the expression levels of single genes, most notably real-time reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and DNA array technology (Lehmann et al. 2001; Lewis et al. 2001; Specht et al. 2001; Walch et al. 2001). A number of studies have shown that such archival material is in principle amenable to the analysis of messenger ribonucleic acid (mRNA) and several protocols have been established for the retrieval of mRNA from routinely processed paraffin-embedded tissues (Ben-Ezra et al. 1991; Foss et al. 1994; Goldsworthy et al. 1999; Lehmann et al. 2001; Specht et al. 2001; Macabeo-Ong et al. 2002; Srinivasan et al. 2002; Abrahamsen et al. 2003). However, the results have also shown that aldehyde-induced cross-linking of mRNA with proteins and degradation of mRNA may cause severe limitations to this approach and that the results have to be interpreted with caution (Ben-Ezra et al. 1991; Masuda et al. 1999).

The parameters of tissue processing that affect the quality of the RNA detectable in the routinely formalin-fixed, paraffin-embedded tissues have been well established in a number of studies. First, the tissues should be fixed as soon as possible to prevent autolysis-induced RNA degradation and loss (Gruber et al. 1994; Srinivasan et al. 2002; Abrahamsen et al. 2003). Second, the duration of fixation should be kept between 0.5 day and 2 days with longer fixation times drastically reducing the number of detectable RNA molecules (Gruber et al. 1994; Macabeo-Ong et al. 2002; Abrahamsen et al. 2003). Third, the formalin used should be buffered to a neutral pH because acidic conditions will lead to enhanced RNA degradation (Impraim et al. 1987; Ben-Ezra et al. 1991; Gruber et al. 1994; Srinivasan et al. 2002). Furthermore,

the size of the nucleic acid fragments amplified by RT-PCR should be kept to a minimum. Amplicons between approximately 80 and 120 base pairs in length usually gave the best results (Ben-Ezra et al. 1991; Koopmans et al. 1993; Specht et al. 2001; Abrahamsen et al. 2003). In contrast to the latter, the first three factors cannot be influenced in retrospective studies and often represent unknown variables when routinely processed archival material is used, resulting in some uncertainty when interpreting the results.

Reduced quality of mRNA is of particular concern when a precise quantification of mRNA copy numbers is desired rather than a qualitative analysis (Bustin 2000). One of the most critical factors in quantitative gene expression studies is the inclusion of one or more reference genes to control for variations due to degradation of mRNA, differences in the amounts of total mRNA analyzed, and variable efficacy of the reverse transcription reaction (Vandesompele et al. 2002). These reference values are usually obtained by measuring the mRNA copy number of a so-called housekeeping gene, such as beta-actin (Vandesompele et al. 2002; Abrahamsen et al. 2003), hypoxanthine phosphoribosyltransferase-1 (HPRT) (Murray et al. 1970; Foss et al. 1994; Vandesompele et al. 2002), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Foss et al. 1994; Sirover 1999; Vandesompele et al. 2002), or elongation factor-1a (EF-1a) (Czworkowski et al. 1996; Gruber et al. 1997; Leverkoehne et al. 2002). Gene expression levels for the gene of interest are then given as copy number per copy number of a housekeeping gene (Leverkoehne et al. 2002; Vandesompele et al. 2002; Abrahamsen et al. 2003). Thus, it appears crucial that the ratio between the detectable copy numbers of different genes remains constant and is not altered by artifacts unique to the experiment performed. For example, it is conceivable that RNA degradation or the degree of cross-linking of RNA molecules with proteins may vary between different mRNA sequences, depending on factors such as base composition, size of full length mRNA, or overall cellular abundance of a given RNA sequence. Moreover, extensive functional interaction of an RNA molecule with cellular proteins may result in a different degree of cross-linking with proteins when compared with RNA molecules that normally do not tightly interact with proteins. For example, the mRNA encoding EF-1a is stored in the cytosol together with several proteins as tightly packed ribonucleoprotein complexes, resulting in a high stability and long half-life of the mRNA molecule (Slobin 1980). The sequence, base composition, length, and cellular function of a given gene transcript make it biochemically unique and different from all other cellular mRNA species. Whether such properties may result in different degrees of RNA degradation or cross-linking with proteins during formalin-fixation, paraffin-embedding, and long-term storage has not been addressed. Importantly, if these factors do play a role, this could potentially influence the ratios between the detectable expression levels of different genes in formalin-fixed, paraffin-embedded tissues, leading to artificial results when expression levels are calculated relative to a housekeeping gene.

The present study was designed to examine the effect of autolysis, formalin-fixation, paraffin-embedding, and long-term storage on the ratio between the expression levels of different genes as detected by real-time RT-qPCR. The expression levels of one gene with highly variable expression levels between different tissues, multiple drug resistance gene-1 (MDR-1) (Endicott et al. 1989; Ginn 1996), and four housekeeping genes of different cellular abundance (beta-actin, HPRT, GAPDH, and EF-1a) were systematically quantified in 576 tissue samples from normal canine liver, heart, spleen, and kidney before and after fixation in formalin and embedding in paraffin. The results indicate that normalization of gene expression levels using any of these housekeeping genes artificially increases or decreases relative expression levels of the target gene in fixed and embedded tissues.

Materials and methods

Tissue collection and processing

Tissue samples of 1×1×0.4 cm in size from liver, spleen, kidney, and heart were taken from three healthy beagle dogs (two male, one female, 3–4 months of age) that were euthanized for other reasons, frozen in liquid nitrogen immediately after removal and stored at −80°C. In addition, one adjacent sample of the same size from each organ was fixed for 1, 2, or 6 days in 10% or 4% neutral-buffered (pH 7.0) formalin. A histological evaluation (hematoxylin-eosin stain) of routinely processed samples failed to detect any significant lesions in the tissues investigated. To examine the effects of autolysis, samples from liver and spleen of a fourth beagle dog (female, 5 months of age) were exposed to autolysis at room temperature for 1, 2, 6, 12, 24, or 100 h, frozen in liquid nitrogen and stored at −80°C. To investigate the combined effect of autolysis and fixation that commonly occurs under routine conditions, samples of similar size from the same organs were subjected to 6 h of autolysis at room temperature before they were fixed and prepared for quantitative gene expression analysis. The effect of long-term storage was examined by analyzing routinely formalin-fixed, paraffin-embedded tissue blocks of the same organs from one healthy dog per time period which had been stored for 5, 10, 15, or 20 years in the diagnostic archive of the Department of Pathology, School of Veterinary Medicine Hannover.

