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REVIEW

The role of polymerase chain reaction and its newer developments in feline medicine

H Lutz*, C Leutenegger, R Hofmann-Lehmann

Clinical Laboratory, Department of Veterinary Internal Medicine, University of Zürich, Switzerland

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We give a brief overview on the principles of the polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), quantitative competitive PCR and real-time PCR (TaqMan technology). The literature dealing with PCR and its role in the diagnosis, pathogenesis and research of infectious diseases of the domestic cat is reviewed. Cross-contaminations which occasionally occur during handling of amplified DNA may be an important problem in the PCR laboratory. In many infectious diseases, PCR results are difficult to interpret as their predictive positive and negative values are not always known. Newer assays, such as TaqMan procedures, are becoming increasingly reliable and cost-effective. It can be expected that additional knowledge on how to interpret PCR results will soon be available.

The development of the polymerase chain reaction (PCR) (Mullis 1987, Mullis & Faloona 1987) has dramatically changed not only many aspects of research in molecular biology but also the diagnosis of infectious diseases in man and animals. Today, PCR can be automated and has therefore also become affordable for clinical feline medicine. In this article we review the basic principles of PCR, its newer developments and current applications in feline medicine.

Principles of nucleic acid amplification

Since the description of PCR (Mullis & Faloona 1987), several modifications of the procedure have been reported with the same goal, to amplify a specific DNA sequence so that it can be easily detected, and in some cases, quantitated. Below, the principles of conventional PCR, competitive PCR and real-time PCR are briefly described. For descriptions of modifications of classical PCR, see the following references: ligase chain reaction (Barani 1991); self-sustained sequence replication (Fahy et al 1991); and Qβ replicase amplification (Shah et al 1994).

Polymerase chain reaction

PCR is an enzymatic procedure which allows the almost unlimited amplification of specific nucleic acid sequences in vitro. During the process, the double-stranded target DNA is multiplied in repetitive cycles (Fig 1). The elements of any PCR are two well-defined primers that bind specifically to one of each of the two DNA strands, sufficient amounts of deoxynucleotides, a heat-stable DNA polymerase and a buffer of well-defined composition. The sequences of the two primers have to be carefully selected for perfect match with the target DNA. Primers which have a typical length of 25–30 base pairs (bp) are synthesised in special instruments.

Typical volumes of a PCR assay are 10–50 µl. The reaction is carried out in PCR tubes specifically designed to fit tightly into cavities of an aluminium block which can be heated and cooled in alternate cycles. The process is usually stopped after 35–40 cycles and the product produced is visualised by gel electrophoresis and subsequent staining. During gel electrophoresis, the amplified DNA is separated according to the size of the molecule, ie small DNA pieces

*Corresponding author at: Veterinärmedizinisches Labor, Universität Zürich, Winterthurerstr. 260, 8057 Zürich, Switzerland. E-mail: hanslutz@vetklinik.unizh.ch

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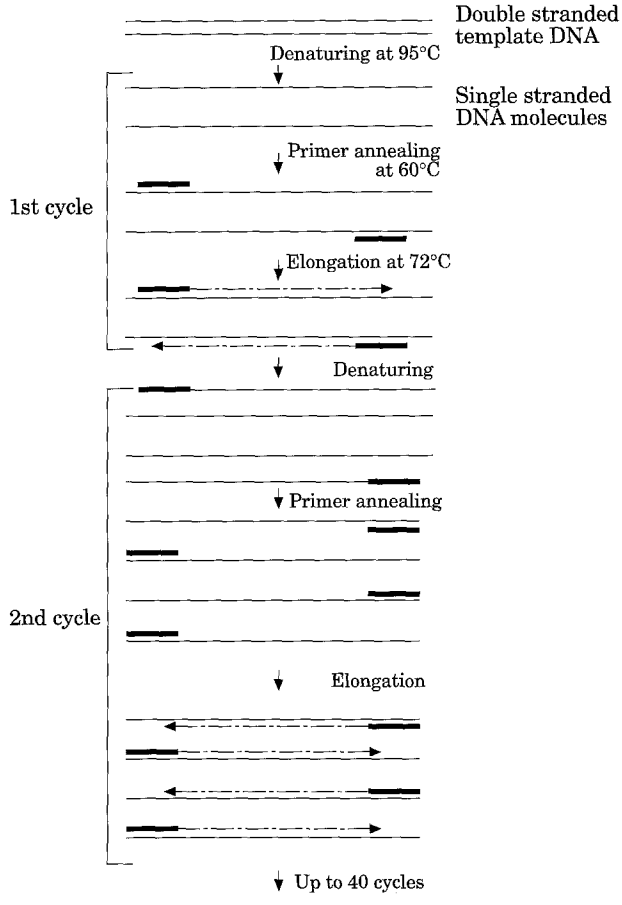

Fig 1. Principle of PCR. A double DNA strand is denatured by heat (95°C) resulting in two single strands. The temperature is then lowered, for example to 60°C, which allows the primers to bind to their complementary sequence. The temperature is then increased to 72°C, the optimal temperature for Taq polymerase which extends the primers at the 3' end. After extension, four strands (two double strands) will have resulted from the two original strands. In cycle 2, the same procedure is repeated, resulting in eight strands and so on.

