Review Article

APPLICATION OF THE POLYMERASE CHAIN REACTION (PCR) IN VETERINARY DIAGNOSTIC VIROLOGY

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ABSTRACT

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The polymerase chain reaction has become an important diagnostic tool for the veterinary virologist. Conventional methods for detecting viral diseases can be laborious or ineffective. In many cases PCR can provide a rapid and accurate test. In this article we explain the basic principles of PCR and supply a reference list of its uses in diagnostic veterinary virology.

Keywords: diagnosis, PCR, polymerase chain reaction, virus

Abbreviations: BLV, bovine leukaemia virus; BVDV, bovine viral diarrhoea virus; DNA, deoxyribonucleic acid; ds, double-stranded; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; RNA, ribonucleic acid; ss, single-stranded; TK, thymidine kinase

INTRODUCTION

The detection of viral infections may be based on direct or indirect diagnostic methods (Bullock *et al.*, 1991; Lennette, 1992).

By direct methods, the virus particles or some of their components, e.g. viral nucleic acids or viral proteins, are detected in the specimens. The most common conventional direct diagnostic methods are the 'gold standard' of virus isolation and also electron microscopy, the detection of viral antigens by antigen-ELISA, the complement-fixation test and the immunofluorescence, immunoperoxidase or peroxidase–immunoperoxidase staining methods. Nucleic acid hybridization is a relatively new detection method that is becoming increasingly popular (Pettersson and Hyypiä, 1985; Viscidi and Yolken, 1987; Gillespie, 1990; Paul, 1990).

Indirect methods can be used to demonstrate antiviral antibodies. Virus neutralization, antibody-ELISA and agar-gel immunodiffusion are the most commonly used indirect methods (Bullock *et al.*, 1991).

In general, a secure diagnosis can be made by the simultaneous application of direct and indirect methods. However, even when applying both approaches, the diagnosis of certain viral infections is ineffective or too cumbersome; for example, during latent herpesvirus infection, when there is little chance of detecting the virus DNA by conventional diagnostic techniques.

The development of molecular biology has contributed to the appearance of highly sensitive, new diagnostic approaches. Various DNA recombinant techniques have

been applied to the rapid detection of viral nucleic acids, used to study viral genes and also to produce new types of vaccines. DNA molecules are cut and gene structures are mapped by restriction endonuclease cleavage. Ligase enzymes are used to insert the DNA fragments into prokaryotic plasmids or bacteriophage vectors. These vectors can be propagated easily, providing a simple means of producing large quantities of the fragment of interest. The large amount and high purity of the DNA molecules produced by these methods allows further studies, for example the nucleotide sequence determination of viral genes. Knowing the sequences, parts of these genes can be artificially produced by a DNA synthesizer machine. The synthetic molecules can be used for many purposes, e.g. as a component of a new DNA recombinant or as a probe in a nucleic acid hybridization test (Gillespie, 1990).

Recently, DNA recombinant techniques have been directly applied to diagnosis and vaccine production. Synthetic oligonucleotide probes have been developed to detect a variety of infectious agents, for example, enteroviruses, rhinoviruses or mycoplasmas (Bruce et al., 1989a,b; Johansson et al., 1992). Recombinant virus vaccines have been produced to control a number of infectious diseases, for example rabies (Wiktor et al., 1988).

In the last decade various molecular biological methods have been introduced into diagnostic virology, the first of them being the nucleic acid hybridization technique mentioned above. The principle of nucleic acid hybridization is that labelled nucleic acid molecules, usually called probes, bind specifically to stretches of target nucleic acids, in this case to viral nucleic acids in the clinical samples. The nucleic acid sequence of the probe is complementary to the target sequence. Accordingly, the probe will only bind (hybridize) to the target if it contains the appropriate viral nucleic acids. In order to demonstrate the hybridization, the probes are labelled in some fashion, for example with radioactive isotopes or, recently, more often with biotin or digoxigenin (Kricka, 1992; Lennette, 1992).

The deployment of various nucleic acid hybridization techniques has considerably increased the arsenal of the diagnostic virologist. The detection of many viral infections, for example that of latent herpesvirus, has become more reliable and more rapid. Various nucleic acid hybridization methods have been demonstrated. These include dotblot, Southern blot, in situ cell and sandwich hybridization. We have recently applied many of these techniques in our laboratory (Belák et al., 1987, 1988, 1989a; Belák and Linné, 1988).