RNA extraction and reverse transcription

Total RNA was extracted from formalin-fixed, paraffin-embedded tissue blocks with a modified proteinase K

digestion protocol as described (Specht et al. 2001). Briefly, 15 slices of 10 μm thickness were deparaffinized for 10 min in 1-ml xylene twice, followed by serial rehydration in 1 ml each of 100, 90, and 70% ethanol and diethylpyrocarbonate (DEPC)-treated water (10 min each). After each step the mixture was centrifuged briefly and the solvent was removed. The tissues were dried at 60°C for 20 min and incubated for 14 h at 60°C in 500 μl of 5 mM EDTA, 50 mM Tris–HCl (pH 8.0), 1% SDS, 0.5 mg/ml Proteinase K (Sigma, St. Louis, MO, USA) and 1 μl poly-A carrier RNA (Qiagen, Hilden, Germany). The RNA was extracted using phenol–chloroform–isoamyl alcohol and a final chloroform extraction step. RNA was finally precipitated by addition of 0.1 volumes of 3 M sodium acetate (pH 5.2), 2 μl of glycogen (10 mM), and 2.5 volumes of ethanol. Total RNA was isolated from the frozen tissues using TRIZOL (Invitrogen, Carlsbad, CA, USA) following the manufacturers protocol. RNA concentration was determined by OD_{260} absorption and purity was verified by OD_{260}/OD_{280} absorption ratio determination (>1.9). To remove traces of genomic DNA, the RNA extracted from both types of samples was then digested using RQ1 RNase-free DNase (Promega, Madison, WI, USA) and purified using the RNeasy Minikit (Qiagen) following the protocols of the supplier. For reverse transcription, approximately 2 μg of total RNA per sample (only exception due to limited amounts available: 1.6 μg from formalin-fixed, paraffin-embedded heart) were reverse transcribed in a 40-μl reaction using the Omniscript RT Kit (Qiagen), 20 units RNase inhibitor (RNase OUT, Promega), and 1.5 μg random primers (Invitrogen).

Standards for real-time PCR

Quantification standards for all mRNA sequences examined were generated by PCR using primers flanking the amplicon of the real-time RT-qPCR (for primer sequences, see Table 1). Primers were synthesized by Invitrogen. The standards were amplified using cDNA generated from fresh canine liver tissue. The PCR products were extracted from the gels using the Qiagen Gel Extraction Kit (Qiagen) following the protocol of the supplier. After determining DNA concentration by OD_{260} measurement, DNA was diluted to concentrations equivalent to 10^8 –10^3 copies/μl in 5 mM Tris–HCl, pH 8.0. Because the sequence of the canine EF-1a mRNA was unknown, it was first established by PCR amplification from canine liver using species-promiscuous primers for EF-1a (Gruber et al. 1997). The PCR product was sequenced and the sequence deposited in the GenBank database (accession No. AY195837) and used for primer selection for this study (Table 1).

Table 1 Sequences and calculated melting temperatures (T_m, calculated for 50-mM NaCl molar strength) of the PCR primers and probes used, sizes of PCR products and GenBank accession numbers of the respective mRNA sequences

Primer and probe names	Sequences in 5′–3′ orientation	T_m (°C)	PCR-product sizes (bp)	GenBank accession numbers
MDR-1 s-up	CAGAGAATCGCCATTGCT	57.1	214	AF045016
MDR-1 s-do	CCACAATGACTCCATCATC	53.9		
MDR-1 for	CAGTTGGTTCAGGTGGCCCT	55.1	79	
MDR-1 rev	CGAACTGTAGACAAACGATGAGCT	55.2		
MDR-1 probe	CCAGAAAAGGCCGGACTACCATTGTGA	65.2		
HPRT s-up	TAAAAGTAATTGGTGGAGAT	48.5	133	CFU16661
HPRT s-do	ATTATACTGCGCGACCAAG	56.1		
HPRT for	GAGATGACCTCTCAACTTTAACTGAAAA	61.5	89	
HPRT rev	GGGAAGCAAGGTTTGCATTG	62.7		
HPRT probe	CTTGATTGTTGAAGATCTCATTGACACAGGCA	65.9		
GAPDH s-up	GTGAAGGTCGGAGTCAACG	59.0	861	AB038240
GAPDH s-do	GTGTCACTGTTGAAGTCACAGGAG	61.0		
GAPDH for	GTCATCAACGGGAAGTCCATCTC	64.2	84	
GAPDH rev	AACATACTCAGCACCAGCATCAC	61.4		
GAPDH probe	TCCAGGAGCGAGATCCCGCCAACAT	68.7		
β-actin s-up	CTACAACGAGCTGCGCGTG	64.1	1137	AF021873
β-actin s-do	GACAGAAATGACAACTGGTTCAGACTC	63.6		
β-actin for	GCTGACAGGATGCAGAAGGAAAT	63.9	101	
β-actin rev	CGCATCTGCTCGCAGTCG	65.5		
β-actin probe	ACAGCCCTGGCCCCAGCAAGGAT	69.4		
EF-1a s-up	AGCCCTTGCGCCTGCCTCTC	70.6	1061	X03558
EF-1a s-do	CAGACACATTCTTGACATTGAAGC	60.9		
EF-1a for	CAAAAACGACCCACCAATGG	63.3	68	AY195837
EF-1a rev	GGCCTGGATGGTTCAGGATA	62.0		
EF-1a probe	AGCAGCTGGCTTCACTGCTCAGGTG	72.7		

Primer names identify the primers used for generation of the quantification standards (*s-up* standard, upstream primer; *s-do* standard, downstream primer) and the primers used for the quantitative expression analyses (*for* forward primer; *rev* reverse primer)

Real-time quantitative PCR

Real-time quantitative PCR was performed as described previously (Leverkoehne et al. 2002) using the Mx 4000 Multiplex QPCR System (Stratagene, West Cedar Creek, TX, USA). Primers and *Taq*-Man probes for the five mRNA sequences examined were designed using the Beacon Designer software (PE Biosystems, Weiterstadt, Germany) and synthesized by Applied Biosystems (Warrington, UK). The reporting dye of all probes was FAM with TAMRA as quencher. The standards, all running with slopes between −3.2 and −3.3, and no template controls were included as duplexes while the samples were measured in triplicates per run. Each run was performed at least twice. Optimized runs started with 10 min at 95°C followed by 40 cycles with 15 s at 95°C and 1 min at 61°C (MDR1, HPRT), 62°C (beta-actin), or 64°C (GAPDH, EF-1a). Primer sequences and sequence sources are shown in Table 1. Optimized reactions were performed under the following conditions: 1-µl cDNA template corresponding to 52.6 ng of RNA, 1× core PCR buffer (Stratagene), 200 µM of each dNTP, 5-mM $MgCl_2$, (GAPDH: 6 mM), 300 nM of each primer, 200 nM of the respective probe (GAPDH: 150 nM, beta-actin: 250 nM), 80-nM ROX as reference dye, 0.625 units of Sure Start Taq-DNA Polymerase (Stratagene) and DEPC-treated water to yield a final reaction volume of 25 µl.