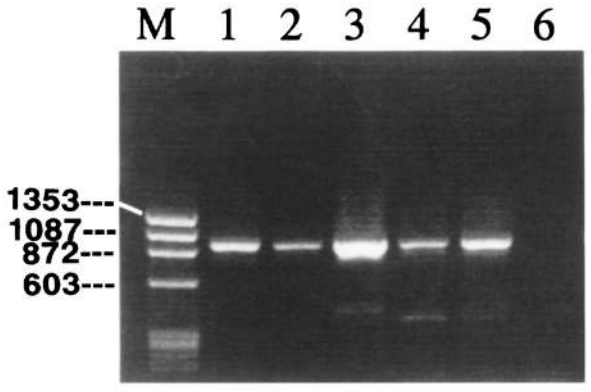

Fig 2. Electrophoresis of amplified products on an agarose gel. Amplified products are loaded onto an agarose gel, separated in an electric field and, after electrophoresis, stained with ethidium bromide. Stained bands are visualised under ultraviolet illumination. DNA fragments migrate according to their size. The size of DNA bands can be determined by comparison with bands of known length. In our example M stands for 'marker' with bands of 1353, 1087, 872 and 603 base pairs. The markers are digestion products of the bacteriophage φX174. Lanes 1–6 show PCR products conducted with plasmids containing a fragment of the feline CCR-5 receptor (lanes 1–5); lane 6 shows no product as the plasmid did not contain the sequence of interest.

migrate far and larger pieces only a small distance from the point of application. After gel electrophoresis, the gel is soaked in a buffer containing a dye that specifically stains DNA, usually ethidium bromide which shows an intense fluorescence when illuminated by ultraviolet light (Fig 2).

With every gel run, a mixture of DNA pieces of different and defined sizes is co-electrophoresed which, by comparison of the migration distance of the calibrator DNA with that of the amplified product, allows the determination of the size of the amplified target DNA (Fig 2). PCR can amplify only DNA as the heat-stable polymerases usually do not recognise RNA as a template. In all cases where RNA is the target, eg in RNA virus detection such as feline coronavirus (FCoV) or feline immmunodeficiency virus (FIV) or when mRNA needs to be quantitated, the RNA has first to be transformed into DNA. This is achieved by a 'reverse-transcription' (RT) step. The RNA is reverse transcribed into DNA by incubating the sample with the retrovirus-derived enzyme 'reverse transcriptase', usually for 45–60 min at 42–48°C. The resulting complementary DNA (cDNA) is then subjected to the PCR run.

There are PCR applications where the amount of target DNA present in the sample is very small. In order to obtain the maximum yield of amplification, a so-called nested PCR (nPCR) can be established in which nested sets of primers are used. PCR is performed for 15–30 cycles with a first set of primers then for another 15–30 cycles with a second set of primers. The second primer set is usually targeted to a fragment within the initially amplified region. Nested PCR not only increases amplification efficiency but also improves the analytical specificity.

The analytical specificity (please note the distinction from diagnostic specificity, below) of the reaction depends on two major factors: the selection and design of (1) the two primers and (2) the assay conditions. To obtain high specificity, the primers must bind to the target DNA only and not to any other, more distantly related, DNA sequence. When primers are designed, their

sequences are usually compared for cross-reactivity with all deposited DNA sequences eg those in the gene bank, a large database which holds all publicly available nucleotide sequences. For instance, if a PCR is established to detect *Bartonella* spp, primers should be selected which recognise a sequence of *B henselae, B quintana* and other closely related Bartonellae which are known to infect cats. The primers should not bind to the respective gene of other bacteria related genetically to *B henselae* such as *Hemobartonella* spp.

However, it is not sufficient to select primers by comparison with sequences deposited in the gene bank alone as several agents may exist in nature from which the gene of interest has not been sequenced and in which the target sequence may deviate from that considered typical for the agent. Therefore, before a PCR assay is used for routine diagnosis, it has to be validated with as many well-defined agents as possible. In infectious diseases, this is usually achieved by testing many well-defined bacterial or viral strains originating from reference laboratories or a depository such as the American Type Culture Collection (ATCC). The specificity of a PCR assay also depends on the assay conditions. 'Mis-priming' which may give raise to the amplification of the wrong sequences, can be decreased by the use of the hot-start technique. This is achieved by pretreatment of the Taq-polymerase, for example by using antibodies or proteins binding to Taq-polymerase which detach from the enzyme only after the DNA-melting temperature has been reached.

In many cases, the evidence of a positive or negative PCR result is not sufficient; information about the quantity of a given target DNA or RNA is also required. Quantitation by conventional PCR is essentially based on the principal of competitive PCR [and reverse-transcriptase PCR (RT-PCR) for mRNA]. A known amount of an in vitro synthesised oligonucleotide (the 'competitor'), comprising the region of the gene segment to be amplified but shorter than its wild-type counterpart, is added to each assay and co-amplified with the DNA to be quantitated. Several tubes with constant amounts of wild-type DNA but varying amounts of competitor DNA are amplified in the same run. After amplification, the products are separated by gel electrophoresis and stained by ethidium bromide. PCR products in the different samples are quantitated by densitometric analysis of a digitised image of the ethidium bromide-stained gel. By comparison of the amount of the competitor with that of the wild-type DNA, it is possible to determine the equivalent point, ie the point where the amount of the competitor equals that of the wild-type DNA (Fig 3).

