The Real-Time TaqMan PCR and Applications in Veterinary Medicine

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Introduction

The polymerase chain reaction (PCR), first described in 1985, is a highly sensitive and specific technique used for the detection of nucleic acids [55]. The inventor of this technology earned a Nobel prize for his achievement [43,44], which has revolutionised research and diagnostic possibilities. Qualitative PCR is a well established and straightforward technology, but quantification of specific nucleic acids present in a sample is a demanding task. Accurate quantification is hampered by a number of variations that may occur during sample preparation, storage or the course of the reaction. Even minor variations in reaction conditions are greatly magnified by the exponential nature of the PCR amplification. These variations may partly be overcome by normalising the amount of PCR products of the specific template with respect to an internal reference template. Considering the hundreds of papers published on the use of quantitative PCR, it is not surprising that a great variety of protocols exist. These methods are almost exclusively restricted to use in research because of two factors they have in common: they are difficult to perform and are costly to run.

In the need for faster, more accurate and more economic systems with a high throughput capacity, three keywords have become important for the development of the next-generation of PCR systems: automation, standardisation and miniaturisation. The development process was accelerated by combining computer-assisted PCR with laser technology so that now the laser-guided detection of PCR products, with the help of a so-called TaqMan probe, and the real-time accumulation of fluorescent data points for every PCR cycle virtually replace the need for a time-consuming post-amplification step. In addition, using an internationally standardised 96-well microtitre plate format enables large numbers of samples to be screened within a few hours. The TaqMan principle is implemented in an Applied Biosystems (ABI) Prism Sequence Detection device (Applied Biosystems, Foster City, California, USA), which is one of the most sophisticated technologies currently available and offers a unique platform for further development.

From PacMan to TaqMan - a computer game revisited

The TaqMan process is based on the PacMan principle - a computer game introduced more than twenty years ago. Who remembers it? PacMan, a fictitious character, was moved with the aid of a joystick through a labyrinth containing thousands of tiny blue ghosts which at the same time had to be caught to add points to the player's score. The TaqMan probe follows the same principle. Continuing the analogy, PacMan is represented by the enzyme Taq DNA polymerase. The internal TaqMan probe has two fluorescent tags and is analogous to the 'target' of the PacMan, thus theTaqMan probe is 'eaten up' by Taq DNA polymerase, causing release of the fluorescence which is coupled to the probe (Figure 1).

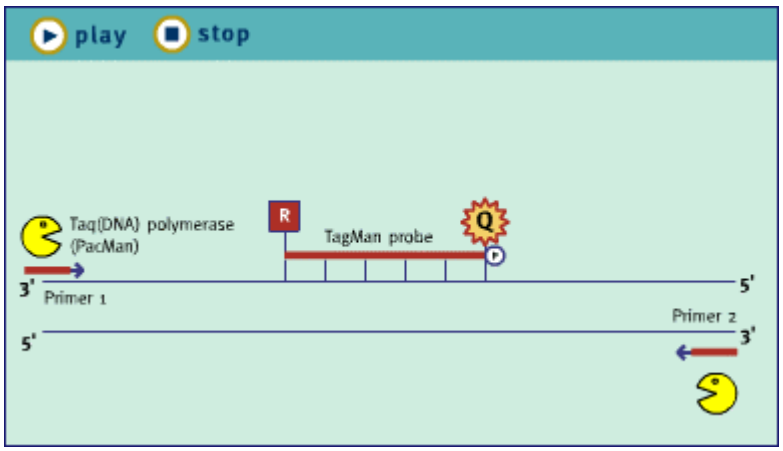

Fig. 1 An Animation of the TaqMan 5'-3' nuclease assay. PCR primers 1 and 2 and a TaqMan probe, labelled with a reporter dye, FAM, (R) and a quencher dye, TAMRA, (Q), bind to the DNA template. The 3' phosphate group (P) prevents extension of the TaqMan probe. The presence of the enzyme, Taq polymerase, enables extension of the primer which displaces the TaqMan probe. The displaced probe is cleaved by Taq DNA polymerase resulting in an increase in relative fluorescence of the reporter. Polymerisation is now complete.