Quantification of gene expression

The mean threshold cycle (Ct) values of each sample were given with the respective standard deviation. Absolute copy numbers of each mRNA analyzed were calculated on the base of the corresponding standard curves via the Mx 4000 software. Means ($\bar{x}$) and standard deviations (SD) between two corresponding runs were calculated. Standard deviations based on Ct values of the triplicates of each run were below 1.0 and the coefficient of variation based on Ct values of the two separate runs with identical sample preparation were below 0.05 in all cases as suggested for reproducible results in a quantitative real-time PCR assay (Bustin 2000).

Statistical analyses

Expression levels between different tissues processed by the same protocol were compared using ANOVA analysis of variance. A paired *t*-test was performed (data were normally distributed) to compare the means from fresh tissues (three dogs) with the means from tissue samples derived from the same organs after fixation and embedding (matched pairs). *P* values ≤ 0.05 were considered statistically significant.

Results

Expression levels of MDR-1 and housekeeping genes in fresh tissues

To establish appropriate reference values, the expression levels of MDR-1, HPRT, GAPDH, beta-actin, and EF-1a were first quantified in unfixed liver, spleen, kidney, and heart tissue from three healthy dogs. All genes examined were found to be expressed in each tissue. As expected, mRNA copy numbers varied markedly between the individual genes as well as between different tissues (Fig. 1). MDR-1 was strongly expressed in the liver with 1.5×10^9 copies per µg of total RNA and weaker expression was detected in the spleen, kidney, and heart with 5.1×10^8, 3.0×10^8, and 1.0×10^8 copies per µg of total

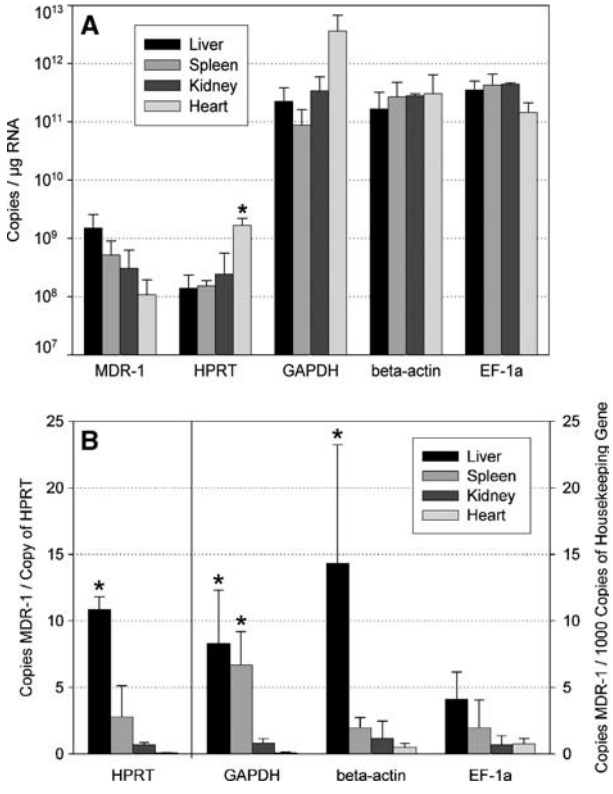


Fig. 1 Quantification of multiple drug resistance gene-1 (*MDR-1*), hypoxanthine phosphoribosyl-transferase-1 (*HPRT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-actin, and elongation factor-1a (*EF-1a*) in four different organs of three healthy beagle dogs. The tissues were frozen in liquid nitrogen immediately after removal and RNA was extracted without formalin fixation. Absolute mRNA copy numbers per µg of total RNA (**A**) and copies of MDR-1 mRNA per copy number of the different housekeeping genes (**B**) are shown. Due to the differences in cellular abundance of the different housekeeping genes, different scales were used in **B** for HPRT (*left panel*; copies of MDR-1 per copies of HPRT) and GAPDH, beta-actin, and EF-1a (*right three panels*; copies of MDR-1 per 10^3 copies of the respective housekeeping gene). *Bars* indicate standard deviations of the means from three dogs. *Asterisks* indicate statistically significant different values among the four organs tested ($P<0.05$; *ANOVA* analysis of variance)

RNA, respectively. However, no statistically significant differences were observed when the organs were compared with each other ($P > 0.05$). Expression levels of the so-called housekeeping gene HPRT were found in a similar range but varied the other way round: highest expression was found in the heart (1.6×10^9 copies per µg total RNA) and approximately tenfold lower expression in the liver (1.3×10^8 copies per µg) with a statistically significant difference only between the heart and the other three organs (Fig. 1A; $P < 0.05$, ANOVA analysis of variance). In contrast, GAPDH was expressed approximately 1,000-fold higher, yet with a greater than 40-fold variation of expression levels between spleen (8.8×10^{10}) and heart (3.6×10^{12} copies per µg). Beta-actin and EF-1a mRNA sequences were detected with high expression levels, comparable with those of GAPDH, but with markedly less variations between the different organs (Fig. 1A). For all results, the standard deviations of the means from three dogs were quite small and ranged from 3.6×10^7 (HPRT, spleen) to 3.1×10^{12} copies per µg of total RNA (GAPDH, heart).

When expression levels of MDR-1 were calculated per copy number of each of the housekeeping genes, much stronger differences were seen between the different organs. Liver had the strongest expression of MDR-1, followed by spleen, and only minimal expression was observed in kidney and heart (Fig. 1B). Of note, the differences between MDR-1 expression levels in the different organs varied significantly depending on the housekeeping gene used as a reference. For example, MDR-1 expression in the spleen was only slightly less (factor 0.84) than in the liver when GAPDH was used as a reference, whereas this difference was much higher (factor 0.14) when beta-actin was used as housekeeping gene. When the different organs were compared with each other using a statistical analysis of variance (ANOVA), only liver expressed significantly more MDR-1 than spleen, kidney, and heart ($P < 0.05$) when HPRT and beta-actin were used as housekeeping genes (Fig. 1B). When GAPDH was used, liver and spleen had significantly stronger expression than kidney and heart. Surprisingly, no significant differences were observed between the different organs with EF-1a as reference.