Real-time PCR (TaqMan procedure)

The TaqMan method is a PCR procedure which not only allows detection but also the quantitation of a given DNA sequence. It measures PCR product accumulation through a dually labelled fluorogenic probe (TaqMan probe; TaqMan, Perkin Elmer Corp/Applied Biosystems, Foster City, CA, USA). It was first described by Holland et al (1991). The procedure is based on the ability of the *Taq* DNA polymerase to not only synthesize a DNA strand complementary to an existing single-stranded DNA but also due to its 5'-3' nuclease activity, to digest an oligonucleotide (the TaqMan probe) bound to the DNA strand (Fig 4). The TaqMan probe must be carefully selected for optimal binding to the sequence of interest; its binding site is located between the two primers. The probe is labelled at the 5' end with a fluorescing dye and at the 3' end with a 'quencher'. When the probe is intact, ie as long as the fluorescing dye and the quencher remain in close vicinity, the emission of the fluorescing dye is quenched upon excitation by ultraviolet light. After binding of the probe during PCR, the 5'-3' nuclease activity of the *Taq* DNA polymerase releases the fluorescing dye from the quencher, resulting in an increased fluorescence signal. The fluorescence intensity is directly related to the amount of input target DNA and can be detected with an automated fluorometer. The quantitation of the amount of DNA copies in an unknown sample is accomplished by comparison of its fluorescence signal with that obtained from samples with known copy numbers.

The advantages of the TaqMan technique over conventional PCR methods are the following. (1) The quantitation of the input target DNA is possible simultaneously with the PCR process itself. (2) No post-amplification steps are necessary. This means that the PCR tube in which the reaction was carried out does not need to be opened after the amplification process. This results in a virtual absence of cross-contamination due to spilling of small amounts of amplified DNA into the laboratory environment. This effect is quite impressive. Let us assume that, in a given PCR tube after amplification, 10^{11} DNA