Important landmarks in the history of DNA diagnosis were the years 1985 and 1987, when the first reports appeared on the polymerase chain reaction method (Saiki et al., 1985; Mullis and Faloona, 1987). None of the basic molecular biological techniques that have been developed in the last decade has had a greater impact. Thousands of articles published since 1985 indicate that PCR has been one of the most rapidly adopted techniques in biochemistry. Reports on the application of PCR to the diagnosis of infectious diseases are also accumulating at an exponential rate (Deacon and Lah, 1989; Erlich et al., 1991; Pershing, 1991). In addition to PCR, other amplification methods have also recently been developed. These include the transcription amplification system (TAS), the ligase chain reaction (LCRTM), the self-sustained replication (3SR), the Ampliprobe system and the Q-beta replicase method (Gillespie, 1990; Gingeras et al., 1990; Birkenmeyer and Mushahwar, 1991).

The purpose of this review article is to briefly describe the PCR method and then to summarize its applications to veterinary diagnostic virology.

POLYMERASE CHAIN REACTION

The polymerase chain reaction is a method used for the *in vitro* amplification of selected target DNA molecules, resulting in a several-million-fold amplification of the target sequence within a few hours. These large amounts of PCR products can then easily be detected and identified (Saiki *et al.*, 1985; Mullis and Faloona, 1987; Erlich *et al.*, 1991).

Components of PCR

Specimens. For viral diagnosis, homogenized organs, mucosal cells collected with swabs or separated leukocytes are the most common specimens. For example, acute Aujeszky's disease (pseudorabies) of pigs is diagnosed in our laboratory by PCR amplification of nasal cells and organ homogenates. The latent infections are detected by testing homogenates of trigeminal ganglia, tonsils and brain (Belák *et al.*, 1989b). Bovine viral diarrhoea virus infection is diagnosed by PCR amplification of organ homogenates (acute disease) or serum specimens (persistent infection). From such specimens, the PCR is able to amplify a few viral nucleic acid molecules amidst a high background of host DNA and RNA.

Before running the PCR, the specimens are prepared for amplification. When seeking DNA viruses, one can purify the specimen DNA prior to amplification, but recent publications demonstrate that good amplification results can be achieved even from unpurified, crude specimens (Belák *et al.*, 1989b; Ballagi-Pordány *et al.*, 1990, 1992). In the case of RNA viruses, the viral RNA has to be purified and transcribed to complementary DNA (cDNA) before starting the PCR.

Primer molecules. The primers are artificially synthesized short (17 to 40 nucleotides long) oligonucleotide molecules that flank the sequence to be amplified (<100 to 2000 bases) and are complementary to opposite strands of the target. The primers are able to find their target in the reaction mixture and specifically anneal to it.

DNA polymerase enzyme. The initial PCR studies utilized the thermosensitive Klenow fragment of *Escherichia coli* DNA polymerase I to synthesize the DNA (Saiki *et al.*, 1985; Mullis and Faloona, 1987). Since this enzyme was inactivated after each amplification step of the PCR (see below), each cycle required the opening of the reaction tube and addition of fresh enzyme. This tedious step was eliminated by the introduction of a thermostable DNA polymerase enzyme, *Taq*DNA polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*. Since this enzyme is not inactivated by heat, it is not necessary to open the reaction tubes between the amplification cycles, which allows execution of the PCR without interruption. Thus, the use of *Taq*DNA polymerase permits automation of the PCR, in addition to decreasing the risk of contamination.

Deoxyribonucleotide triphosphates (dNTPs). The four free dNTPs (dATP, dCTP, dGTP and dTTP) are the building blocks for the new DNA strands.

Reaction buffers. Various buffers and inorganic ions aid the PCR, e.g. KCl promotes the *Taq* polymerase activity and free magnesium ions are essential for its function.

Equipment and tools required

DNA thermocycler machines, laminar flow cabinets, and electrophoresis and hybridization equipment are the main accessories of a PCR laboratory. Many laboratories use specific PCR tube holders and tube openers, as well as specific micropipette tips in order to prevent cross-contamination and carryover of specimens (Gingeras *et al.*, 1990; Belák and Ballagi-Pordány, 1993).

The steps in the PCR

The components of the PCR, described above, are mixed in reaction tubes. The mixtures are covered with mineral oil or wax to avoid evaporation and the tubes are placed into the thermoblock of a DNA thermocycler machine. The thermoblock is controlled by a microprocessor in order to increase and to decrease the temperature rapidly, in accordance with a preselected program.

