Nucleic acid hybridisation and polymerase chain reaction in the diagnosis of infectious animal diseases

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Summary: The authors describe and summarise the use of nucleic acid hybridisation and polymerase chain reaction (PCR) technologies in the diagnosis of animal diseases.

PCR is a powerful biological tool which enables exponential enzymatic amplification in vitro of a given deoxyribonucleic acid sequence. This technique is currently used to study the molecular pathogenesis of many infectious diseases and also for diagnosis. PCR is usually more sensitive than conventional methods and does not require complex facilities, and will therefore soon become the preferred technology of laboratory diagnosticians, field veterinarians and health officers.

KEYWORDS: Animal health – Diagnosis – Polymerase chain reaction – Probes.

INTRODUCTION

In the past, the diagnosis of infectious animal diseases has been hampered by the complexity of the technology, the facilities required and the amount of time needed for detection and characterisation of most pathogens. With the advent of probe technology, new methods are available to field veterinarians, diagnosticians and animal health officers. These new methods offer specificity and sensitivity equal to those of conventional pathogen detection procedures, while possessing the advantage that they are often able to produce results in a single day, thus providing the opportunity of taking more effective control measures.

The presence of infectious agents in fluid or tissue samples can be revealed by microscopic visualisation, or by detection of biological activity or structural components (antigenic proteins and nucleic acids). The nucleotide sequence of the nucleic acid of each pathogen is different to that of other micro-organisms and susceptible hosts. This uniqueness forms the basis of two recently-developed diagnostic tools: nucleic acid hybridisation and polymerase chain reaction (PCR). The characteristics and application of these techniques in the diagnosis of infectious diseases of domestic animals are considered below. Excellent earlier analyses of the technology should be consulted in order to obtain a broader view (15, 20, 24, 27, 52, 56, 57, 61, 67).

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TECHNICAL CONSIDERATIONS

Nucleic acid detection relies on the ability of complementary single-stranded (ss) chains to react with each other. If one of these chains (probe) is labelled in some way, it becomes possible to unveil the presence of the other chain in a hybridisation test. Alternatively, using two short probes (primers) and adequate reaction conditions, minute quantities of a given pathogen can be detected through PCR (Fig. 1).

**Hybridisation**
- Dot blot
- *In situ*
- Radioactive labelling
- Non-radioactive labelling
- Biotin
- Digoxigenin
- Sulfonylation

**Amplification**
- Polymerase chain reaction
- Results can be read in agarose gels or by hybridisation

**FIG. 1**
The dominant nucleic acid detection techniques currently available for the detection of infectious agents

**Extraction of nucleic acids**

The initial step in the detection of nucleic acid is extraction of the acid from the sample. Usually, ground tissue or fluid samples are incubated with detergents and digested with proteinase to release the nucleic acids which are separated from proteins, lipids and cell debris by a phenol extraction. The nucleic acid is then recovered by an ethanol precipitation and bound to a solid support such as a nylon or nitrocellulose membrane for hybridisation; alternatively, the nucleic acid is used directly for PCR.

Meticulous care should be taken when dealing with ribonucleic acid (RNA) targets, to avoid degradation by ribonucleases (RNases). All glassware, water and materials which are to be in contact with the sample should be treated. Autoclaved disposable plasticware should be used, and disposable gloves should be worn at all times. Samples should be processed as quickly as possible and at low temperature. RNase inhibitors such as vanadyl ribocomplexes and human placental ribonuclease inhibitor (RNAsin) can help to avoid degradation (for greater detail, see 64).

In some instances, traditional extraction procedures have been successfully replaced by more straightforward protocols. Examples of such protocols are the boiling of serum for hepatitis B virus detection (F.E. Baralle, personal communication) and the purification of cytomegalovirus (CMV) deoxyribonucleic acid (DNA) from urine specimens by binding to glass powder (11).

**Hybridisation**

Classically, probes are produced by inserting sequences of the gene of interest into the DNA of plasmid or bacteriophage vectors which can be propagated to obtain high DNA yields (2). From the “library” of genomic sequences obtained, clones are selected which suit the specific needs of a given test (specificity, sensitivity, ability to detect a
broad spectrum of field strains). For diagnostic purposes, the purity of any nucleic acid to be used as a probe is of prime importance, in order to avoid background signals which would complicate the interpretation of results. Background noise due to non-specific hybridisation to host or contaminant sequences is reduced by the excision of the cloned fragment.