Briefly, the method is based on the 5'-3' exonuclease activity of the Taq DNA polymerase, which results in cleavage of fluorescent dye-labelled probes during PCR; the intensity of fluorescence is then measured by a Sequence Detection System (SDS). The TaqMan probe is located between the two PCR primers and has a melting temperature 10°C higher than that of the primers: binding of the TaqMan probe prior to the primers is crucial because without it PCR products would be formed without generation of fluorescence intensity and thus without being detected. The TaqMan probe has two fluorescent tags attached to it. One is a reporter dye, such as 6-carboxyfluorescein (FAM), which has its emission spectra quenched due to the spatial proximity of a second fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). Degradation of the TaqMan probe, by the Taq DNA polymerase, frees the reporter dye from the quenching activity of TAMRA and thus the fluorescent activity increases with an increase in cleavage of the probe, which is proportional to the amount of PCR product formed (Figure 2). The ABI Prism 7700 is a laser-coupled spectrophotometer which monitors the position of the 96-well microtitre plate, 8 times per minute. Most of the data are stored in a true real-time determination and at the end of 40 cycles all the data for quantitative analysis are stored in a SDS file. A positive TaqMan PCR result may be visualised by at least two means (Figure 2 and 3). The amplification plot reflects the generation of the reporter dye during amplification and is directly related to the formation of PCR products (Figure 2).

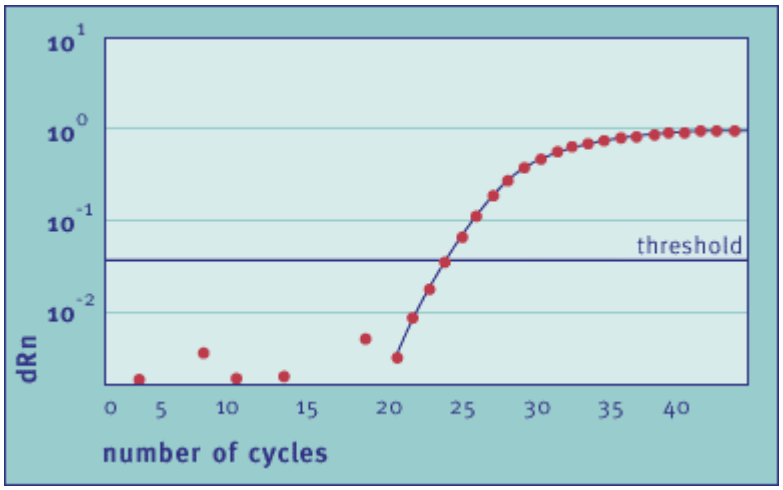

Fig.2 Positive results from a TaqMan PCR are visualised by at least two means: the amplification plot reflects the generation of the reporter dye during amplification and is related directly to the formation of PCR products. The intersection between the amplification plot and the threshold is defined as the cycle threshold, or CT, value. The CT value is related directly to the amount of PCR product and, therefore, related to the original amount of target present in the PCR reaction.

The intersection between the amplification plot and the threshold, where the threshold is defined as 10 times the standard deviation of the background fluorescence intensity and which is measured between cycle 3 and 15, is known as the cycle threshold, or CT, value (default settings of the SD software may be changed manually). The CT value is directly related to the amount of PCR product and therefore related to the initial amount of target DNA present in the PCR reaction. Figure 3 illustrates the single fluorescent components of the reaction.

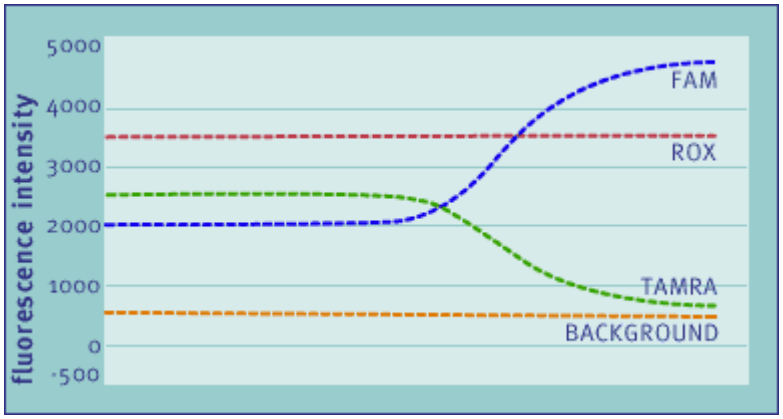

Fig.3 The second of two ways in which a positive result from a TaqMan PCR analysis is visualised. The graph reflects the single fluorescent component of the reaction. The two fluorescent tags bound to the TaqMan probe are 6-carboxyfluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA). A positive TaqMan result is reflected by an increase in the fluorescent intensity of FAM and a decrease in the fluorescent intensity of TAMRA.