The PCR is run by repeated cycles of heating and cooling. Each cycle consists of three steps: denaturating double-stranded (ds) DNA; annealing primers to the now single-stranded (ss) DNA template; and extending nascent strands using *Taq* polymerase enzyme (Figure 1). The principles of the steps are as follows:

1. *Denaturating.* The reaction mixtures are heated to approximately 90–95°C. At this temperature the ds DNA is denatured, i.e. the double-stranded molecules are separated to form single strands.

2. *Primer-annealing.* The temperature of the thermoblock is programmed to a predetermined value between 37°C and 70°C to allow specific annealing between the primers and the single strands of the target DNA. A temperature is selected that allows only specific annealing (by base pairing) between exact matches of primer and target sequences and does not allow non-specific annealing to other sequences. The optimal annealing temperature mainly depends on the length and on the guanine/cytosine (GC) content of the primers; accordingly it is empirically determined for each primer pair.

3. *DNA-synthesis (primer extension).* The temperature is increased to between 67°C and 72°C. At this temperature *Taq* polymerase extends the primers by using each single-stranded target as a template for the construction of a complete complementary strand. This results in duplication of both original DNA strands. One PCR cycle is completed within 3–5 min.

Repeating the cycles. On completing the first cycle, the thermoblock is heated up again and begins the second round of amplification. In this cycle the double strands will separate and serve yet again as templates for new DNA synthesis. Accordingly, each PCR cycle results in the duplication of the DNA target (see above). If the number of cycles is n, then the amplification results in a 2^n exponential increase in DNA. By performing 25 to 30 cycles, a 33.6-million-fold amplification of the target could theoretically by achieved within 3 h. However, due to decrease of enzyme activity and other factors, one cannot expect more than an approximately 10^6-fold amplification of the target in a single PCR assay.
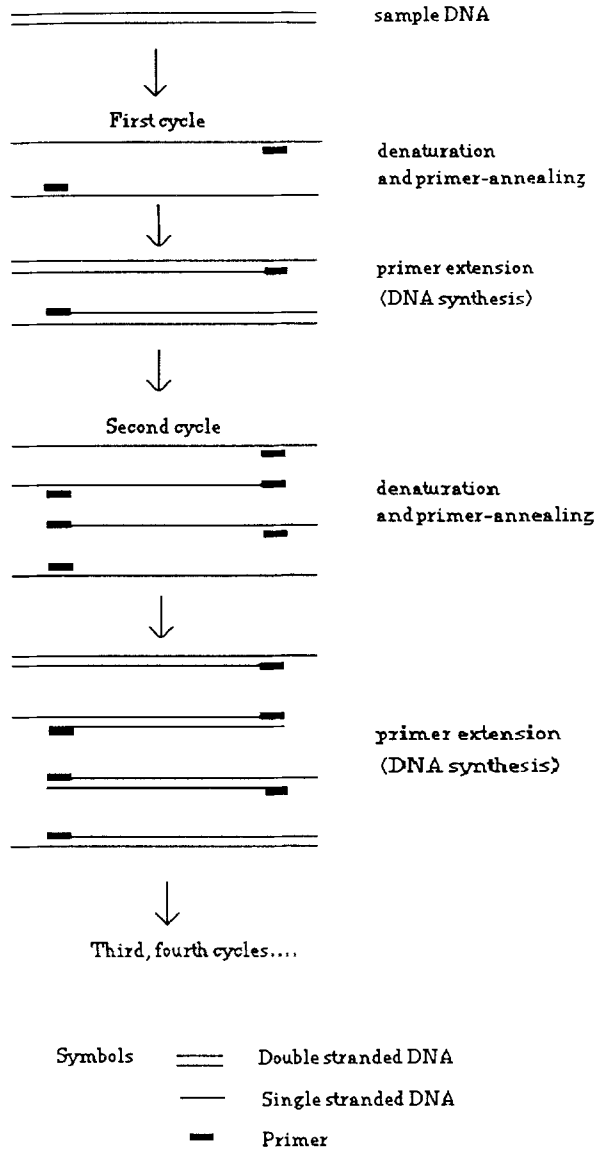
Figure 1. Cycles of the polymerase chain reaction

Double PCR with nested primers. When compared to single PCR, a further increase of the DNA yield can be achieved when double PCR is run with nested primers. In this case, a first PCR is run for some 20–25 cycles by using two external primers. Subsequently, a small amount of the first PCR product is transferred into a fresh amplification tube containing two internal primers. The second PCR is run for a further 20–35 cycles. The internal primers are closer to each other on the DNA strand and therefore produce a shorter second PCR product. Double PCR has an amplification ability of approximately 10^{11}–10^{12}, and is thus much more sensitive than the single reaction.

