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Personal View

Improving the analysis of quantitative PCR data in veterinary research

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In recent years we have seen the emergence of molecular veterinary medicine, prompted by the availability of sequence information on pathogens, as well as on mammalian species relevant for economic (e.g. chicken, pig, cattle) and social (e.g. horse, dog, cat) purposes. Molecular biological tools are being integrated rapidly in the diverse specialities of veterinary research, allowing diseases to be described in molecular terms, both at the genetic level (DNA) and at the functional genomics level, including mRNA, non-coding RNA (ncRNA) and microRNA. Species-specific single nucleotide polymorphism (SNP) platforms are available to resolve the genetic background of inherited diseases.

Expression profiling or functional genomics aims to identify differential gene signatures or ncRNA profiles associated with various experimental and/or clinical conditions. This is commonly carried out using species-specific micro-array platforms that measure relative expression levels of large numbers of gene products or miRNAs. Results need to be verified using independent techniques; the most popular validation technique is real-time, fluorescence based, reverse transcription quantitative PCR (RT-qPCR).

The highly sensitive RT-qPCR technique is a complex, multi-step procedure that can be prone to numerous errors, potentially leading to misinterpretation of data. Hence, it is important that the technical information provided in a manuscript is complete, that protocols are validated and that results and conclusions are based on appropriate methods of analysis. Numerous publications, mainly in non-veterinary journals, have argued for the need for appropriate validation of the reference genes used to report relative expression levels of specific gene products with accuracy. It is abundantly clear that calculations of gene expression levels relative to a single, unvalidated, reference gene, without any stability expression evaluation, can be highly misleading (Dheda et al., 2005).

A quick survey of papers published in *The Veterinary Journal* in 2009 and 2010 revealed only two papers in which several reference genes were used to calculate relative gene expression. Veronica Spalenza and colleagues at the University of Turin evaluated several reference genes in bovine peripheral lymphocytes (Spalenza et al., 2011). In a paper by Eric Zini and colleagues at the Vetsuisse Faculty, University of Zürich, three reference genes were used for relative gene expression calculations (Zini et al., 2010).

Very few veterinary publications provide information on RNA quality and integrity and there is little information on the reverse transcription step or on the efficiency of the subsequent PCR. This makes it difficult to evaluate the relevance of any data reported in these journals or to attempt to reproduce the data in a different laboratory. Standardisation of reporting procedures and, indeed, reporting of a minimum amount of relevant technical information of molecular strategies is of paramount importance if gene expression studies are to be more reliable.

The so-called '*Minimum Information for publication of Quantitative real-time PCR Experiments*' (MIQE) guidelines aim to improve the standard of publications utilising qPCR by providing a checklist of the critical information required to enhance readers' appreciation of the data, facilitate the repeatability of these experiments and enhance the comparison between different studies (Bustin et al., 2009). These guidelines are gradually being put into practice, with more than 300 citations appearing in the peer-reviewed literature in the last year; their general adoption by veterinary researchers would serve to strengthen the research field of veterinary medicine.

A recently published modified standardisation approach, MIQE-précis (Bustin et al., 2010) offers simplified guidelines and a Microsoft Excel-based checklist aimed at improving transparency and

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coherent reporting of qPCR data. A set of key criteria is provided to help standardise the separate steps in a qPCR experiment, starting with the initial sampling, assay design and optimisation, through to data analysis. This list is detailed and stringent, but provides an essential guide that allows reviewers and readers to judge a manuscript's merits. Here, we wish to highlight best practices for designing, conducting and reporting RT-qPCR experiments in veterinary medicine.

Sampling

- Biological variation in clinically derived biopsies is unavoidable and, in contrast with standardised designed experiments, is difficult to control. Sampling conditions can greatly affect variation, but can be standardised by the investigator. Therefore, detailed information about sampling techniques, storage conditions and storage times, as well as information on pre-biopsy medication, are essential.
- Following the isolation of RNA, empirical information about potential interfering contamination with traces of genomic DNA is of importance. This is especially so for species with large numbers of processed pseudogenes or when information about intron/exon structure is lacking. This issue can be addressed by the inclusion of 'no-RT controls' (a PCR reaction on samples without reverse transcriptase treatment).
- The amount, quality and integrity of RNA must be recorded. The use of degraded RNA increases variability and can generate false results. The availability of microfluidics-based devices for nucleic acid quality assessment allows automated, rapid and standardised quality assessment of very small amounts of total RNA. These use quality metrics, such as the RNA integrity number (RIN; Agilent), RNA quality indicator (RQI; BioRad) or ScreenTape degradation value (SDV; Lab901) to represent the level of degradation in a sample. However, an assessment of rRNA integrity does not necessarily compare with an assessment of mRNA or miRNA integrity. Importantly, inhibition of reverse-transcription or PCR should be checked by dilution of the sample or use of a universal inhibition assay. Results from samples showing large variations in integrity may not be comparable.

Table 1
Checklist of key criteria for essential information required for proper assessment of reverse transcriptase quantitative PCR experiments.