A positive TaqMan result is reflected by increasing the fluorescent intensity of the reporter dye, FAM, and by decreasing the fluorescent intensity of the second fluorescent tag, TAMRA. Other fluorescent components present in this procedure are ROX, which is mixed in the PCR buffer to a constant concentration and therefore may be used to normalise fluorescent signals when subtle differences in the volume of the PCR reaction mix occur. Background fluorescence is produced by the plastic of the 96-well plate as well as the optic devices of the detection unit.

The advantages of real-time TaqMan PCR over conventional quantitative PCR

The titration assay based on competitive PCR was first described in 1990 [25]. Though this method is very accurate for DNA measurements, there are several pitfalls that should be considered when using this technique for the quantification of low abundance mRNA [14]. Differences in reverse transcriptase (RT) efficiency can vary up to 50% and are overcome by the addition of an homologous internal control RNA to the RT reaction. Any modification of this technique involves two rounds of PCR, which include a titration and a quantification assay [45,68]. By using the results obtained from the initial titration assay, a known quantity of RNA sample is mixed with the internal control so that the quantities of both molecules are equal. This mixture is then reverse transcribed and a PCR is performed on samples that have been diluted serially. The PCR products are separated by gel-electrophoresis and the band intensities are quantified by video imaging and densitometry. This assay is accurate and sensitive, but involves the definition of very stringent limits [61].

In contrast, the kinetic ELISA-PCR is based on the measurement of the amount of amplicons produced by PCR during each successive cycle. It is a technique that has been applied in many fields [3,63,65] and even analysis at the single cell level has been carried out [31]. It is based on a liquid-phase hybridization step after PCR amplification for the detection and quantification of the PCR products, and therefore internal standards can be used. Biotinylated primers, incorporated in the PCR products, are caught by avidin bound to the ELISA-plate. After binding of amplification products, quantification is achieved by using digoxigenin-labelled internal probes. A colour reaction is induced by adding an anti-digoxigenin alkaline phosphatase-coupled antibody (anti-DIG-AP) and paranitrophenyl (PNP) as the substrate. The optical density (OD) can be measured in any ELISA reader, at a wavelength of 405 nm. This method allows direct quantification of PCR products and provides the results in a digital format.

Kinetic real-time TaqMan PCR, implemented in the ABI Prism 7700, is the method of choice for quantitative PCR because it uses internal probes for the quantification of PCR products; the hybridisation step is carried out during amplification and does not require post-amplification handling and thus reduces the overall manual handling and the risk of carry-over. Real-time TaqMan PCR, on the other hand, has the wide dynamic range and robustness of kinetic PCR and the advantages of the liquid hybridization assay, but lacks the time consuming post-amplification steps involved in kinetic ELISA PCR. The TaqMan system was thought do be less sensitive [15], but in our hands, and in many others, it will repeatedly achieve an absolute sensitivity of 5-10 molecules [26,28,40,67].

The greatest problem facing the diagnostic application of PCR is the production of false-positive results. They are attributable to contamination by nucleic acids, particularly from previously amplified material (carry-over). Any contaminant, even the smallest airborne remnant carried over from the previous PCR procedure or from a strongly positive sample (contamination), may be multiplied and produce a false-positive result. In the TaqMan system, the problem of carry-over is significantly reduced because of the real-time measuring principle, which is based on a closed-tube detection system. The probability of carry-over can be decreased further, or even eliminated, by inclusion of the AmpErase UNG system [47].