In order to compare the sensitivity of the two assays, both single and double PCR tests were constructed in our laboratory to diagnose various animal diseases (Belák *et al.*, 1989b; Ballagi-Pordány *et al.*, 1990, 1992; Belák and Ballagi-Pordány, 1991). To take pseudorabies virus as an example, we found that the specimens had to contain at least 10^4 virus particles/mg tissue to yield a positive result in the single PCR. In contrast, the double PCR assay detected as few as 10 to 50 genome copies of the viral DNA/mg tissue.

By using double PCR, not only the sensitivity but also the specificity of the assay is increased. The reason for this is that, in double PCR, all four primers have to find specific nucleic acid regions, instead of just the two.

Visualization of the PCR products. A frequently mentioned statement regarding the amplification rate of PCR is that 'the PCR finds a needle in a haystack and then produces a stack of needles' (DeMarchi, 1990). This principle indicates that, by running a PCR, a large number of copies are produced from a minimal amount of target DNA. The large quantities of the PCR products (so-called amplicons) can easily be identified by various methods.

(1)   The characteristic size of the product can be determined by electrophoresis and ethidium bromide (EtBr) staining.
(2)   The nucleotide sequence of the product can be identified by nucleic acid hybridization (gene probes).
(3)   The product can be identified by simple colorimetric methods, e.g. the DIANA (detect immobilized amplified nucleic acids; Wahlberg *et al.*, 1990) or the CODAF (colorimetric detection of amplicons on filter; Belák and Ballagi-Pordány, 1993) procedures.

The PCR products are rapidly identified by these methods, since the results can be read within an hour (electrophoresis, CODAF) or at most several hours (hybridization, DIANA). However, when the methods are compared, nucleic acid hybridization appears to be the most specific technique available for the identification of PCR amplicons (Belák and Ballagi-Pordány, 1993).

PCR AS A DIAGNOSTIC METHOD

If the diagnostic applicability of the PCR is compared to that of nucleic acid hybridization, one important difference is immediately evident. The nucleic acid hybridization test at best detects only that amount of viral nucleic acids that accumulated in the examined specimens during the viral infection. In certain infections the amount of such nucleic acids may be very low and, since the hybridization technique does not amplify its target, it frequently fails to detect these cases. In contrast, the PCR method amplifies its target and therefore detects even very low numbers of viral nucleic acid molecules in the specimens.

Owing to this unique ability, the PCR has been accepted as a common method in medical diagnostic work. This is quite a remarkable achievement for a technique first reported in 1985.

In human medicine the PCR has been applied to the diagnosis of many viral

diseases, including herpesviral, papillomaviral and retroviral infections (Eisenstein, 1990; Gingeras *et al.*, 1990; Coutlée *et al.*, 1991).

Practical questions concerning routine diagnostic application of the PCR

The system of patenting and the costs of the test are the most common questions concerning the application of the PCR for routine diagnosis. The aim of this article is not to discuss these in detail, but the following is a brief summary of the present situation. The Perkin-Elmer Cetus company, the original owner of the PCR patent, sold the rights to F. Hoffmann-La Roche Ltd. Subsequently, the new owner expanded the patent rights to Europe. However, customers purchasing commercial PCR diagnostic kits do not have to deal with patent questions, since these are arranged between the owner of the patent and the producer of the commercial kits.

There is a general opinion around the world that the costs of the PCR are extremely high. However, owing to the introduction of inexpensive preparations of *Taq* polymerase enzyme, these costs have markedly decreased in the last years. In our laboratory the PCR is run as a routine diagnostic procedure to detect 12 viral diseases of domestic animals and we have run several thousand PCR procedures in the last three years. Our experience is that the costs of PCR diagnosis are lower than those of conventional virus isolation.

PCR IN VETERINARY VIROLOGY

The first reports of the use of the PCR in veterinary diagnostic practice appeared in the late 1980s (for example Belák *et al.*, 1989b; Deacon and Lah, 1989; Gould *et al.*, 1989; Nunberg *et al.*, 1989). PCR diagnosis of many animal viral diseases followed rapidly. The technique seemed to be especially applicable where traditional diagnostic methods required long and complex culturing procedures or intricate processing, for example in co-cultivation or in electron microscopy (Deacon and Lah, 1989). In the following section we consider several diseases as examples to indicate the importance of the PCR as a new and powerful diagnostic method in veterinary virology.