More recent approaches for probe production are the use of synthetic oligonucleotides, RNA probes and PCR products. The design of oligonucleotide probes is based on available sequence information. Base-pair mismatches might occur when such probes are used for the detection of field isolates, leading to reduced interaction between probe and target. Several reports indicate that when highly-conserved regions of the genome are selected and hybridisation conditions are optimised, oligonucleotide probes can be useful for viral detection (9, 10, 30). RNA probes, which are more difficult to handle because of the widespread presence of RNases, can be synthesised in vitro in large amounts using specially-constructed recombinant plasmids such as those containing the SP6 promoter. RNA probes are single-stranded (ss) and thus there is no competing reassociation during hybridisation; they are therefore more sensitive than double-stranded (ds) DNA probes (1). If the PCR has been optimised for a particular pathogen, probes for detection of the pathogen can be prepared very easily using labelled primers or deoxyribonucleotides linked to a biotin or digoxigenin residue.

Probes can be labelled with isotopes or non-radioactive markers such as biotin or digoxigenin. Labelling involves replacement of a proportion of nucleotides in the probe with labelled nucleotides (nick translation and random priming) or end labelling. The nucleic acid can also be chemically modified (sulfonylated) in such a way that it becomes detectable by specific antibodies. Although $^{32}$P has been the label of choice when sensitivity is of prime importance, many reports (and the experience of the present authors) indicate that $^{32}$P can be replaced, with important operative advantages, by non-radioactive markers which do not constitute a health hazard to operators and have a long shelf life.

Biotin has a high affinity for avidin and, when coupled to peroxidase or alkaline phosphatase, serves to detect hybrids through a colorimetric reaction. Similarly, the hapten digoxigenin is used to label probes which are detectable by means of specific antibodies conjugated with an enzyme. Fluorochromes conjugated with biotin or antibodies have also been used to label probes for in situ hybridisation, allowing the location of target sequences in tissue sections and cell monolayers.

Hybridisation is carried out by incubating the labelled probe, under appropriate conditions, with the membrane in which the nucleic acids extracted from the sample have been spotted. After several washes to remove unbound probe, the membrane is exposed to X-ray film (if the probe is labelled with an isotope) or processed for colorimetric visualisation of results if non-radioactive radiation is used (27).

**Polymerase chain reaction**

This technique, first described by Saiki and colleagues (63), enables the amplification of a specific DNA sequence a million-fold or more. If the target is an RNA sequence, a copy DNA has to be synthesised by reverse transcription before the PCR is carried out. The reaction requires a cyclic, three-step process:

- **a)** denaturation of DNA
- **b)** annealing of primers
- **c)** primer extension.
Denaturation by heating at 93-95°C separates the two DNA strands. In the annealing process, primers attach to complementary regions on either the left (5') or the right (3') side of the sequence of interest. During primer extension, new strands of DNA are produced by the addition of nucleotides (starting at the 3' end of the annealed primer), to the unpaired DNA strand. This process takes place by the catalytic effect of a thermostable DNA polymerase. The number of DNA strands doubles upon completion of each cycle, and after 30-45 cycles millions of copies of the target sequence (known as amplicons) are present in the test tube (Fig. 2).

![Diagram of PCR process](image)

**Fig. 2**

**The polymerase chain reaction process**

The deoxyribonucleic acid (DNA) strands of the pathogen nucleic acid (a) are separated (denatured) at 94°C, and the primers hybridise (anneal) to the complementary sequences at 37-60°C (b). In a third step (extension), a thermostable DNA polymerase produces complementary strands at 72°C (c). If this three-step process is repeated 30-40 times, an exponential accumulation of DNA copies occurs (d) and the original target sequence is amplified millions of times.

**Selection of primers**

Primers are synthetic ss oligonucleotides, the sequence of which is complementary either to the left side of one strand of the target sequence (upstream primer) or to the right side of the other strand (downstream primer). In order to select primers for detection of a particular pathogen, sequence data relating to the genome of the primers must be available. Usually, the most suitable genomic portions for PCR tests intended for detection of field strains are those which are conserved among isolates, although some primer/template mismatches do not necessarily prevent amplification.
After a genomic portion is chosen, a search is conducted for pairs of 18-24 mer oligonucleotides with several characteristics:

- 40-60% G+C content
- absence of polypurines or polypyrimidines
- absence of secondary structure
- absence of complementarity, particularly at the 3' end, which may promote the formation of an artifact called "primer dimer".

Computer programmes, available upon request, can help to perform this task (62). Since the efficiency of the PCR is lower when long genomic fragments are amplified, the selected primers should be less than 600 bases pairs (bp) apart.