Influence of tissue autolysis

When the influence of different periods of autolysis on the number of detectable mRNA molecules was examined, a marked reduction in the number of detectable mRNA molecules was not observed until 24 h of autolysis (Fig. 2). A marked decline was observed only after 100 h with an approximately tenfold reduction of the copy numbers in the spleen (Fig. 2A) and a 500-fold reduction in the liver (Fig. 2B). Surprisingly, the number of detectable mRNA copies in the liver dropped for most sequences at 2 and 6 h and then increased again. Importantly, all five mRNA sequences tested were similarly affected by degradation during the course of tissue autolysis.

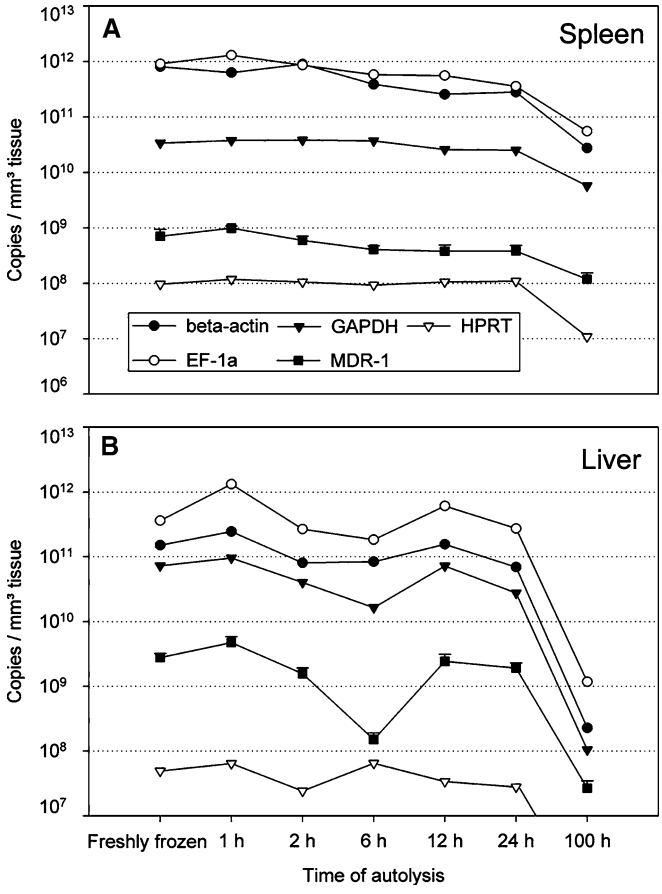


Fig. 2 Influence of tissue autolysis at room temperature on the detectable copy numbers of MDR-1 and four housekeeping genes in spleen (**A**) and liver (**B**). Copy numbers were measured using real-time reverse transcriptase-quantitative polymerase chain reaction. For the sake of clarity, standard deviations of the means are only shown for MDR-1

Influence of formalin fixation and different fixation times

The most significant loss of detectable total RNA was observed after 1 day of fixation (Fig. 3A; 10% formalin) with a reduction of extractable RNA to 51.4% (kidney), 45.9% (heart), 28.4% (liver), or 19.2% (spleen; values are means ± standard deviations from three dogs) compared to the same tissue without fixation and embedding. Similar reductions were detected when 4% formalin was used as fixative (heart: 65.9%, kidney: 49.4%, liver: 31.5%, and spleen: 16.7%; Fig. 3C). Statistically significant differences were only seen between unfixed tissues and the same tissues after fixation. However, due to relatively large standard deviations that were present in most cases, not all declines in extractable RNA after fixation proved statistically significant. Prolonged fixation for 2 or even 6 days had no significant further detrimental effects on the amount of extractable total RNA. The slight variations seen between days 1, 2,

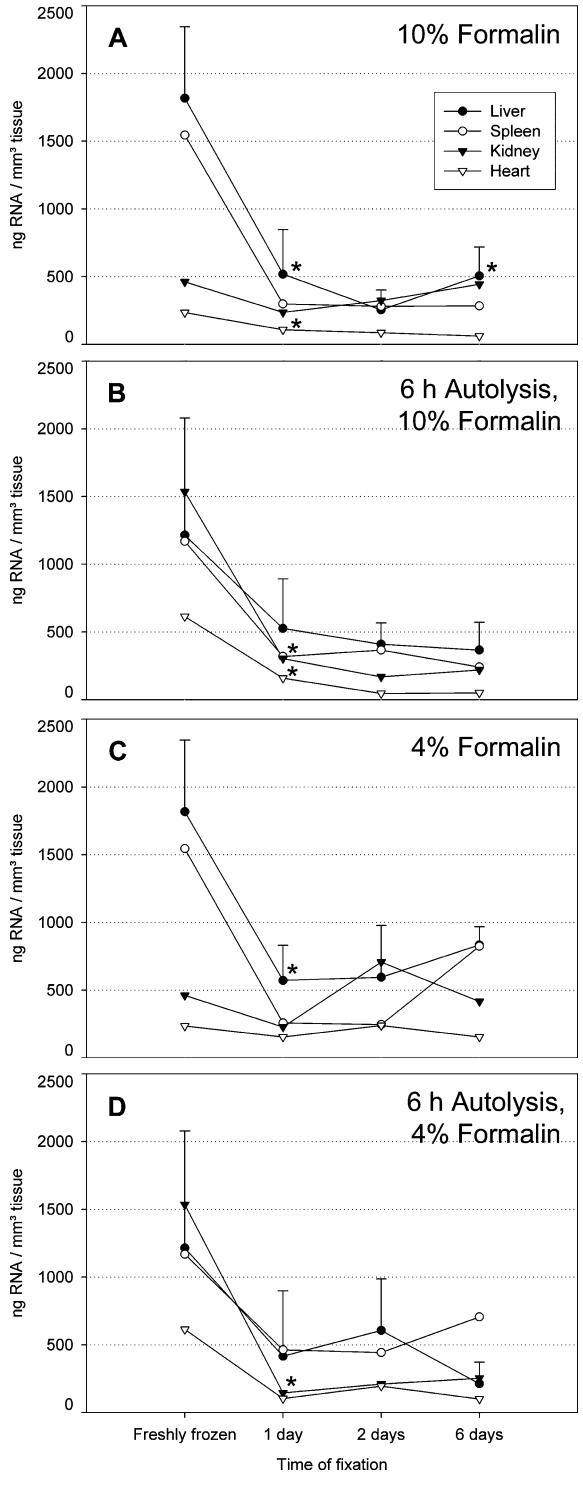


Fig. 3 Markedly reduced amounts of total RNA extractable from formalin-fixed, paraffin-embedded tissues. Freshly frozen tissue was compared with tissue that had been fixed in 10% (**A**, **B**) or 4% (**C**, **D**) formalin for 1, 2, or 6 days. To simulate conditions of routine tissue processing, the effect of tissue autolysis (6 h, room temperature) prior to formalin fixation was examined (**B**, **D**). Purified RNA was quantified by measuring the optical density (*OD*) at 260 nm. For the sake of clarity, standard deviations of the means from three dogs are only shown for liver. *Asterisks* indicate statistically significant declines only after 1 day of fixation ($P < 0.05$; ANOVA)