Examples of the application of the PCR in diagnosis in veterinary virology

Aujeszky's disease (pseudorabies) virus of pigs

The pseudorabies virus may establish latent infection in swine following a primary acute infection. The latently infected animals are symptomless and do not shed virus. However, virus can periodically be reactivated from these carriers, spreading to susceptible animals and causing new outbreaks. Thus, the presence of latently infected pigs may hinder the control of the disease and the completion of eradication programmes.

The acute form of the disease is easily diagnosed by conventional virus isolation. However, there are no practical and sensitive conventional methods for detecting the latent cases. After the acute infection disappears and latency has been established,

infectious virus is difficult to detect by standard techniques. Viral DNA molecules persist in several organs of the pigs, including the trigeminal ganglia and tonsils. To detect latency, one can try to apply explant cultures, co-cultivation or nucleic acid hybridization techniques. Co-cultivation is rather cumbersome and slow, and its sensitivity is uncertain. Nucleic acid hybridization is more rapid but, as mentioned above, this method may also face problems of low sensitivity. In latent cases the number of ganglia and tonsil cells that harbour viral DNA may be very low ($\leqslant 1\%$) and these cells frequently contain such a low copy number of viral DNA molecules that it remains below the detection level of the nucleic acid hybridization tests.

Owing to its capacity to amplify the target, the PCR proved to be a practical method for detecting latent cases of Aujeszky's disease. Diagnostic PCR assays have been developed, in Sweden (Belák et al., 1989b), in France (Jestin et al., 1990), in the USA (Maes et al., 1990; Lokensgard et al., 1991; Wheeler and Osorio, 1991; Dangler et al., 1992; Volz et al., 1992), and in Taiwan (Chang, 1991). These assays selected in the first instance the gII, the gp50, the gX or the TK (thymidine kinase) gene regions of the viral DNA as amplification targets.

Several groups are working on the simultaneous application of two PCR assays, which will amplify essential (e.g. gII) and non-essential (e.g. gI or TK) regions of the viral genome. In countries that use deletion mutants as live vaccines (such as gI- or TK− mutants), genetic recombinations between field strains and vaccine strains may cause confusion in the diagnosis of Aujeszky's disease. The circulation of these recombinant viruses potentially hinders eradication efforts. PCR assays have been developed to define the genotypic status of virus isolates with regard to the presence or absence of deletions. These PCR assays will determine whether normal pseudorabies virus vaccine usage can lead to the development of such genotypic recombinants (Dangler et al., 1992).

A further practical application of PCR will be to determine the infectious status of the so-called single reactor pigs. During eradication programmes the serological surveys occasionally detect a single positive pig in a herd. This animal is referred to as single reactor. The seropositive status of single reactors may be due to previous vaccination against Aujeszky's disease, exposure to a field strain of the virus, or possibly to a false positive reaction in the serological assay (Annelli et al., 1991). A common assumption is that the single reactors are false positives, but successful isolation of the virus from immunosuppressed single reactors indicated that in certain cases this assumption is incorrect and that some of the single reactors are seropositive due to a latent PRV infection. To assess the infection status of single reactors by classical means requires the cumbersome and expensive method of immuno-suppression followed by virus isolation attempts. We have found that latent PRV infection in single reactors can be safely detected by the PCR method. Moreover, an accurate diagnosis can be obtained within 24 h.

Bovine leukaemia virus

Bovine leukaemia provides another example of the application of the PCR to veterinary diagnostic virology. Bovine leukaemia virus (BLV) is the causative agent of enzootic bovine leukosis. At present, indirect (serological) detection methods (most commonly agar-gel immunodiffusion and various ELISA methods) are used for the

diagnosis of BLV infection. These methods are easy to perform and they are excellent tools for estimating the prevalence of BLV infection in a herd. However, in individual cases serology may yield false negative results (Rogers *et al.*, 1988). The serological tests may also fail to detect antibodies in early stages of infection (Florent, 1988) or in animals persistently infected with bovine viral diarrhoea virus (BVDV; Roberts *et al.*, 1988). Furthermore, these tests are of no value for diagnosis of BLV in young calves born from infected cows. By suckling colostrum, these calves receive antibodies from their dams and the serological tests cannot differentiate this passive immunity from the active immunity which develops after infection. Serology should therefore be reconsidered as the exclusive method for assessing BLV infection.

As a complement to serological techniques, direct diagnostic methods should be applied to obtain a reliable BLV diagnosis, but the conventional direct detection techniques, e.g. *in vitro* cultivation of lymphocytes with or without stimulation, electron microscopy and inoculation of sheep, are expensive, laborious and/or require special facilities. The time required for diagnosis may extend to weeks or even to several months.