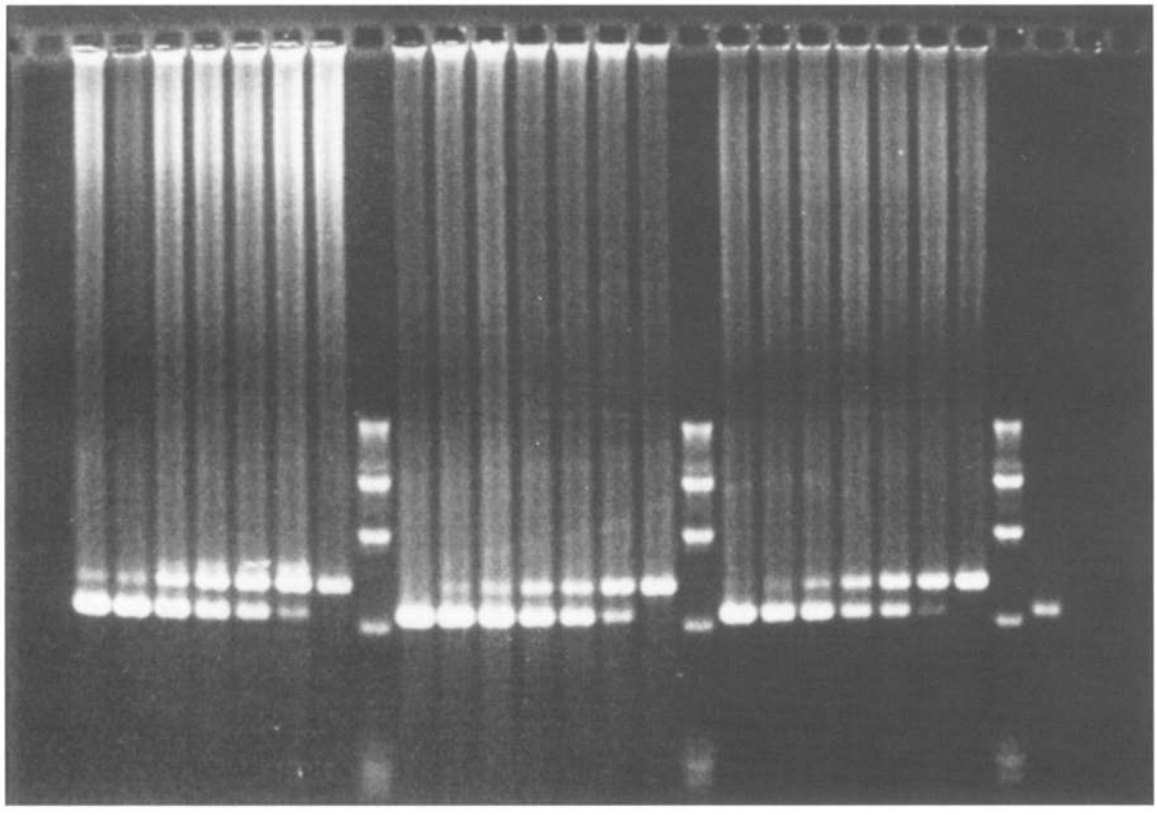

Fig 3. Competitive quantitative FIV provirus PCR. Constant amounts of host DNA are co-amplified with varying amounts of a competitor. The competitor contains the same primer binding sites as the wild-type DNA but is shorter so that it can be separated clearly on the gel. The number of copies present in the wild-type DNA can be determined by calculation of the 'equivalence point', which is defined as the concentration at which the amount of competitor equals that of the wild-type DNA. At the equivalence point, the bands of the competitor and of the wild-type DNA are of identical density. In our example, the FIV proviral load of three cats was determined. DNA of each cat was co-amplified with (from left to right) 10 000, 5000, 1000, 500, 100, 50 and 0 copies of the competitor. The lanes to the right of the panel of each cat contain marker molecules. In the far right lane, 100 molecules of competitor DNA were amplified and loaded onto the gel. From visual inspection, the equivalence points for the three cats can be estimated to be between 500 and 1000 copies (first cat from left), between 50 and 100 copies (middle cat) and between 100 and 500 copies (right cat). For details, see text and (Pistello et al 1994, Allenspach et al 1996).

copies are present in the usual 50 µl of the assay. In conventional PCR, a gel electrophoresis step has to be carried out after each PCR run. It is easy to speculate that, during pipetting of the sample, 1/1000 (0.05 µl) of the original volume is spilled into the air surrounding the PCR instrument. Although this 'spill' is small, it still contains 10^8 DNA copies; if some of these DNA molecules inadvertently contaminate a new PCR tube, a false-positive result will arise. There are procedures that will help minimise the risk of this type of contamination in conventional PCR, but the TaqMan technology avoids this risk completely. (3) The TaqMan procedure is fast and highly efficient. It has been adapted to the Microtiter system allowing the simultaneous amplification of 96 samples, therefore it can be performed fast and at relatively low cost.

PCR-based tests for the detection of feline infectious diseases

Due to its analytical sensitivity and specificity, PCR is considered to be the best method for the diagnosis of infectious diseases, especially of viral infections. Accordingly, PCR-based procedures have been described for most feline infections. These are briefly reviewed below.

Feline parvovirus

A PCR assay which allows the detection of parvovirus in faeces of dogs and cats at a high sensitivity was described by Schunck et al (1995). The sensitivity was about 10- to 100-fold more sensitive than electron microscopy, the standard method for parvovirus diagnosis. So far, no evaluation of this assay under field conditions has been published.

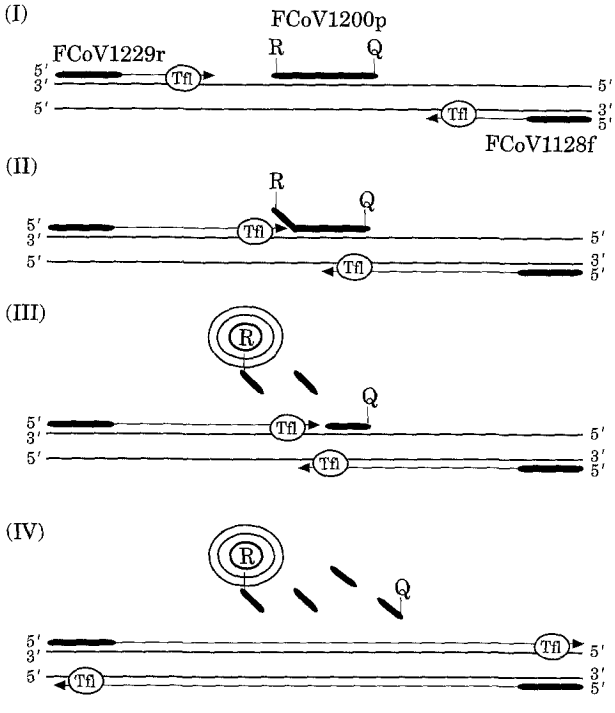

Fig 4. Principle of the TaqMan PCR. In a first step (I) the probe labelled with a fluorescing marker, R, and a quencher, Q, binds to a stretch between the two primers. As long as R is covalently bound to Q, illumination by UV does not result in fluorescence. During extension (II), the 5'-3' exonuclease activity results in hydrolysis of the probe which leads to liberation of R (III). The amount of R released during PCR can be measured directly during the entire amplification process. The amount of fluorescence that accumulates in a tube is proportional to the amount of amplified DNA. Our example shows the FCoV TaqMan principle. FCoV1229r—first primer, FCoV1128f—second primer, FCoV1200p—probe, Tfl—heat stable *Thermus flavus* polymerase. For details see text and Gut et al 1999.

Feline herpesvirus (FHV-1)

Reubel et al (1993) reported a PCR assay which allowed detection of FHV-1 in oral and ocular swabs and other materials at a high sensitivity (3×10^3 particles). They were able to detect latent FHV-1 in head tissues, especially the trigeminal ganglia, optic nerves, olfactory bulbs and corneas. The oral fauces, salivary glands, lacrimal glands, cerebellum and conjunctivae were less consistently positive. The cerebral cortex, thymus, trachea, lung, liver, spleen, kidney and peripheral blood mononuclear cells were consistently negative for the FHV-1 genome. Infectious virus was not recovered from tissue homogenates, regardless of the PCR status of the tissues. Hara et al (1996) described a nPCR which readily allowed detection of FHV-1 in ocular and nasal swabs.