Taken together, the quantitative real-time TaqMan PCR technique has several advantages over the classical quantitative PCR system. The use of fluorescent dye-labelled probes increases the sensitivity of the system by at least 7 orders of magnitude and gives rise to a linear relationship between copy number and CT values. In addition, the liquid hybridization assay adds further specificity to the system, comparable to hybridization techniques using blotted PCR products. The elimination of post-amplification steps increases reliability and reproducibility of the assay [26,32]. A major factor responsible for the accuracy of the kinetic PCR method is the determination of the CT value within the logarithmic phase of the amplification reaction, instead of the endpoint determination

used by conventional systems. The Sequence Detection Software (SDS) calculates the CT value when amplification of PCR products is first detected, in other words at the beginning of the exponential phase of amplification, when accumulation of inhibitory PCR products is unlikely to occur. This system offers great potential for automation. Standardisation is achieved by using specific software for primer-probe design. The default settings of the Primer Express Software (Applied Biosystems, Foster City, CA), designs oligonucleotide triplets (two primers and a matching TaqMan probe) that can all be amplified with the same protocol and universal mastermix.

Applications in Veterinary Medicine

Applications of the TaqMan principle are extremely wide. There are three principle fields of interest for the real-time TaqMan PCR user: pathogen detection (i.e. viruses, bacteria, fungi, etc), gene expression (i.e. cytokines, growth factors, transcription factors, etc.) and allelic discrimination (detection of single nucleotide polymorphism, SNP). A few selected examples will illustrate the potential of the real-time TaqMan PCR technique.

Pathogen detection using TaqMan PCR

Feline immunodeficiency virus (FIV), a lentivirus isolated first in California (USA) [48], is similar morphologically and genetically to the human immunodeficiency virus (HIV) [22]. Because FIV is a naturally occurring pathogen which induces an AIDS-like disease in cats, it is considered an important animal model for the study of AIDS in human beings. Furthermore, the FIV model has proven to be useful for studying AIDS pathogenesis, for evaluating new anti-lentiviral drugs and for establishing criteria for the development of safe and efficacious vaccines against lentiviral infections [4,9]. To study the effect of candidate vaccines or therapeutics, highly sensitive and specific test systems were successfully established to quantify the FIV RNA and DNA load for vaccine and therapy studies [32,35]. Quantitative assays for both FIV provirus and viral RNA have a similar absolute sensitivity of 10 molecules.

The level of HIV-1 RNA in serum has the highest predictive value with regard to disease progression [20,42] and sensitive virus load assays have been critical in monitoring the status of HIV-1 infection [18]. Animal models provide great potential for research into such regimes; the most promising for studies of AIDS therapy is infection of rhesus macaques with simian immunodeficiency virus (SIV) [17,54] or with chimeras of SIV containing HIV-1 targets (SHIVs), such as reverse transcriptase (RT-SHIV) [64]. One considerable limitation of the SIV model in HAART-related research is the lack and/or expense of highly sensitive assays to measure viral burdens in plasma. The current test for detection of SIV has been the branched-chain DNA assay (Bayer, Emeryville, CA), which is expensive and not sensitive enough (1500 viral RNA copies/ml) to detect very low viral loads in the SIV system. Moreover, the assay is not adapted to all strains of SIV or to RT-SHIV [60]. Several assays with greater sensitivity than existing quantitative ones have been established [28,62]. In our laboratory, we have optimised a real-time TaqMan RT-PCR assay for SIV RNA which was more sensitive (50 vs 1,500 RNA copies/ml) and had fewer false positives and negatives than the current version of the SIV branched-chain DNA assay [36].

Feline coronavirus (FCoV) is known to be highly prevalent in the cat population, especially in catteries [1]. It is the most important fatal infectious disease in cats, with about 5 - 12% of seropositive cats developing lethal FIP [2]. The pathogenicity of FCoV leading to the FIP syndrome may be linked to mutagenesis, due to increased viral replication. Successful management of FIP may eventually consist of better methods of disease prevention, as well as management of the disease after it occurs. Prevention of FIP can be accomplished by detection and separation of FCoV shedding from non shedding cats, resulting in the reduction of coronaviral load or even the elimination of FCoV from a