### Optimisation of PCR

The basic components of a PCR reaction are the primers, deoxynucleotides (dNTPs), a buffer containing magnesium chloride, a thermostable DNA polymerase and target DNA. The concentration of reaction components and time/temperature parameters must be adjusted for efficient amplification of specific targets. A suitable starting point would be 200 µM of each dNTP, 0.2 µM of each primer, a Cl$_2$Mg concentration of 1.5 mM, 2.5 units of enzyme and 10,000 copies of semi-purified target DNA. The annealing temperature is initially defined by the characteristics of the primers, while denaturation and extension are usually performed at 94°C and 72°C, respectively. Thirty-five cycles with 90 sec for each step usually allow the visualisation of a reaction product in agarose gels. The optimal concentration of Cl$_2$Mg, in the range of 1-6 mM, should be subsequently determined. If smears are detected in the gel, in addition to the expected fragment size, it might be necessary to raise the annealing temperature, optimise the primer concentration or use other primers. Similar tests should then be conducted with fewer target molecules and using artificial samples. In this process, positive and negative controls should be included in each experiment. Finally, to determine the specificity and sensitivity of the test, a significant number of field and control samples should be examined and the results correlated with those obtained using conventional techniques.

### Amplicon detection

The PCR product (amplicon) is usually visualised as a distinct band in agarose gels stained with ethidium bromide. The size of the amplicon, compared with molecular weight standards, corresponds to the length of the target sequence defined by the primers. The identity of the amplified fragment is confirmed by hybridisation with an oligonucleotide probe complementary to an internal area of the amplicon and the presence of restriction enzyme sites. The hybridisation is performed using a PCR product spotted on membranes (dot blot), or after transfer of the nucleic acids present in the agarose gel to a solid support (Southern blot). Restriction enzymes cut the amplicons into fragments of defined size which are visible in agarose gels.

These procedures are relatively complex and are excessively time-consuming for the clinical laboratory. An interesting alternative which might allow the processing of many samples simultaneously, and even automation, is the specific capture of amplified material onto solid phases, followed by colorimetric assay. This approach uses 96-well microtitre plates to which a "capture probe" is covalently attached (55). Amplicons
generated using biotin-labelled primers are trapped, and the presence of hybrids detected by a streptavidin-peroxidase conjugate and a chromogenic substrate (35).

**Laboratory procedure**

False-positive reactions can arise due to contamination of a sample being examined by PCR products of previous tests. Since millions of copies of target DNA are produced in each positive reaction and only a few are needed to produce a false positive, precautions should be taken to avoid this "carryover". The incorporation of positive, negative and "no DNA" controls in each experiment is essential to detect this phenomenon. Good general laboratory practice should be followed to avoid this, along with observance of the following guidelines (37):

- different laboratories and equipment should be used for pre- and post-PCR manipulations
- water, buffers, tips and tubes should be autoclaved, and work surfaces exposed to ultraviolet light
- aliquots of reagents should be prepared daily for use in a PCR product-free environment
- the DNA sample should be the last element added to each tube.

Critical experiments should be repeated and, whenever possible, more than one pair of primers should be used to amplify disparate genomic fragments.

**DIAGNOSTIC APPLICATIONS**

While hybridisation tests serve some specific purposes quite well, relatively low sensitivity and technical difficulties have limited the application of these tests in clinical laboratories. This issue has been extensively reviewed by Knowles and Gorham (36). Nevertheless, the high sensitivity of PCR, which is usually more sensitive than conventional methods, makes this technique a true candidate to replace many of the procedures currently in use for the rapid detection of pathogens. The fact that very few copies of the nucleic acid of a micro-organism are needed for rapid detection has produced a marked increase in the number of laboratories implementing PCR for diagnostic purposes.

In contrast with conventional techniques, which demand a different procedure for each pathogen, the implementation of PCR requires similar procedures and reagents for all pathogens. After optimisation, PCR can be performed in low-complexity facilities using relatively low-cost equipment.

**Detection of foot and mouth disease virus by PCR**

The following provides an illustration of a strategy to develop and assess a PCR assay for the detection of foot and mouth disease virus (FMDV). The gene coding for the viral polymerase (located near the 3' end of the viral genome) was chosen for PCR because it is highly-conserved among serotypes and knowledge of the sequence is available (45). Oligonucleotides with suitable characteristics, including 50-60% G+C content and the absence of secondary structure, were selected using a computer programme (62). The reagent concentrations and time/temperature parameters were optimised using as
template a plasmid in which the sequence of interest was inserted. The conditions for reverse transcription were defined using purified viral RNA. Nucleic acid extraction was performed following the method described by Meyer and colleagues (47). Results were read on agarose gels stained with ethidium bromide and confirmed by hybridisation of the PCR products spotted on nylon membranes with a 30 mer internal oligonucleotide labelled with digoxigenin (M. Rodriguez and colleagues, unpublished findings).