Stiles et al (1997) compared the results obtained by nPCR, virus isolation (VI), and fluorescent antibody (FA) testing to detect FHV-1 in 46 cats with naturally acquired conjunctivitis or respiratory tract disease, or both. PCR was found to be around five times more frequently positive than VI or FA testing. Of 15 clinically normal cats, conjunctival and throat specimens from two cats were FHV-positive by PCR. None of the samples tested from these cats were positive by VI or FA testing. The authors concluded that in cats with respiratory tract disease and conjunctivitis, or with conjunctivitis only, nPCR was more sensitive for detecting FHV than VI or FA testing. The same authors evaluated nPCR for the detection of FHV-1 in conjunctivae (in 50 cats with conjunctivitis and 50 normal cats) and corneas (26 keratectomy samples from cats with sequestra and 13 samples from normal cats). Fifty-four per cent of the conjunctival specimens from cats with conjunctivitis and 12% of specimens from clinically normal cats were PCR-positive. The difference was statistically significant. PCR was positive in 18% of corneal specimens from cats with sequestra and 46% of the clinically normal cats. The difference was not significant. The authors concluded that cats with conjunctivitis were more likely to have a positive PCR result than clinically normal cats, making it probable that FHV-1 was associated with the disease state. They further concluded that as FHV-1 DNA could not be detected in most corneas from cats with sequestra, FHV-1 may not be an important factor for sequestration.

Nassisse et al (1998) also studied the involvement of FHV-1 in sequestra and eosinophilic keratitis using a PCR protocol. They came to somewhat different conclusions. As they detected FHV-1 DNA in 5.9% of corneas from clinically normal cats, in 55.1% of corneal sequestra and in 76.3% of scraping specimens from cats with eosinophilic keratitis, they concluded that FHV-1 may play a role in the pathogenesis of eosinophilic keratitis and corneal sequestration. In comparative evaluations of VI by cell culture and PCR, two groups also came to the conclusion that PCR is more sensitive than VI (Sykes et al 1997a, Weigler et al 1997). In these studies, where PCR was found to be more sensitive than VI, theoretically the discrepancy could be explained by false-positive results due to laboratory contamination. However, there is no reason to readily accept this as an explanation. PCR demonstrates the DNA coding for a given virus in the absence of viral replication. Successful VI requires that the virus can replicate to levels that allow detection in cell culture. Hence, the two

tests do not necessarily demonstrate the same biological state; a positive PCR can be taken as evidence of prior infection but not necessarily as proof for the presence of infectious virus.

Feline calicivirus (FCV)

Sykes et al (1998) described a RT-PCR for the detection of the capsid gene of FCV. The PCR results from conjunctival swabs were compared with those of VI by cell culture during the course of experimental infection of five vaccinated and three non-vaccinated cats. There was good correlation between the two techniques but virus detection correlated poorly with clinical signs regardless of the assay used. The authors concluded that the RT-PCR assay described was as sensitive as cell culture for detection of FCV in conjunctival swabs and that a broad range of field isolates was detected. The assay was found to be suitable for large-scale epidemiological studies of FCV infections in cats. However, it was also shown that even the use of RT-PCR on conjunctival swabs alone is insufficient for accurate diagnosis of FCV infection in cats with conjunctivitis. For the purpose of diagnosis of FCV infection, the use of oropharyngeal swabs might have been preferable.

Feline leukaemia virus (FeLV)

For detection of FeLV infection, two principle approaches can be selected. (1) FeLV requires integration of its genome into the DNA of the host cells for replication. The integrated form of FeLV, 'provirus', can be directly amplified by PCR. A considerable obstacle to direct PCR amplification of the exogenous (infectious) FeLV provirus is the multiple copies of the so-called endogenous FeLV sequences which are present in every cell of the cat's body and which share sequence homologies with exogenous FeLV. In order to detect exogenous FeLV, it is necessary to design primers which amplify sequences not present in the endogenous FeLV. (2) In principle, FeLV RNA can also be detected in the plasma by RT-PCR, although this is somewhat more complicated. PCR has been used for the detection of FeLV infection for several years. In 1993, Jackson et al published a study in which they determined the prevalence of FeLV envelope glycoprotein 70 (gp70) and FeLV gp70 DNA in formalin-fixed, paraffin-embedded tumour tissues from 70 cats with lymphosarcoma (LSA). Tissue sections were tested for gp70 antigen using avidin–biotin complex immunohistochemistry (ICH); DNA was extracted and purified from the same tissue blocks for PCR amplification of a 166-base pair region of the one end of the FeLV genome, the 'long terminal repeat' (LTR).