cattery [21]. A commercial FIP vaccine is available and consists of a temperature-sensitive mutant form of FIP virus, delivered through the mucosa. The vaccine is supposed to undergo replication only in low temperature, outer oronasal cavities and thus triggers protective antibodies but not FIP. A few controversial studies have recorded a reduction in FIP as a result of vaccination, especially in FCoV naive cats at the time of vaccination [19,24,41,50,58]. It becomes evident that quantification of coronaviral load in FCoV positive shelter cats, or the development of strategies for the prevention or elimination of FCoV in catteries, will depend on PCR procedures that allow the reliable and fast analysis of large numbers of samples. A sensitive real-time TaqMan RT-PCR should enable this type of research [26]. The FCoV real-time TaqMan RT-PCR assay is based on the reverse transcription and amplification of a portion of the FCoV 7b gene, which is known to be highly conserved among coronavirus isolates. This assay, adapted from a previous study [27], has an analytical sensitivity between 10 and 100 times better than a nested RT-protocol. Real-time TaqMan RT-PCR detected most of the important laboratory and field strains of FCoV, including FIPV 204859, FIPV UCD1, UCD 5, FeCV UCD 1, FeCV RM but not the human coronavirus (HCV) strain 229E [26]. The assay allowed absolute quantification with high sensitivity, it was reliable, rapid, easy to use and enables a high sample throughput, making it an excellent tool for diagnostics and FCoV research.

Tick-borne zoonotic pathogens are well known in many areas of the world. Among the tick-borne diseases in Europe, Lyme disease (caused by Borrelia burgdorferi), ehrlichiosis (caused by various species of Ehrlichia) and tick-borne encephalitis (caused by the tick-borne encephalitis virus, TBEV) are the most important zoonotic diseases. Early diagnosis and treatment is necessary to prevent fatal infections and chronic damage to various tissues. Due to the variety of uncharacteristic clinical signs, tick-borne diseases are not easily recognised. Diagnosis is based on clinical findings, a history of exposure to ticks, and direct or indirect detection of the pathogen. The design and optimisation of real-time TaqMan PCR systems for a range of tick-borne pathogens has proved to be important for diagnosis and research and has initiated a series of exciting new projects in this field [33,38,51,52,53,69].

Quantification of gene expression

RT-PCR is the technique of choice for analysing extremely low abundance mRNA derived from cells or tissues. PCR is a well established method, the sensitivity of which is a principal advantage over other techniques, such as Northern blots or RNase protection assay. Once again, the competitive approach is the most commonly used in this field; it ensures normalisation of differences in the kinetics of the reverse transcription reaction by using an internal control, known as the competitor. In due course, the real-time TaqMan PCR will replace many of the conventional systems because of these advantages. We have established real-time TaqMan systems for quantification of gene expression in different species. Inevitably, these assays have received great interest for their use in veterinary research and have been introduced into a number of different projects. Three ongoing veterinary research projects have been chosen to exemplify the usefulness of quantification of gene expression using real-time TaqMan PCR systems.

Cytokines play a central role in the regulation of cell differentiation, proliferation and cell-cell communication [5]. In addition, some cytokines have important effector functions via activation of cytotoxic compounds (eg. perforin, oxygen and nitric oxide radicals). Therefore, these hormones of the immune system are important for the definition of correlates of protective immunity, evoked by viral and bacterial infections or by vaccines. Cytokine induction in cats immunized with an experimental FIV DNA subunit vaccine, which used feline IL-12 as an adjuvant, has shown the involvement of IL-12 p40, IFN-g, and IL-10. Up regulation of mRNA for these cytokines was observed in cats with complete protection against a FIV challenge infection but not in control cats immunized with a placebo vaccine, consisting of uncoated gold particles and FIV gp140 coated with gold particles [35] (Figure 4).

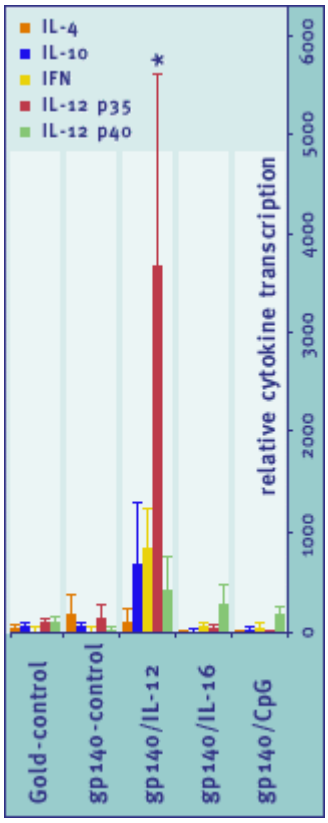

Fig.4 Transcription of cytokines after immunising cats with a DNA vaccine. Cytokine transcriptions are shown 8 weeks after FIV infection challenge. Each cytokine signal is normalised against the GAPDH signal and then calibrated against the lowest normalised cytokine signal at 8 weeks. Bars represent ± standard error (SE) of normalised cytokine signals of 4 cats per vaccine group and control group.
* Cytokine transcription of cats from the vaccine group, FIV gp140, given IL-12 as the adjuvant, differed significantly from transcription of control cats (PMWU<0.05).