In order to determine the sensitivity and specificity of the assay, experiments were carried out using dilutions of viral stocks of serotypes A, O and C, artificial samples, and bovine tissues taken at necropsy from experimentally-infected cattle (Tables I and II). In addition, further proof of the specificity of the test was obtained by examining fifty tissue samples (tongue epithelia, lung, soft palate, oesophagus, kidney and lymph node) from uninfected and experimentally-infected cattle (P. Murphy and colleagues, personal communication).

**TABLE I**

### Assessment of the sensitivity of the polymerase chain reaction for the detection of foot and mouth disease virus using dilutions of viral stocks of serotypes A, O and C

<table>
<thead>
<tr>
<th>Viral stock</th>
<th>Serotype</th>
<th>Titre ($\log_{10} LD_{50}/ml$)</th>
<th>Sensitivity in gels ($LD_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 644</td>
<td>O1</td>
<td>6.90</td>
<td>1.90</td>
</tr>
<tr>
<td>No. 547</td>
<td>O1</td>
<td>7.04</td>
<td>0.27</td>
</tr>
<tr>
<td>No. 622</td>
<td>A87</td>
<td>7.04</td>
<td>0.27</td>
</tr>
<tr>
<td>No. 578</td>
<td>C85</td>
<td>6.80</td>
<td>0.19</td>
</tr>
</tbody>
</table>

LD$_{50}$: 50% lethal dose for newborn mice
Results were read directly on agarose gels stained with ethidium bromide and confirmed by hybridisation

The experimental data indicates that the test is specific and that sensitivity is at least of the same order of magnitude as conventional methods when results are read directly on agarose gels. Increased sensitivity is observed after hybridisation of the PCR product. Many samples may be processed simultaneously in less than 24 h, and once the test is optimised complex laboratory facilities are not required.

**OTHER APPLICATIONS OF POLYMERASE CHAIN REACTION FOR DETECTION AND CHARACTERISATION OF INFECTIOUS ANIMAL DISEASE AGENTS**

**Viruses**

*African swine fever virus*

Four PCR primers and one oligonucleotide probe were designed and synthesised to amplify a 740 bp fragment corresponding to the conserved region of the genome of African swine fever virus (ASFV). A specific 640 bp PCR product was amplified using
TABLE II

Evaluation of the sensitivity and specificity of the polymerase chain reaction test for the detection of foot and mouth disease virus using beef samples made artificially (a), or beef and tongue epithelia taken at necropsy from experimentally-infected cattle (b)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Serotype</th>
<th>Titre (LD&lt;sub&gt;50&lt;/sub&gt;/0.25 ml)</th>
<th>Result of PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef homogenate + stock no. 567</td>
<td>O&lt;sub&gt;i&lt;/sub&gt;</td>
<td>54.00</td>
<td>Positive</td>
</tr>
<tr>
<td>Beef homogenate + stock no. 567</td>
<td>O&lt;sub&gt;i&lt;/sub&gt;</td>
<td>5.40</td>
<td>Positive</td>
</tr>
<tr>
<td>Beef homogenate + stock no. 567</td>
<td>O&lt;sub&gt;i&lt;/sub&gt;</td>
<td>0.54</td>
<td>Positive</td>
</tr>
<tr>
<td>Beef homogenate + PBS</td>
<td>–</td>
<td>–</td>
<td>Negative</td>
</tr>
<tr>
<td>b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected beef&lt;sup&gt;(1)&lt;/sup&gt;</td>
<td>A</td>
<td>1.98</td>
<td>Positive</td>
</tr>
<tr>
<td>Infected beef&lt;sup&gt;(1)&lt;/sup&gt;</td>
<td>O</td>
<td>15.70</td>
<td>Positive</td>
</tr>
<tr>
<td>Infected beef&lt;sup&gt;(1)&lt;/sup&gt;</td>
<td>C</td>
<td>1.98</td>
<td>Positive</td>
</tr>
<tr>
<td>Uninfected beef</td>
<td>–</td>
<td>–</td>
<td>Negative</td>
</tr>
<tr>
<td>Infected epithelium&lt;sup&gt;(2)&lt;/sup&gt;</td>
<td>A</td>
<td>1.00</td>
<td>Positive</td>
</tr>
<tr>
<td>Infected epithelium&lt;sup&gt;(2)&lt;/sup&gt;</td>
<td>O</td>
<td>1.24</td>
<td>Positive</td>
</tr>
<tr>
<td>Infected epithelium&lt;sup&gt;(2)&lt;/sup&gt;</td>
<td>C</td>
<td>1.00</td>
<td>Positive</td>
</tr>
<tr>
<td>Uninfected epithelium</td>
<td>–</td>
<td>–</td>
<td>Negative</td>
</tr>
</tbody>
</table>