Among others, results were related to antemortem FeLV ELISA for serum p27 antigen. Viral DNA was detected by PCR in 80% of cases and viral antigen by IHC in 57% of cases. Seventeen cases were PCR-positive and IHC-negative; one case was PCR-negative and IHC-positive. Clinical records included FeLV ELISA results for 30 cats. All 19 ELISA-positive cats were positive on PCR and IHC; of the 11 ELISA-negative cats which were negative on IHC, seven were positive on PCR.

From these findings it can be concluded that the majority of LSA is associated with FeLV infection, that expression of the FeLV envelope is almost always paralleled by presence of FeLV provirus and that presence of p27 in the serum (by ELISA) was always associated with the presence of FeLV provirus, which is proof of infection. In another study, Jackson et al compared the results of the p27 ELISA in serum with those of FeLV PCR performed on DNA from blood leukocytes from cats with low and high suspicion of having an FeLV-related disease (Jackson et al 1996). There was a good correlation between PCR and ELISA and the authors concluded that PCR can be adapted for diagnostic purposes using peripheral blood samples.

In a recent study, Hofmann-Lehmann et al (1998) established a TaqMan method for the detection and quantitation of FeLV provirus and compared it with conventional nested PCR. The specificity of both methods was excellent. Both procedures amplified a short sequence located in the LTR; exogenous FeLV was detected only. Uninfected SPF cats (n=45) always gave negative PCR results. The sensitivity was good; as few as one to two copies of FeLV-DNA were detected readily and highly reproducibly with both PCR systems (coefficient of variation for the Ct values of the TaqMan method, within-run precision=0.9–1.0%).

Using the methods above, Gruber et al (1998) studied the prevalence and the load of FeLV provirus in 594 healthy and sick cats in Switzerland. The PCR results were evaluated with respect to FeLV p27 positivity in serum, rearing conditions and clinical findings. Without exception, all cats with p27 antigenaemia (7%) were highly positive for FeLV provirus and displayed a high proviral load. Eleven per cent of all cats were p27-negative but provirus-positive.

However, these cats had a significantly lower proviral load (*c.* 300×) than p27-positive cats. Among sick cats, there was a significantly higher proportion of (1) FeLV p27-positive and (2) provirus-positive cats (negative for p27) than among healthy animals. From these results it was concluded that (1) p27 antigenaemia is closely associated with high proviral load; (2) a significant portion of the cats studied (11%) had been in contact with FeLV without displaying antigenaemia at the time of test; (3) the p27 negative/FeLV provirus positive status characterised by low proviral load may be associated with latent FeLV-infection; and (4) FeLV provirus positivity is somehow associated with disease even at low proviral loads.

Feline immunodeficiency virus (FIV)

In 1992, Rimstad & Ueland described a nPCR assay which allowed the detection of FIV provirus. In their procedure, sequences of the genes coding for the core protein gag and polymerase were amplified in the same tube. Lymphocytes of six out of 14 seropositive cats were PCR-positive. Hohdatsu et al (1992) described a similar approach and were able to show that the percentage of PCR-positive tests in FIV-infected cats could be increased by culturing the lymphocytes before PCR was done. It was concluded that FIV-infected lymphocytes are present at very low frequency. Interestingly, in the same year, Dandekar et al (1992) reported that FIV-naive SPF cats living in an experimental setting in close contact with FIV-infected (PCR- and seropositive) cats became PCR-positive but remained sero-negative for a prolonged time. The phenomenon of FIV PCR positivity in the absence of FIV specific antibodies was later confirmed (Leutenegger et al 1998a).

Lawson et al (1993) studied the proviral load during the course of experimental FIV infection. They found that the proportion of peripheral blood mononuclear cell (PBMC) containing provirus in individual cats ranged from one in 70 to one in 99 600 PBMC. Momoi et al (1993) described the use of mixed primers specific for the so-called gag gene in the detection of the Japanese TM2 and the prototype Petaluma. Matteucci et al (1993) studied the rate of FIV infection in different material from FIV-infected cats by comparing results from isolation with those from PCR. PCR showed that FIV genomes were present in saliva and plasma more frequently than expected on the basis of virus isolation data. Saliva was also found to contain viral DNA, indicating that it may harbour virus-infected cells as well as free virus.

Feline coronavirus (FCoV)

FCoVs are RNA viruses that must be reverse-transcribed before DNA PCR is possible. The first PCR assay for FCoV was decribed by Li and Scott (1994). In 92 clinical cats, seven of the eight FIP-suspected cats (87.5%) and 51 of the 84 non-FIP-suspected cats (60.7%) were positive for FCoV in PCR. From this observation it must be concluded that PCR is not suitable for the determination of FIP. A nested RT-PCR was described by Herrewegh et al (1995) which amplified a highly conserved 3'-untranslated region of the 7b gene of FCoV. FCoV RNA was detected in faeces, serum, plasma or ascitic fluid samples from 14 of 18 cats (78%) with naturally occurring feline infectious peritonitis (FIP). However, FCoV PCR was not considered as useful in the diagnosis of FIP as many apparently healthy cats were also shown to be PCR-positive.