PCR assays have been developed to detect BLV in clinical specimens in Australia (Naif *et al.*, 1990, 1992; Brandon *et al.*, 1991), in the USA (Murtaugh *et al.*, 1991; Sherman *et al.*, 1992) and in Sweden (Ballagi-Pordány *et al.*, 1992). The majority of the BLV PCR systems are based on the demonstration of the proviral DNA in blood leukocytes. Some of these methods can provide a safe diagnosis within 24 h (Ballagi-Pordány *et al.*, 1992).

Bovine viral diarrhoea virus (BVDV)

BVDV can also be regarded as an important candidate for PCR diagnosis. This virus is one of the most important pathogens of cattle, causing economic losses of considerable importance throughout the world. Propagation of BVDV in cell cultures allows the differentiation of cytopathic and non-cytopathic biotypes (cp-BVDV and noncp-BVDV). Both biotypes are pathogenic for cattle, causing two disease entities. Acute bovine diarrhoea is characterized by high morbidity and low mortality, whereas the acute or chronic mucosal disease is manifested by low morbidity and high mortality. Both entities have suggestive clinical signs and lesions.

The virus is frequently excreted by persistently infected animals. These are the offspring of cows in which, after a primary infection, transplacental spread occurred during pregnancy. The *in utero* infection may result in abortion or stillbirth, in malformation of the fetus or in immunotolerance. The calves, born with immuno-tolerance, carry a persistent, latent infection throughout their life. These animals are virus carriers and excretors but are usually seronegative. After a recurrent infection they may develop mucosal disease.

Owing to *in utero* infection, BVDV is a frequent contaminant of fetal calf serum used in cell culture work. This may lead to BVDV contamination of biological products, such as vaccines and pharmaceuticals.

There is a great need for sensitive and rapid methods for detecting BVDV in various specimens. Conventional diagnosis is based on the direct demonstration of the virus in clinical specimens (virus isolation, immunohistochemistry) or on indirect detection of infection by assessment of the specific antibody response. These methods

are either insensitive, time-consuming or unsuitable for large-scale screening. For example, the conventional virus isolation method encounters difficulties in maintaining cell cultures free of BVDV, it requires a long time and high costs to detect the virus. The non-cytopathic BVDV variants may require two or three passages before detectable amounts of viral antigens accumulate in the cell cultures. Another problem of virus isolation is that it frequently fails to detect the virus in toxic specimens, such as semen. In such cases the cells are destroyed before propagation of the virus occurs.

PCR detection of the virus proved to be a specific, rapid and highly sensitive method of diagnosis and an improvement over the existing technology (Schroeder and Balassu-Chan, 1990; Belák and Ballagi-Pordány, 1991; Boye *et al.*, 1991; Brock, 1991; Lopez *et al.*, 1991; Hertig *et al.*, 1991; Roehe and Woodward, 1991; Ward and Misra, 1991; Hooft van Iddekinge *et al.*, 1992).

Review of viral diseases of animals diagnosed by PCR

The articles available today on the application of PCR in veterinary diagnostic virology are listed in Tables I and II. However, with a large subject such as PCR diagnosis some authors providing significant contributions in this field may unfortunately and inadvertently have been omitted. Articles in which PCR is used to achieve other scientific aims, such as genetic or gene expression studies and the characterization of DNA regions, are not included. Articles concerned with PCR in relation to the diseases of laboratory animals are also excluded, but this group will be discussed in a later paper.

The lists presented are intended to contribute to the introduction of PCR assays in routine diagnostic veterinary virology laboratories.

Practical application of PCR in routine diagnosis

It should be noted that, despite the enthusiasm surrounding this technique, PCR is routinely performed as a clinical service in only a few laboratories. The 'Achilles heel' of this technique is its extreme sensitivity, which can result in nonspecific positive results. The reason for this is that instruments, air or solutions may carry over minute amounts of amplified DNA fragments, which can serve as PCR templates. These forms of contamination will lead to the generation of false positives and confusing results that may hamper the introduction of this new technique into routine diagnostics. The risk of cross-contamination is extremely high when a very sensitive double PCR with nested primers is undertaken.

Double PCR assays have been established in our laboratory for the detection of several important animal viruses, such as pseudorabies virus, equine herpesviruses (EHV-1 and 4), bovine leukaemia virus, bovine viral diarrhoea virus, equine arteritis virus and feline coronaviruses. During the developmental stage of these PCR assays, many technical problems, such as PCR product carryovers and sample cross-contaminations, hindered the introduction of PCR as a routine diagnostic method. By constructing special tube-holders and tube openers and also by applying a simple technique of pipetting, such false positive results were eliminated. Simple methods

have been applied to visualize and identify the PCR products. The details of procedures designed to eliminate carryovers and simple visualization procedures are described by Belák and Ballagi-Pordány (1993).