DNA and RNA extraction from archived formalin-fixed and paraffin-embedded (FFPE) tissues are of considerable interest for retrospective studies. Embedding bone and other calcified tissues in paraffin requires prior decalcification, a process that takes several weeks or months to complete and increases the risk of RNA degradation. As an alternative, bone and calcified tissues may be embedded in a hydrophobic acrylic resin, based on methyl methacrylate (MMA) [10]. The embedding process is completed within three weeks and does not involve a long process of decalcification. These final sections demonstrate excellent morphological preservation and are ideal for computerised image analysis. Furthermore, in situ hybridization has demonstrated that cellular RNA is still accessible for hybridisation [13]. While DNA extracted from FFPE tissue has been used successfully for many applications, reports describing the analysis of RNA extracted from FFPE tissues or MMA embedded tissues have been rare, or even absent [16,30,56,57].

Because of this situation, we tested the feasibility of quantitative real-time TaqMan RT-PCR in MMA-embedded compared to fresh samples. Biopsies of subchondral cystic lesions (SCL) were taken from horses either during arthroscopic surgery or post mortem; these were fixed in paraformaldehyde and embedded in MMA. Though RNA extracted from MMA embedded bone sections was severely degraded, it could be reverse transcribed with random hexamer primers and amplified, using real-time TaqMan PCR systems for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the proinflammatory cytokines interleukin (IL) 1-b and IL-6 and the inducible form of NO synthase (iNOS) (Figure 5), which show distinct differences of gene expression in different areas of the SCL34. Analysis of MMA-embedded tissues with real-time TaqMan PCR allows an additional refinement of the method. Tissue structure is preserved excellently in MMA blocks, and sawed sections from these blocks can be dissected further to allow localised measurements of gene expression.

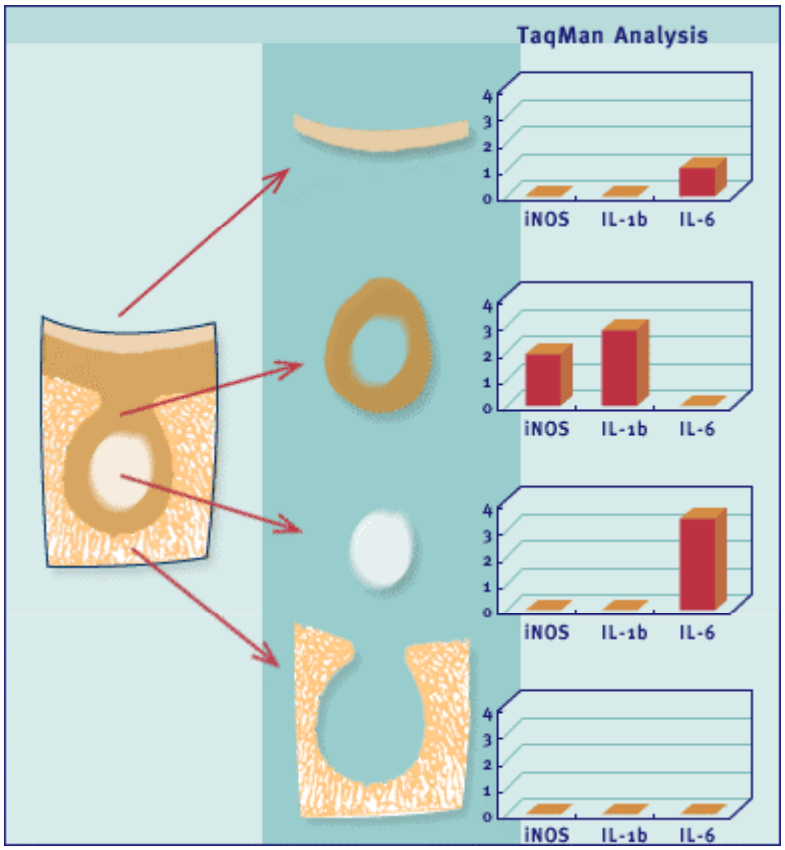

Fig.5 Quantitative mRNA analysis of IL-1b, iNOS and IL-6, all normalised to mRNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 50-micrometer sections from methyl methacrylate (MMA) embedded tissues were cut on a sawing microtome. Specific areas of the subchondral cystic lesion (SCL) were excised using a micro-scissor; total RNA was extracted and reverse transcribed into cDNA for further analysis and quantification of mRNA for IL-1b, iNOS and IL-6. The signal of GAPDH mRNA was used to normalise for differences in RNA extractions and for different efficiencies of cDNA synthesis.