and 6 when 4% formalin was used (panels C and D in Fig. 3) were not statistically significant ($P > 0.05$; ANOVA).

Influence of combined autolysis and fixation

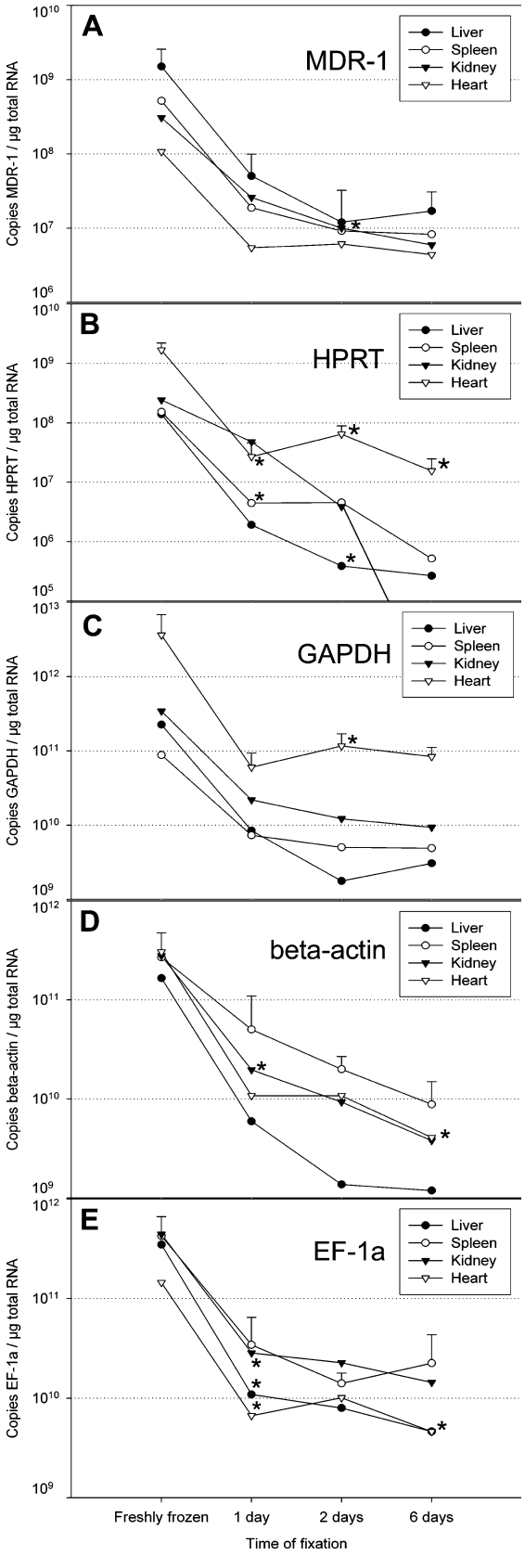
When samples were subjected to 6 h of autolysis at room temperature before fixation for different time periods, the strongest effect was again observed after 1 day of fixation with no significant further loss after 2 or 6 days of fixation (Fig. 3B, D). As seen before, the effects were similar for 4 and 10% formalin used as a fixative. Surprisingly, more total RNA was seemingly obtained from unfixed heart and kidney after 6 h of autolysis when compared to directly frozen fresh tissues, but these differences were not statistically significant ($P > 0.05$).

RNA yields from fixed tissues

The total tissue mass used for RNA extraction from the paraffin-embedded tissues corresponded to a total volume of approximately 15 mm^3. The amount of total RNA obtained from these samples ranged from 1.6 µg (heart) to 7.8 µg (liver) after 1 day of fixation. Following transcription, the cDNA derived from this material was sufficient for approximately 37 (heart) to 150 (liver) real-time RT-qPCR reactions with the protocols used in this study. The calculated absolute mRNA copy numbers detectable in these samples ranged from 2.8×10^6 for HPRT mRNA in the spleen and liver after 6 days of fixation to 2.0×10^{10} for beta-actin mRNA in the spleen after 1 day of fixation (see also Fig. 4B, D).

Conservation of individual RNA sequences in fixed tissues

To assess the degree of individual degradation for different mRNA sequences, the mRNA copy numbers for MDR-1, HPRT, GAPDH, beta-actin, and EF-1a were quantified per µg of total RNA extracted from fresh or fixed tissue using real-time RT-qPCR. As seen for total RNA, the most significant loss was observed after 1 day of fixation for all mRNA sequences analyzed (Fig. 4A–E). Moreover, the approximately 100-fold reduction after 1 day of fixation was remarkably similar for all mRNA sequences tested. Again, not all declines proved statistically significant due to large standard deviations. In most cases, prolonged fixation for 2 or 6 days resulted in a minor further loss of detectable copy numbers with slight variations between the different sequences examined. For all tissues and mRNA sequences analyzed, the copy numbers detected after 6 days of fixation ranged between 15- and 1,400-fold lower compared to the numbers detectable in the same tissues prior to fixation and embedding. Very similar results were obtained when



the same experiments were performed with 4% formalin as fixative or with autolysis (6 h, room temperature) and fixation in 4% or 10% formalin (not shown).