For testing, 0.25 ml aliquots of a 1/10 homogenate were used and results were read directly on agarose gels and confirmed by hybridisation.

LD<sub>50</sub>: 50% lethal dose for newborn mice

PCR: polymerase chain reaction

PBS: phosphate-buffered saline

(1) three days post-infection

(2) sixteen days post-infection

oligonucleotides 1 and 5 as primers and assayed with templates extracted from organs and plasma of ASFV-infected pigs. PCR was demonstrated to be quicker and more sensitive than conventional methods (73).

**Avian endogenous leukaemia virus**

By the use of DNA amplification methods, envelope genes of avian endogenous leukaemia virus structures have been partially sequenced, showing that subgroup-specific sequences of the endogenous loci were largely homologous with those of Rous-associated virus type O (60).

**Avian polyomavirus**

A PCR assay was developed for detection of an avian polyomavirus, budgerigar fledgling disease virus (BFDV), using a single set of primers complementary to sequences located in the putative coding region for the BFDV VP1 gene. The assay was very specific for BFDV and highly sensitive, being able to detect as few as twenty copies of the virus. Using these methods, it was suggested that persistent infection may occur in adult birds, and further evidence was provided of possible in ovo transmission (58).
**Bluetongue virus**

PCR was adopted for the identification of a globally-conserved, serogroup-specific nucleic acid sequence (210 bp) of bluetongue virus (BTV) RNA in BTV serotypes from the United States of America (USA). Preliminary results indicate detection of target RNA sequences at a level of less than 2 fg, equivalent to 7,500 viral particles (16). When segment 7 primers (from BTV-ISA) were used, it was possible to detect as few as six molecules of segment 7 ds RNA per sample and also to detect purified ds RNA from different isolates of other serotypes. Positive results were obtained on red blood cells from infected cattle containing as much as 16 TCID\(_{50}\), but not those containing 1.6 TCID\(_{50}\) (81). Primers which encode virus reactive protein VP3, constructed using sequences based on RNA segment 3, were used to detect serogroups of BTV on platelet, buffy coat and packed red cells of BTV-infected sheep (43).

**Bovine coronavirus**

PCR was used to synthesise ds and ss probes for detection of bovine coronavirus (BCV) using recombinant plasmids as template molecules. After fragment-specific amplification by PCR, approximately ten viral genomes were detected by agarose gel analysis (78). Diagnosis of BCV in 134 clinical samples by hybridisation was more effective with ss than with either ds probes produced by PCR, or a combination of six recombinant plasmid probes containing non-overlapping sequences (79).

**Bovine herpesvirus-4**

PCR was used and compared with hybridisation and cell-culture isolation of samples from rabbits experimentally infected with bovine herpesvirus-4 (BHV-4). Virus was detected by PCR in several organs (including nervous tissues) which were found negative by other techniques (50).

**Bovine immunodeficiency virus**

Infection of embryonic bovine lung cells by bovine immunodeficiency virus was monitored both by syncytia formation and by reverse transcription followed by PCR, showing the advantages of the latter (33).

**Bovine leukaemia virus**

Proviral bovine leukaemia virus (BLV) DNA was detected in lymphocytic and tumour DNA at all stages of infection in cattle, by the use of a proviral BLV DNA probe and amplification of the DNA (51). PCR amplification (gag-p24, env-gp51, polymerase reverse transcriptase) was used to examine seropositive and seronegative cattle for the presence of BLV DNA in peripheral blood mononuclear cells. This protocol enabled the detection of one viral genome per 100,000 cells (49). When primers for the polymerase and px regions of BLV were used, as few as ten copies of BLV DNA were consistently amplified. This technique was successfully used in a blind study on blood mononuclear cells of eighteen cows (72).