TABLE I

Detection of DNA viruses with the polymerase chain reaction

Virus	Gene region[a]	Reference
Porcine parvo	VP2	Molitor *et al.* (1991)
Bovine papilloma type 1	Nt 3759–4002	Von Teifke and Weiss (1991)
Bovine papilloma type 2	Nt 3760–4006	Von Teifke and Weiss (1991)
Bovine polyoma	Nt 436–721	Schuurman *et al.* (1991)
	Nt 2744–3130	Schuurman *et al.* (1991)
Avian polyoma	VPI	Phalen *et al.* (1991)
Bovid herpes type 4	EcoRI L	Naeem *et al.* (1991)
Alcelaphine herpes type 1	Nt 1549–2576	Hsu *et al.* (1990)
	ND	Katz *et al.* (1991)
Pseudorabies (Aujeszky's) disease	gII	Belák *et al.* (1989b)
	gII; gX	Maes *et al.* (1990)
	gII; IE180	Lokensgard *et al.* (1991)
	gII	Volz *et al.* (1992)
	gp50	Jestin *et al.* (1990)
	gp50	Wheeler and Osorio (1991)
	gp50	Osorio and Rock (1992)
	BamHI 15; gX, IE	Chang (1991)
	TK	Dangler *et al.* (1992)
	gX	Scherba *et al.* (1992)
Equine herpes type 1 (and 4)	gp13	Ballagi-Pordány *et al.* (1990)
	gp13	Von Hardt *et al.* (1992)
	gp13 (gC); gH	Sharma *et al.* (1992)
	gB	O'Keefe *et al.* (1991)
	gB	Welch *et al.* (1992)
	env	Khun *et al.* (1992)
Feline herpes	TK	Nunberg *et al.* (1989)
Infectious laryngotracheitis	BHI 1.4	Shirley *et al.* (1990)
	gB	Poulsen *et al.* (1991)
Channel catfish	Eco RI A	Boyle and Blackwell (1991)
African swine fever	Ca. Nt % 52–56	Steiger *et al.* (1992)[b]
Duck hepatitis	Nt 2594–3000	Qiao *et al.* (1990)
Chicken anemia	*caps*	Todd *et al.* (1992)

Abbreviations: BHI 1.4, BamHI "1.4 kb" fragment; *caps*, capsid protein; EcoRI A or L, EcoRI cleavage, "A" or "L" fragment; g and gp, glycoprotein; IE, "immediate early" gene; Mu, genetic map unit; ND, not determined; Nt, nucleotide number; TK, thymidine kinase; VP, virus protein

[a]Several authors cleaved the DNA, selected a fragment, sequenced it and selected primers without determining the exact position of the selected sequence in the virus genome or its function in the virus replication (see Shirley *et al.*, 1990; Naeem *et al.*, 1991). Other authors provide more accurate information by determining the location of the selected primers in the nucleotide number of the virus genome (see Hsu *et al.*, 1990). The third group of authors reports on the function of the gene regions used for primer selection; e.g. Molitor *et al.* (1991) selected primers from the gene region coding for the protein 2 of porcine parvovirus. The gene regions are referred to here using the original nomenclature of the various authors; no attempt was made to apply a unified nomenclature

[b]These authors refer to genetic map units in percentages, taking the entire virus genome as 100%