An understanding of immune regulation during infections of the mammary gland in dairy cattle is important for the design of prophylactic vaccines and for the optimization of therapeutic protocols. Using real-time TaqMan PCR, we addressed gene transcription in milk cells of Holstein cows during mid-lactation to define the cytokine profile of these cells in a healthy mammary gland [37]. Cows selected for this study showed relatively low somatic cell counts of <105/ml milk, however, transcription of IL-8, TNF-a, IFN-g, and GM-CSF mRNA was detected in all samples and IL-12 p40 in 6 of 7 samples (Figure 6). Holstein cows are bred for high milk production causing a permanent state of stress in these animals. As a result, a certain amount of tissue damage and possibly low-grade bacterial presence is common. The assumption of the apparent healthy state of the animals is important in order to understand the expression of cytokines indicating a certain level of inflammation. In addition, this study revealed differences between cytokine profiles determined with conventional quantitative RT-PCR and with real-time TaqMan PCR systems. Real-time TaqMan PCR systems are a promising alternative to conventional quantitative PCR systems and could provide a better understanding of immuneregulatory cytokines and their potential use for diagnosis, prophylaxis and immunotherapy.

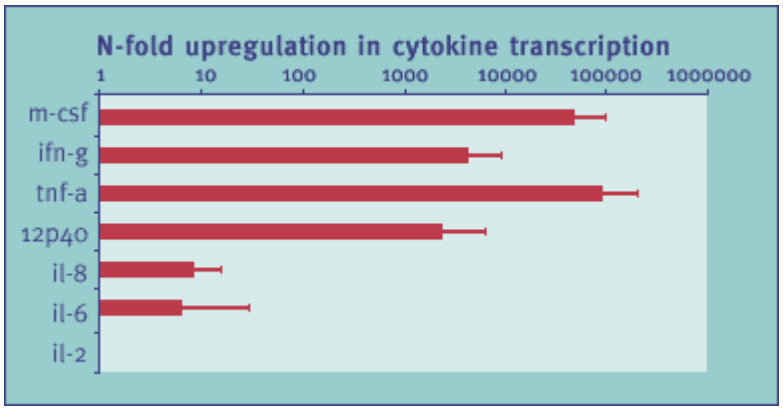

Fig.6 Upregulation in transcription was measured for six different cytokines present in milk cells collected in mid-lactation from seven healthy Holstein cows. Bars represent the means of cytokine transcription. Cytokine transcription is expressed as n-fold differences to the calibrator, which was the lowest detectable cytokine signal. Note the semilogarithmic scale.

Allelic discrimination

A fascinating application of the real-time TaqMan PCR is the detection of gene mutations and genome instabilities. The detection of gene mutations is based on the design of two TaqMan probes, specific for the wildtype allele (A) and the mutant allele (B) [39]. Both probes are labelled with two different fluorescent tags. The TaqMan probe is designed with the gene mutation affecting the middle part of the probe sequence. Binding of the wildtype TaqMan probe to the mutant allele and vice versa is suboptimal because of the mismatch within the TaqMan probe and the target sequence. As a consequence, binding of the TaqMan probe to the unmatched target sequence is highly reduced, or even completely abrogated. Reduction of the binding capacity decreases the difference of the reporter fluorescence in the sample and that in the no template control, which is defined as the ΔRn value. Running appropriate controls for AA, AB and BB, the SDS algorythm for allelic discrimination generates three clusters, and unknown sample DNA will be clearly identified as AA, AB, or BB. A protocol for analysing bovine leucocyte adhesion deficiency (BLAD) has been established [23] and found the system to be highly reliable (observations made by the author; unpublished).