Using the same technique, Fehr et al (1996, 1997a) tested cats for the presence of FCoV in connection with vaccination against FCoV infection. They showed that cats could only be protected against FIP by vaccination with an attenuated FCoV vacine if they were not infected by FCoV at the time of vaccination. Gamble et al (1997) described a PCR assay which amplified a sequence of the gene coding for the E2 protein. Interestingly, the assay was positive with three FIP-derived strains and several naturally occurring FIP strains, but negative with one enteric coronavirus, one canine (CCV) strain and a porcine (TGEV) coronavirus. So far, there have been no published reports that confirm the FIP-specificity of this assay. Gunn-Moore et al (1998) described a RT-PCR assay for the detection of FCoV and a cell culture method for the isolation of field strains of FCoV. Using RT-PCR, they found around 80% of FIP cases as well as healthy cats from households with endemic FCoV to be FCoV-viremic, irrespective of their health status. They also showed that, over a 12-month period, a similar percentage of healthy cats remained viraemic. In addition, in their study the presence of viraemia did not appear to predispose the cats to FIP development. From these experiments, it can be concluded that detection of FCoV in the blood of cats cannot be considered a marker for FIP and therefore that demonstration of FCoV in the blood of cats, even if they show clinical signs

of FIP, is not diagnostic for FIP. In the same study, these authors demonstrated that there was a good correlation between the results of RT-PCR and those of VI by their cell culture system. From this it may be concluded that PCR and cell culture detect the same in cases of FCoV infection and that a positive PCR is an indicator of the presence of infectious virus.

A TaqMan assay for the detection and quantitation of FCoV was recently described by Gut et al (1999). This assay amplifies a conserved portion of the 7b gene and detects FCoV, CCV and TGEV, but not bovine and human coronaviruses. It is ideally suited for the detection of FCoV-shedding cats and the quantitation of their viral load. Using this method, we were able to show that there is a very strong correlation between viral load in faeces and the frequency of FCoV shedding (unpublished results). Therefore, this technique may be used to detect cats which are shedding high amounts of FCoV. It would be interesting to investigate whether removing of these 'high shedders' decreases the frequency of FIP in a multi-cat household situation. Whether this TaqMan assay has any use in the diagnosis of FIP is unknown but unlikely.

Bacterial infections

A PCR procedure amplifying the citrate synthase gene of *B henselae* was described which detected *B henselae*, *B quintana* and *B prowazekii* (Norman et al 1995, Chomel et al 1996). Using a PCR detecting *Helicobacter* spp, 38 of 49 (78%) clinically healthy pet cats were positive for *H heilmannii*; no *H pylori* or *H felis* were found (Neiger et al 1998). All cats showed mild gastritis. *H heilmannii* was suspected to be of zoonotic importance; it was detected in a human subject with acute gastric erosions and in his two cats (Dieterich et al 1998). A PCR method detecting a region of the major outer membrane protein gene of *Chlamydia* was described which detected *Chlamydia* of feline origin as well as several other strains (Rasmussen & Timms 1991). In another study, 13% swabs collected from cats with upper respiratory diseases were positive for Chlamydia in PCR (Sykes et al 1997). Restriction endonuclease analysis revealed that chlamydial isolates from cats in Japan, France, the USA and the UK had identical restriction patterns and therefore appeared to be very similar. PCR amplification of different chlamydial genes and subsequent analysis by endonuclease digestion and electrophoresis allowed detection and characterisation of different chlamydial isolates (Sayada et al 1995, Everett & Andersen 1997, Martin and Cross 1997, Meijer et al 1997).

PCR techniques in the research of feline diseases

Viral vaccine and pathogenesis studies, quantitation of viral load

In the context of pathogenesis and vaccine studies, quantitation of input target DNA is often required in addition to positive or negative PCR results. In this context, several methods have been published which allow quantitation of FIV as provirus or as viral RNA (Pistello et al 1994, Diehl et al 1995, Vahlenkamp et al 1995, Allenspach et al 1996, Dean et al 1996). Competitive PCR and RT-PCR have been used as tools to study the pathogenesis of FIV infection and the efficacy of FIV vaccines (Pistello et al 1994, Diehl et al 1995, Cammarota et al 1996, Leutenegger et al 1998). A TaqMan-based PCR for the quantitation of the FIV load in cats was recently developed (Leutenegger et al 1999). This procedure was shown to be fast (90 samples within 2 h), highly sensitive, reproducible and relatively inexpensive. Quantitative aspects of viral load have also been studied in FeLV and FCoV infections (Gruber et al 1998, Hofmann-Lehmann et al 1998, Gut et al 1996). In addition to quantitation, PCR methods have been essential in the study of viral pathogenesis and vaccine efficacy (Brojatsch et al 1992, Boomer et al 1994, Bishop et al 1996, Fehr et al 1997, Foley et al 1997). Determination of PCR-amplified sequences can also be used for characterisation of viruses. In a recent study, a 235-bp hypervariable region of the capsid protein gene of the feline calicivirus was used to compare viruses of three attenuated vaccines to viruses isolated from vaccinated cats with clinical signs of FCV infection (Radford et al 1997). Some isolates were found to be quite similar to the virus strains used in the vaccines and it was concluded that these isolates may have originated from the vaccine.