TABLE II
Detection of RNA viruses with the polymerase chain reaction

Virus	Gene region[a]	Reference
Foot and mouth disease	1A; P2A	Hofner et al. (1990)
	RNA pol	Meyer et al. (1991)
	RNA pol	Rodriguez et al. (1992)
	RNA pol, VP1	Laor et al. (1992)
Rabbit haemorrhagic disease	Nt 57–74; 645–628	Meyers et al. (1991)
Bluetongue	VP7	Gould et al. (1989)
	RNA segment no. 6	Dangler et al. (1990)
	RNA segments nos 1–3, 5–8, 10	Wade-Evans et al. (1990)
	RNA segment no. 3	McColl and Gould (1991)
Bovine rota	gene no. 8	Xu et al. (1990)
Bovine rota	gene nos. 3, 11	Eiden et al. (1991)
Infectious bursal disease	Ca. Mu 570–620	Davis and Boyle (1990)
	Nt 1730–1879	Wu et al. (1992)
Infectious pancreatic necrosis	Nt 1839–2324	Rimstad et al. (1990)
Bovine viral diarrhoea	Nt 386–861;5001–5600;9001–9300	Schroeder & Balassu-Chan(1990)
	gp48	Belák and Ballagi-Pordány (1991)
	Nt 6322–7475	Brock (1991)
	Nt 9893–10098	Lopez et al. (1991)
	gp53	Roehe and Woodward (1991)
	gp53, p80	Hertig et al. (1991)
	p80	Ward and Misra (1991)
	p80	Hooft van Iddekinge et al. (1992)
	Nt 98–3490	Boye et al. (1991)
	Nt 231–248; 12 434–12 451	Brock et al. (1992)
	Nt 4546–4564; 7545–7564	Qi et al. (1992)
Hog cholera	gp55	Roehe and Woodward (1991)
	Nt 1189–1488	Liu et al. (1991)
	Nt 98–3490	Boye et al. (1991)
Equine arteritis	LS; pol, N	Chirnside and Spaan (1990)
Duck influenza	HA	Wang and Webster (1990)
Newcastle disease	F[b]	Jestin and Jestin (1991)
Phocine distemper	F[b], P	Haas et al. (1990, 1991)
Rabies	121–140 bAG; 428–450 b AM	Ermine et al. (1990)
	NPC, PG	Sacramento et al. (1991)
	N	Shankar et al. (1991)
Bovine corona	N	Verbeek and Tijssen (1990)
Porcine respiratory corona	1b, S, 3a, b, 4, M, N, 7	Britton et al. (1991)
Infectious bronchitis	M, N	Andreasen et al. (1991)
	S2	Lin et al. (1991)
	N	Zwaagstra et al. (1992)
	Me, N	Jackwood et al. (1992)
Bovine leukaemia	env-gp51	Naif et al. (1990, 1992)
	env-gp51	Murtaugh et al. (1991)
	env-gp51	Ballagi-Pordány et al. (1992)
	Nt 5099–5542	Brandon et al. (1991)
	gag-p24	Murtaugh et al. (1991)
	pol	Murtaugh et al. (1991)
	pol, pX	Sherman et al. (1992)
Feline leukaemia	U3	Schrenzel et al. (1990)
Avian leukosis	gp85	van Woensel et al. (1992)
Bovine immunodeficiency disease	pol	Kashanchi et al. (1991)
Feline immunodeficiency disease	gag	Hohdatsu et al. (1992)
	gag/pol	Rimstad and Ueland (1992)

TABLE II (cont)

Virus	Gene region[a]	Reference
Maedi/visna	Nt 180–1370	Haase *et al.* (1990)
	gag	Zanoni *et al.* (1990, 1991)
	gag	Staskus *et al.* (1991)
Caprine arthritis–encephalitis	*gag/pol*	Zanoni *et al.* (1990)
Equine infectious anaemia	*gag*	Whetter *et al.* (1990)
	Nt 6626–6856	O'Rourke *et al.* (1991)
	LTR	Carpenter *et al.* (1991)
	LTR, *gag, pol*	Kim and Casey (1992)

Abbreviations: AG, antigenomic; AM, antimessenger; *caps*, capsid; 3D, RNA replicase; *env*, envelope; F protein, fusion protein; *gag*, group-specific antigen; gp, glycoprotein; HA, haemagglutinin; LS, leader sequence; LTR, long terminal repeat; M, matrix; Me, membrane glycoprotein; N, nucleocapsid; NPC, nucleoprotein cistron; Nt, nucleotide number; p, protein; PG, pseudogene; *pol*, polymerase; *prot*, protease; S2, spike protein no. 2; VP, virus protein
[a]As in Table I, several authors determine the nucleotide numbers while others indicate the function of the selected regions. The gene regions are referred to here using the original nomenclature of the various authors, no attempt was made to apply a unified nomenclature
[b]Primers were selected from the F region of the rinderpest virus genome

PCR kits for routine diagnosis

The routine diagnostic application of PCR is facilitated by the availability of diagnostic PCR kits. Thus, kits are available from SVANOVA Biotech (Uppsala, Sweden) for the PCR diagnosis of Aujeszky's disease of pigs (pseudorabies), diseases of horses caused by equine herpesvirus types 1 and 4, bovine leukaemia and bovine viral diarrhoea/mucosal disease. No doubt, other companies will soon be providing similar complete PCR diagnostic systems.

CONCLUSION

We believe that the methods and tools developed in the last few years will enhance the acceptance of the PCR technique as a reliable complement to conventional diagnostic methods in the routine diagnostic laboratories.

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