Genome instability includes amplification or deletion of part of the genome resulting in dysmorphology, trisomy or monosomy for parts of the genome. In tumors, the amplification of oncogenes, for example HER2/neu59, or the deletion of tumor suppressor genes, for example p53 [46], are frequently found. Currently, detection of genome instability is accomplished via restriction fragment length polymorphisms (RFLP) or by the different size of PCR products for analysis of a loss in heterozygosity. More laborious methods allow the detection of deletions and amplifications and rely on either quantitative Southern blotting or fluorescent in situ hybridization (FISH). All these methods are difficult to perform, are time-consuming, use large amounts of material or specialised tissue samples and may offer limited resolution.

These difficulties point towards the use of quantitative PCR. Two breast tumor cell lines, MCF-7 and T-47D, were analysed using this method. The T-47D cell line has only one defective copy of p53 [46] and the MCF-7 cell line has only one copy of erbB-211. Both the p53 and the erbB-2 are located on chromosome 17; the S100ß marker located on chromosome 21 was used as a reference. Assays for p53 and S100ß were run in parallel reactions on the same genomic DNA (gDNA) and compared to a control gDNA. By using the ratio between the target and the reference marker, relative ratios of copy numbers can be calculated. Using this technique, gene deletions that had been detected previously, using other techniques, could be successfully detected [12].

Discussion

PCR has proved to be a useful tool in research and diagnosis. However, its use has also brought new challenges to research. The sensitivity found in PCR technology and the availability of quantitative results will bring new problems to the interpretation of these results. As illustrated with feline infectious peritonitis, a fatal disease caused by mutant feline coronavirus (FCoV) strains [66], its diagnosis is difficult, especially when patients lack typical clinical signs of the disease. The causative agent, FIP virus (FIPV), is a macrophage-trophic mutant of the ubiquitous feline enteric coronavirus (FECV) [49, 66] and can be detected in blood and faecal samples from healthy cats [27]. Interpretation of positive PCR results in these cats is difficult, especially when the cat appears to be healthy. In this situation, the positive and negative predictive values are the basis for correct interpretation. By testing large numbers of blood samples from healthy cats, cats with non-FIP disease and cats with FIP-related disease, diagnostic values can be calculated; the diagnostic specificity gives the percentage of negative FCoV-PCR tests in healthy cats and the diagnostic sensitivity is the percentage of positive FCoV-PCR tests in sick cats. If the diagnostic specificity is 100%, then no FCoV has been found in a healthy cat and therefore any positive results must be associated with disease, either with non-FIP or with FIP. If this were the case, the FCoV PCR would be easy to apply. But this is rarely the case and, therefore, intense investigations are necessary to get these values. So far, the positive predictive values for FIP RT-PCR have not been determined carefully, which makes interpretation difficult. Every new PCR test to be used for the purpose of diagnosis should be evaluated carefully according to this procedure. The same information is lacking for FeLV, FHV and many other infections. A great deal of work is needed to generate a basis of knowledge for correct interpretation of these tests. In veterinary medicine, PCR-based diagnostics are just becoming widely used and because of the increased cost-effectiveness of the newer assays, knowledge for their interpretation will soon become available.

The veterinarian and his relationship with the next-generation PCR technology

As with any methodology, work on quantitative PCR is a continuous progress. Although all of the methods described in this review make use of the Nobel prize awarded PCR technology, the pros and cons of the subtle differences in assay format, accuracy and reliability of quantification, all have to be rigorously compared. The next generation of PCR quantification techniques will be more automated, standardised and compact than their predecessors. The time taken for preparation and amplification will be reduced significantly by the use of small volume glass capillaries and silicon-based microchip technology [7,8,29]. Amplification times have been decreased to several minutes by using an Advanced Nucleic Acid Analyzer (ANAA) that consists of a battery-powered array of silicon-based PCR microchips with thin-filmed resistive heaters [6].

How can these new systems be incorporated into the life of the average veterinarian? Future veterinarians may be equipped with affordable, battery-powered, hand-held devices for the diagnosis of infectious diseases, based on the old PCR principle but embedded in technology which is already available, albeit very expensive. These applications will not be restricted to the detection of infectious pathogens but will include the detection of genetic diseases and the monitoring of therapeutic success. Whether or not this futuristic scenario becomes a reality, the PCR technology will undoubtedly experience a widening of its applications in the field of veterinary diagnosis of pathogens, gene expression and the detection of genetic diseases.

Acknowledgements

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