Cloning and sequencing of feline genes

A vast number of genes and their functions have been and are currently being studied in the cat. If the sequence of a gene of interest in the cat has already been published in other species, such as mice or humans, it is usually relatively easy to amplify the corresponding gene from feline DNA

or complementary DNA by PCR. This is done by designing primers which recognise sequences that are conserved among different species ('consensus sequences'). These primers are then used to amplify the gene of interest in the cat. The resulting PCR product can then be cloned into plasmids, expanded in bacterial cultures and finally sequenced. By sequencing overlapping segments of a gene, the whole sequence can eventually be determined. If the whole sequence is known, the gene can usually be expressed as protein in bacteria, yeast or feline cell cultures or be used as a DNA vaccine. It is not the goal of the present article to discuss all published reports that deal with PCR and cloning of feline genes; at the time of writing of this article, there were almost 300 papers that involved PCR in one way or another. A few developments may be of direct interest for veterinary clinicians and are therefore mentioned here. Many of the feline cytokines and other molecules of importance in feline medicine have now been sequenced (McGraw et al 1990, Cozzi et al 1993, Ohashi et al 1993, Gallagher et al 1995, Mayr et al 1995, Rimstad et al 1995, Dunham & Onions 1996, Pecoraro et al 1996, Dean et al 1998, Lutz & Leutenegger 1998). If sequences are known, they can readily be used for the quantitation of mRNA expression as it was the case for IL-2, IFN gamma, IL-4, IL-6, IL-10, IL-12 and IL-16 (Rottman et al 1995, Dean et al 1998, Fehr et al 1997, Leutenegger et al 1998b).

Detection and characterisation of viruses in non-domestic felids

Similarly to the strategy of the characterisation of feline genes, unknown viruses can be amplified and sequenced using PCR and consensus sequences recognising genes of the virus of interest. Using this approach, several viruses were characterised in non-domestic felids such as pumas (Langley et al 1994), lions (VandeWoude et al 1997a, 1997a,b) and the pallas cat (Barr et al 1997).

Discussion

PCR has proven to be a very useful tool in research and diagnostics. However, its use has also brought new challenges. PCR is so sensitive that how to interpret results obtained by this technique has to be carefully evaluated. Interpretation of a PCR result can be very difficult, as illustrated by the example of FIP. The diagnosis of FIP is often quite difficult, especially if the cat does not have typical clinical signs of FIP (ascites, emaciation etc.). FCoV can readily be detected in the blood of cats which are completely healthy and which will not die from FIP (Herrewegh et al 1995, Fehr et al 1997, Gunn-Moore et al 1998). If this is the case, then how do we interpret a positive FCoV PCR in a sick cat? Many laboratories offer FCoV PCR with the claim that a positive test performed using blood in a sick cat is diagnostic of FIP. In order to know the true value of the test, the positive and negative predictive values need to calculated. In the case of FIP, this would ideally be done by testing a large number of blood samples collected from healthy cats, cats with a variety of diseases but not with FIP (non-FIP) and from cats that clearly have FIP. Ideally, this evaluation is performed in the area from which most of the samples analysed by a given laboratory originate. From this analysis, the diagnostic specificity and sensitivity can be calculated. The diagnostic specificity gives the percentage of negative FCoV-PCR tests in healthy cats; the diagnostic sensitivity 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 any positive result must be associated with disease, either with non-FIP or with FIP. If this was the case, the FCoV PCR would be easy to use.

To determine the positive predictive value for FIP which corresponds to the probability that a positive FCoV-PCR originates from a cat with FIP, we have to know the prevalence of positive FCoV-PCR results in the healthy and the non-FIP sick cats of a population. Let us assume that in 100 cats (healthy and sick) living in a certain area during a given time the frequencies of FCoV-positive PCR tests performed with blood are three for healthy, three for non-FIP cats and three for FIP cases. That means that nine are positive, but only 33% of all positive tests are associated with FIP; in other words, that the probability for FIP being present in a cat with a positive test is only one-third. The true positive predictive value may be even lower, as we had assumed in our example that at any given time three out of 100 cats have FIP, which is likely to be too high. So far, the positive predictive value for FIP RT-PCR has not been carefully determined, which makes the test very difficult to interpret. It is important that these aspects are clearly answered for every new test before it is widely used in the field.

The same information is lacking for FeLV and FHV infections. FeLV genes are integrated into the DNA of infected cells. Although the presence of FeLV proviral DNA is statistically related to 'disease' (Gruber et al 1998), the presence of FeLV proviral DNA may not be of importance in an individual cat. It is well-known that FHV can cause latent infections. Again, the significance of a positive FHV PCR in a healthy cat is not really clear. Therefore, much research needs to be done before we can correctly interpret some of these assays. PCR-based diagnostics in feline medicine are only now becoming widely used and, due to the cost-effectiveness of some of the newer assays, the required knowledge for interpretation of the test results will soon become available.

Many hereditary diseases have been characterised in the domestic cat. Knowledge of the nature of the genetic changes involved will make it possible in the future for the establishment of PCR-based assays to detect such mutations in pedigree animals and will therefore help in breeding healthy animals. Occasionally, PCR-based assays could also be used in forensic veterinary medicine. Recently, a murder case was solved in which the DNA of the cat belonging to a relative of the murderer helped in the conviction (Menotti-Raymond et al 1997).

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