◀

Fig. 4 Degradation of the mRNA sequences encoding MDR-1 (**A**), HPRT (**B**), GAPDH (**C**), beta-actin (**D**), and EF-1a (**E**) in 10% formalin-fixed, paraffin-embedded tissues. For the sake of clarity, standard deviations of the means from three dogs are only shown for the mRNA with the highest copy number. *Asterisks* indicate statistically significant differences when compared to the preceding data point ($P < 0.05$)

Comparison of ratios between different RNA sequences in fixed tissues

To examine whether the ratios between different mRNA sequences may be affected by formalin-fixation and paraffin-embedding, the ratios between MDR-1 and each of the housekeeping genes were compared with each tissue before and after fixation and embedding ("matched pairs"). Ideally, if no change was present after fixation and embedding compared to the ratio obtained from an unfixed sample from the same tissue, the factor would be 1.0. The actual factors obtained for all 576 matched pairs analyzed are presented in Fig. 5. For example, 13 out of 288 matched pairs (no autolysis prior to fixation, black bars in Fig. 5, Panel A) and 18 out of 288 matched pairs (6-h autolysis prior to fixation, black bars in Fig. 5, Panel B) had changes in their respective ratios between 0.9 and 1.1, i.e., had mRNA copy numbers of MDR-1 per copy of each of the housekeeping genes that were between 90% and 110% after fixation and embedding compared to that ratio before fixation. Conversely, 154 (=53.4%; no autolysis, black bars in Panel A) and 155 matched pairs (=53.8%; with autolysis, black bars in Panel B) had factors of below 0.9 whereas 121 (=42.0%; no autolysis) and 115 (=39.9%; with autolysis) had factors of above 1.1. Thus, slightly more numbers of matched pairs had decreased ratios after fixation and embedding. These results were virtually identical for the matched pairs without autolysis and those with 6 h of autolysis prior to fixation (Panel A vs. Panel B). Importantly, significant numbers of the "matched pairs" suffered from drastically reduced or increased ratios of copy numbers after fixation and embedding. For example, 75 of the 576 matched pairs (=13.0%) had gene expression ratios of MDR-1 per housekeeping gene that were tenfold or more reduced compared to the same tissue prior to fixation and 14 out of 576 pairs (=2.4%) had ratios increased tenfold or greater. When these factors were compared between the different housekeeping genes used, those housekeeping genes with high copy numbers (GAPDH, beta-actin, and EF-1a; white bars in Fig. 5) tended to have more decreased (253 out of 432; =58.5%) than increased ratios (154 out of 432; =35.6%), whereas more increased (82 out of 144; =56.9%) than decreased ratios (56 out of 144; =38.8%) were obtained after fixation when the low-abundance housekeeping gene HPRT was used (gray bars in Fig. 5). No differences were seen between the different organs, fixatives, or fixation times used (not shown).

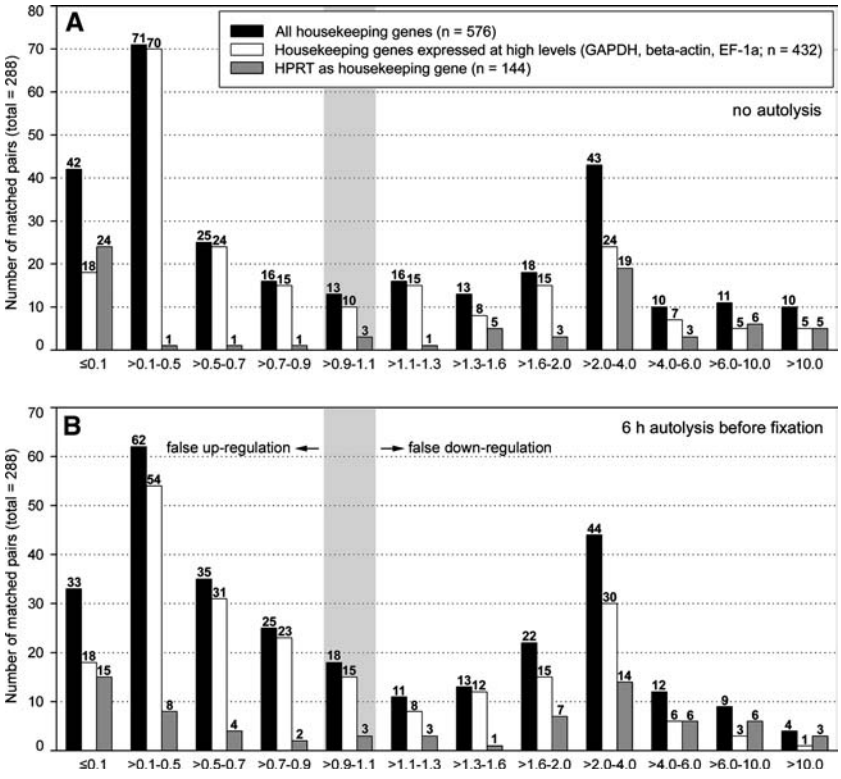


Fig. 5 Impact of formalin-fixation and paraffin-embedding on the ratio between MDR-1 mRNA copy numbers and copy numbers of HPRT, GAPDH, beta-actin, and EF-1a as housekeeping genes in 576 matched tissue sample pairs. The ratio obtained after fixation and embedding was divided by the ratio obtained from the same tissue sample before fixation (matched pair) and the resulting factors are given as groups at the abscissa. Ideally, if no change was present after fixation and embedding compared to the ratio obtained from an unfixed sample from the same tissues, the factor would be 1.0. The numbers of matched pairs per group are given on top of the bars (ordinate). The tissues were fixed without (**A**) and with 6 h of autolysis at room temperature (**B**) prior to fixation

Influence of long-term storage

Another important factor when using archival material is the possible influence of long-term storage on the preservation of RNA. When total RNA was extracted from paraffin blocks after long-term storage (5, 10, 15, and 20 years), the results indicated that the amount of RNA was not necessarily correlated with storage time (Fig. 6A). A slight but clearly time-dependent decrease was only observed in the liver. When the mRNA copy numbers of MDR-1 and the four housekeeping genes were quantified in the archival material, the detected copy numbers were slightly variable between the tissues and the different mRNA sequences, but clearly not strictly dependent on the duration of storage (Fig. 6B–E). Of note, few mRNA sequences with otherwise low copy numbers were undetectable in select cases (HPRT after 5 years in the liver, MDR-1 after 10 years in the spleen, and HPRT after 10 years in the heart).