	Information required
Sample/template	
Source	If cancer, was biopsy screened for adjacent normal tissue?
Method of preservation	Liquid nitrogen; RNAlater; Formalin
Storage time (if appropriate)	If using samples >6 months old
Handling	Fresh; Frozen; Formalin
Extraction method	TriZol; Columns
RNA: DNA-free	Intron-spanning primers; No RT control
Concentration	Nanodrop; Ribogreen; Microfluidics
RNA: Integrity	Microfluidics; 3′:5′ assay
Inhibition-free	Method of testing
Assay optimisation/validation	
Accession number	RefSeq XX_1234567
Amplicon details	Exon location; Amplicon size; Amplicon sequence
Primer/probe sequence	Even if previously published
Amplicon verification	Melt curves
In silico	BLAST; Primer-BLAST; m-fold
Empirical	Primer concentration; Annealing temperature
Priming conditions	Oligo-dT; Random; Combination; Target-specific
PCR efficiency	Dilution curve
Linear dynamic range	Spanning unknown targets
Limits of detection	Accurate quantification
Intra-assay variation	Copy numbers not Cq
RT-PCR	
Protocols	Detailed description; Concentrations; Volumes
Reagents	Supplier; Lot number
Duplicate RT	ΔCq
NTCs	Cq and melt curves
NACs	ΔCq beginning: End of qPCR
Positive control	Inter-run calibrators
Data analysis	
Statistical justification	Biological replicates
Validated normalization	GeNorm summary
Specialist software	QBAsePlus

Cq, Quantification cycle (former terminology was Ct, threshold cycle); ΔCq, Difference in Cq; NAC, No amplification controls, only needed for probe-based measurements; NTC, No template controls; RT, Reverse transcriptase.

Assay optimisation

- Target accession numbers, amplicon locations and sizes, primer (and if used probe) sequences (or commercial assay catalogue numbers) and experimental conditions must be listed.
- Routine melt curve analyses for DNA binding dye assays or, preferably, sequencing of amplicons during the optimisation and validation steps, is required to verify the amplicons' specificity.
- The efficiency of the PCR dramatically affects relative expression; therefore crucial information on PCR efficiency contains optimal annealing temperature, $MgCl_2$ concentrations and calibration curves over a dynamic range that must include the concentrations of the unknown samples.

Reverse transcriptase-PCR

- The production of cDNA with a reverse transcriptase enzyme is probably the most variable step in the molecular enzymatic reaction sequence. Ideally, biological repeat samples are analysed in parallel or, as a minimum, two independent reverse transcriptase reactions should be carried out, especially when the differences in expression are small (<10-fold).
- Information about cDNA synthesis priming must be supplied, i.e. did the reaction make use of oligo-dT primers, of random primers, a combination of both or target-specific primers?
- The type and supplier of reverse transcriptase enzymes must be listed.
- 'No template controls' are essential, since they provide information about PCR contamination.
- Ideally, measurements for one specific gene product from all samples should be carried out on a single plate. This minimises variation due to absence of inter-run variation. If this is not possible (e.g. because the study is prospective or there are too many samples), then identical samples ('inter-run calibrators') must be included on the different plates. These allow measurement of inter-run variation and so allow plate-to-plate comparison.

Data analysis

- mRNA and miRNA must be normalised against appropriate reference targets, which should be stably expressed under the conditions of the experiment. This stability must be validated experimentally for each tissue or sample. Primer pairs of numerous candidate reference genes are available on the Internet. Freeware and commercial software is available that allows optimal and correct normalisation. The best known are GeNorm,[1] Bestkeeper[2] and Normfinder,[3] which analyse the stability of candidate reference genes and rank them according to their relative stability. In addition, GeNorm will provide information on the number of reference genes needed. As a rule of thumb,

[1] See: medgen.ugent.be/~jvdesomp/genorm/.
[2] See: www.gene-quantification.de/bestkeeper.html.
[3] See: www.mdl.dk/publicationsnormfinder.htm.

at least three reference genes are needed, but even this may not always be sufficient. It is obviously more time-consuming and expensive to quantify expression of three or more reference genes compared to only one. However, if expression data based on inappropriate normalisation are wrong and misleading, the amount of time wasted, both by the researchers and anyone reading their publication, along with the additional costs incurred, will be substantially greater. Once the reference genes are statistically justified, it is important to provide information on the software used to quantify the gene expression levels.

A checklist, useful for authors and reviewers, is depicted in Table 1. At first sight, this list appears to create a Herculean task. Actually, once experiments are properly designed and assays performed, each one of the tasks will have already been carried out, allowing each item on the list to be ticked. In fact, many researchers already implement the MIQE-précis system in their experimental design, but space limitations imposed by journals result in the omission of this crucial information in the printed publication. However, most journals now have on-line supplements, which are the ideal repository for this information. Adherence to the guidelines will also speed up the review process of a manuscript and hence is likely to be of direct benefit to the researcher.

In summary, adherence to the MIQE guidelines helps with assay design, improves transparency, allows for accurate evaluation and comparison of different research papers and prevents waste of precious clinical or experimental samples in substandard experiments. This is of benefit to the individual researcher, reduces the number of experimental animals required and so has tremendous benefits for the advancement of veterinary research in general.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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