Discussion

Quantification of the mRNA copy numbers of MDR-1, HPRT, and GAPDH showed remarkable variations between the different organs. This finding was expected for MDR-1 which is known to be differentially expressed in canine tissues (Ginn 1996), but unexpected for HPRT and GAPDH which are frequently used as housekeeping genes to control for variations in RNA template amounts. In contrast, only minor variations were observed between the different organs for beta-actin and EF-1a, which thus seem to be more evenly expressed between different organs. The expression levels of canine MDR-1 with strongest expression in the liver are consistent with the results of a previous study in which the MDR-1 protein was only detected in the liver but not in the heart, spleen, and kidney (Ginn 1996). However, when the copy numbers were normalized relative to each of the four housekeeping genes (Fig. 1B), the differences between the four organs varied drastically. Clear and statistically significant differences between liver and the three other organs were present only when HPRT or beta-actin was used as a reference. In contrast, when GAPDH was used for normalization, MDR-1 expression seemed to be similarly strong in liver and spleen, and weak or absent in kidney and heart. Even more unexpectedly, no statistically significant differences of MDR-1 expression were present between the different organs when EF-1a was used for normalization. It thus seems that the use of different housekeeping genes may lead to different results and interpretations of the expression data as to their biological significance, although the tendency of MDR-1 being most strongly expressed in the liver was conserved regardless of the housekeeping gene used. These differences are obviously caused by the varying expression levels of the four housekeeping genes in the four organs tested. Taken together, these results and a comparison with the MDR-1 protein expression data (Ginn 1996) suggest that beta-actin and HPRT may serve as suitable references for a comparison of MDR-1 expression in different tissues.

A second important observation was the relatively low overall expression of HPRT, which was approxi-

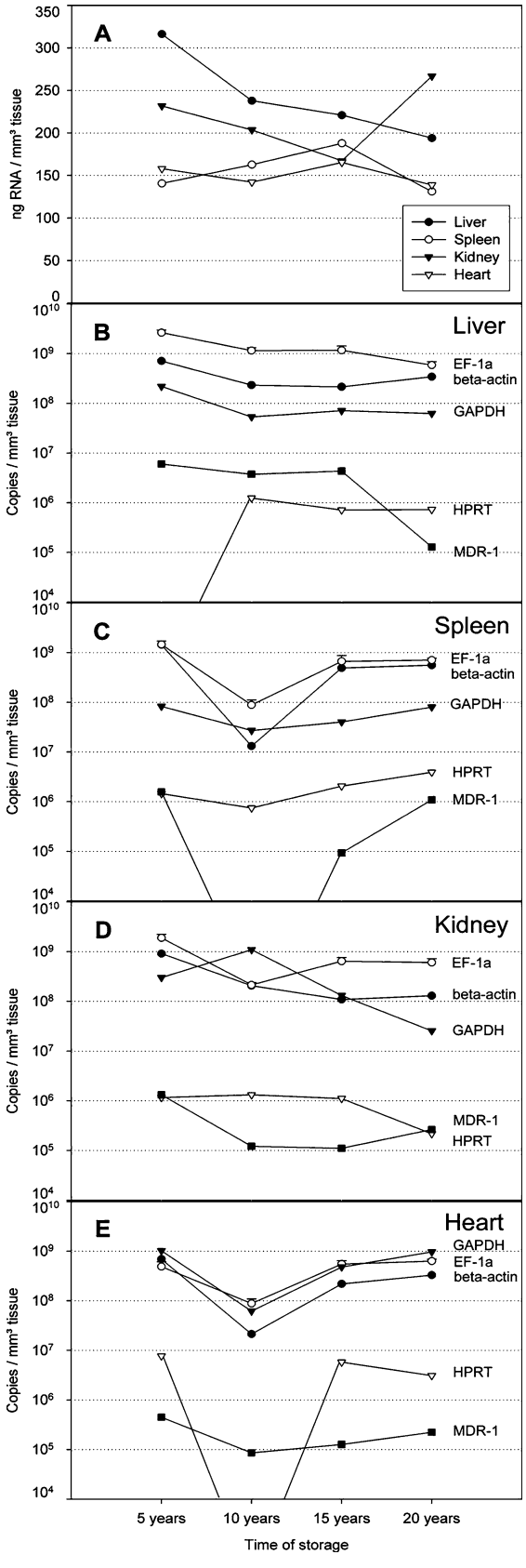


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Fig. 6 Influence of long-term storage of formalin-fixed, paraffin-embedded tissue blocks on the integrity of total (**A**) and sequence-specific (**B** to **E**) RNA. Missing data points: although repeatedly tested, some mRNA sequences were undetectable in some tissues

mately 1,000-fold lower than the copy numbers of GAPDH, beta-actin, and EF-1a. HPRT copy numbers in the liver and spleen were similar to the copy number of MDR-1 in the heart where the MDR-1 protein had not been detected previously (Ginn 1996). Clearly, due to the higher sensitivity of the RT-PCR assay, when compared to immunohistochemical protein detection, these results argue for weak but present expression of MDR-1 in the canine spleen, kidney, and heart. It would thus appear that HPRT, despite its variable copy numbers in different tissues, may serve as an appropriate control when low-copy number mRNA sequences are examined. This notion is further supported by data obtained from experiments using archived tissue with or without long-term storage in which HPRT was undetectable in a small number of samples (see below), warranting caution for the detection of low-copy number messages in archival tissues.

It is generally believed that any delay in processing fresh tissues before formalin-fixation under routine conditions will result in significant and time-dependent damage to the mRNA of interest (Srinivasan et al. 2002). Dramatic biochemical alterations occur in tissues as early as 10 min after anoxia (Kingsbury et al. 1995; Srinivasan et al. 2002), a time period which is frequently exceeded before postmortem material or biopsy samples are fixed in formalin. As a consequence, most authors recommend the prefixation time to be as brief as possible (Bustin 2000; Srinivasan et al. 2002; Abrahamsen et al. 2003). The analyses of the effect of prolonged prefixation time (autolysis) on the detectable mRNA copy numbers in the present study stand in sharp contrast to this notion. Tissue autolysis of spleen and liver samples of up to 24 h did not result in a significant loss of copy numbers in all five mRNA sequences tested (Fig. 2). These results are very similar to observations on the stability of total RNA and mRNA isolated from bovine reproductive tissues following autolysis (Fitzpatrick et al. 2002). Although encouraging, caution is warranted before these results are universally applied for other tissues because many different factors may play a role here, most importantly different levels of RNase activity in different tissues. Accordingly, the results obtained from liver samples in the present study were more variable than spleen which is most likely due to the higher amounts of RNases in the liver. The drop of mRNA copy numbers in the liver at 2 and 6 h followed by an increase at 12 h cannot be easily explained, but may be due to complex interactions between RNA and protein molecules during the process of degradation and decay. Since this effect was not observed in the spleen, its relevance remains unclear. More importantly, when the ratios between the copy numbers of different mRNA sequences were analyzed in formalin-fixed tissues, the results were virtually identical, regardless of whether or not the fixation was preceded by 6 h of autolysis. In conclusion, a delay in

tissue processing with prefixation times of up to a few hours does not appear to be problematic for quantitative gene expression studies.

The results of this study as well as previous studies (Specht et al. 2001; Abrahamsen et al. 2003) show that mRNA sequences can readily be quantified by real-time RT-PCR in the vast majority of fixed and embedded tissue samples. As expected and as reported previously (Foss et al. 1994; Goldsworthy et al. 1999; Srinivasan et al. 2002; Abrahamsen et al. 2003), formalin-fixation and paraffin-embedding resulted in a marked reduction of detectable mRNA molecules, although only short target sequences were amplified here (68–101 bp). In this study, the strongest effect was seen after 1 day of fixation with a reduction of extractable total RNA between twofold and fivefold. A much higher loss was observed for the individual mRNA sequences with a loss of amplifiable copy numbers of approximately 100-fold. This difference was most likely caused by less degradation of the more stable ribosomal RNA, which constitutes 95–99% of the total cellular RNA (Alberts et al. 2002; Fitzpatrick et al. 2002). Surprisingly, the strong declines did not always prove to be statistically significant which was most likely due to large standard deviations in some data groups. Importantly, the reduction of individual mRNA sequences appeared to be very similar for the different genes and organs examined. Prolonged fixation times of up to 6 days caused no further significant loss of quantifiable mRNA molecules. This is of practical relevance because under routine conditions, fixation times may occasionally be increased up to a few days due to a delay in automated processing during weekends or holidays.

As the central and novel aspect of this study, we hypothesized that the ratio between the copy numbers of the different mRNA sequences is unchanged after formalin-fixation and paraffin-embedding when compared to mRNA isolated from the same tissue samples without fixation and under optimal conditions. Surprisingly, this ratio was found to be largely altered in most of the samples in which the relative quantification of MDR-1 expression obtained from the fixed tissues was far from reflecting the true expression levels measured before fixation. Importantly, these alterations would have been large enough to yield significant artifacts in an assay designed to identify differentially regulated genes. Among the 576 matched pairs analyzed, 208 (=36.1%) yielded results after fixation and embedding that would have artificially indicated a twofold or higher downregulation of MDR-1, whereas 143 (=24.8%) of the samples would have artificially suggested a twofold or higher upregulation of MDR-1. Moreover, a more than tenfold down- or upregulation would have been falsely simulated in 13.0% or 2.4% of the samples, respectively. These deviations were due to similarly altered copy numbers of both the MDR-1 mRNA and the housekeeping mRNA and were not attributable to either the numerator or the denominator alone when the relative expression ratio of MDR-1 per housekeeping gene was calculated. In general, calculating the ratio between two slightly distorted copy numbers obviously results in the potentiation of the deviation, which was to be expected from a mathematical point of view. In conclusion, differentially regulated genes, even if there are only slight differences, may lead to artificial misinterpretation of the data if used as reference genes for normalization. It would thus appear that none of the four housekeeping genes tested can serve as a reliable reference for normalization of quantitative gene expression studies using formalin-fixed, paraffin-embedded tissues. Of note, none of the four housekeeping genes tested proved superior to the others with regard to variability of the results from formalin-fixed tissues. The altered gene expression ratios measured after fixation and embedding may thus be inherent to RNA molecules in general and not specific for certain mRNA sequences. It therefore appears questionable whether the "ideal" reference mRNA can be found at all for normalization of expression data obtained from partially degraded RNA as template.

Long-term storage of archived tissue blocks obviously had no additional detrimental effects. The quantification of total RNA and of the mRNA copy numbers of MDR-1 and four housekeeping genes in routinely formalin-fixed, paraffin-embedded tissue blocks after 5, 10, 15, and 20 years of storage failed to identify a clear time-dependent loss of RNA. Rather, the somewhat variable results that were obtained from the archival material in a few cases may have been due to differences in the tissues investigated such as age, breed, gender, health status of the dogs, or the quality of the formalin used. Clearly, the lack of available matched pairs, i.e., RNA extracts from the same tissues before and after long-term storage makes a statistical analysis impossible and limits the conclusions to be drawn from this part of the study. Nevertheless, the data obtained here suggest that archived tissue blocks are in principle amenable to RT-PCR studies even after long-term storage of up to 20 years.

One important observation of this study is the lack of detectable specific mRNA sequences in a small number of formalin-fixed, paraffin-embedded tissues. This lack was mostly seen for the mRNA of the low-abundance housekeeping gene HPRT (Foss et al. 1998) and only in a few cases for MDR-1 in the spleen and heart where MDR-1 protein is thought to be not expressed (Ginn 1996). Obviously, these cases fall below the detection limit of the RT-qPCR method used and can clearly be explained by the approximately 100-fold reduction of mRNA copy numbers due to fixation and embedding. The housekeeping gene HPRT would thus appear to be an excellent and sensitive internal control for establishing the preservation of low-copy number mRNA molecules in a given sample. On the other hand, lack of detectable HPRT mRNA in 23.6% of the formalin-fixed samples (Table 2) suggests that the quantification of low abundance mRNA molecules such as HPRT may be particularly critical in fixed and embedded tissues.

In this study, quantitative RT-PCR reactions between two and ten with triplicates per run could be performed with the RNA extracted per mm^3 of formalin-fixed, paraffin-embedded tissue. Undoubtedly, this figure is influenced by many factors, including the type of organ used and the abundance of the mRNA sequence of interest. However, such a prediction would be valuable for planning experiments using archived tissue samples, especially when the amount of the material available is limited. Such limitations particularly apply for laser-capture microdissected (LCM) tissues in which only a small subset of the cells of interest are present (Goldsworthy et al. 1999). Consistent with the results obtained in this study, we routinely use between 0.01 mm^3 and 0.001 mm^3 of freshly frozen LCM tissue and between 1 mm^3 and 0.1 mm^3 of formalin-fixed, paraffin-embedded LCM tissue for real-time RT-qPCR detection of a high abundance mRNA sequence such as EF-1a (not shown).

In summary, the results of this study show that quantification of mRNA copy numbers is in principle possible using formalin-fixed, paraffin-embedded tissue samples. However, fixation and embedding artificially increased or decreased the relative expression ratios of MDR-1 after normalization with housekeeping genes to a degree that would make any biological interpretation of these expression data problematic. Thus, it is questionable if quantification in fixed and embedded tissues based on corrections using housekeeping genes makes sense at all.

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