**Bovine papillomavirus**

Using PCR, bovine papillomavirus (BPV) DNA was detected in 70% of commercial calf serum batches and 100% correlation was established with virus isolation (69). BPV DNA was detected by PCR using a set of primers located in the E5 open reading frame fitting BPV-1 and BPV-2, after extraction from formalin-fixed frozen sections of twenty equine sarcoids. These results support the general view that BPV plays an important role in equine sarcoids (74).
Bovine rotavirus

A full-length complementary DNA (cDNA) copy of the gene encoding the major neutralising antigen of bovine rotavirus was amplified, enabling the detection of this virus in infected faeces with 100,000 times greater sensitivity than the conventional electropherotype method and a 5,000-fold increase in sensitivity over hybridisation (89).

Bovine virus diarrhoea virus

By the amplification and sequencing of the p125 coding regions of a base pair homologous to the bovine virus diarrhoea virus (BVDV) biotype, cytopathogenic and non-cytopathogenic Pe515 strains were detected (18). Using a set of 20 bp primers located in the conserved 3' region of the BVDV genome, it was possible to amplify a 205 bp target sequence from BVDV DNA. The amplification assay was sensitive enough to detect one molecule of cloned BVDV cDNA, showing that the sensitivity of the test is higher than 1 TCID\textsubscript{50} (42). PCR for BVDV was also shown to be ten times more sensitive than virus isolation, by Vaniddekinge and colleagues (77). On the basis of published sequence data on the NADL (National Animal Disease Laboratory, USA) strain of BVDV, primers from nucleotide 6322 to 7475 were extracted from serum and white blood cells of persistently-infected heifers and used for PCR (8). Six 20 bp sequences from across the viral genome were selected by homology analysis of the published genomic sequence of the NADL and Osloss isolates of BVDV. The probe originating nearest the 5' end of the viral RNA, ND001, detected 86% of the cytopathic and non-cytopathic viral isolates analysed (38). By using primers from the gp48 region of the cytopathic NADL strain, it was possible to detect BVDV infection in serum samples of persistently-infected animals (6). Degenerate oligonucleotide primers designed on the basis of the sequence of two strains of BVDV and one strain of hog cholera virus were used in the PCR to detect viral RNA in cells infected \textit{in vitro} or in circulating lymphocytes (83).

Caprine arthritis-encephalitis

Caprine arthritis-encephalitis virus and maedi-visna virus were detected by PCR (90).

Distemper virus

Morbillivirus from \textit{Phoca vitulina} was identified using PCR with cDNA derived from rinderpest morbillivirus (25).

Equine arteritis virus

Three different primer pairs were used for reverse transcriptase PCR genomic RNA amplification of clinical samples of equine arteritis virus, detecting as few as 600 plaque-forming units (PFU) per ml in seminal plasma (14).

Equine herpesvirus-1

PCR was performed on infected and uninfected cultured cells and on 63 specimens from 29 aborted equine foetuses. Results were evaluated by electrophoresis and dot-blot hybridisation using an oligonucleotide probe labelled with biotin. Results were obtained in 24 h and the close correlation with virus isolation results was established (3). Unpurified DNA derived from cultures of equine foetal kidney cells infected with equine herpesvirus (EHV)-1 or EHV-4 was amplified by PCR using one pair of oligonucleotide primers. Restriction endonuclease digestion of the amplified segments with PVuII, followed by electrophoresis, revealed restriction fragment length polymorphisms which enabled the two virus types to be differentiated (53).
Equine infectious anaemia virus

PCR was used to detect the presence of equine infectious anaemia virus (EIAV) genomes in peripheral mononuclear cells of horses experimentally infected with the CL 22 strain of EIAV (87). Proviral sequences in the peripheral blood mononuclear cells of three horses with acute EIAV infection were monitored using PCR. Provirus was detected in the initial viraemic episode in each horse and during each of three relapsing viraemic cycles. Following each viraemic episode, provirus levels in the peripheral monocytes decreased to less than one copy per $5 \times 10^6$ cells (54).

Feline leukaemia virus

PCR was used to study the pathogenesis of experimental co-infection of cats with feline influenza virus and feline leukaemia virus (FeLV) (76). PCR has also been used for identification of FeLV infection on feline olfactory neuroblastoma (68).

Foot and mouth disease virus

Rapid and sensitive detection of FMDV in bovine and porcine tissues was successfully achieved by enzymatic RNA amplification of the polymerase gene. This method was able to detect amounts smaller than 1 TCID$_{50}$ when combined with hybridisation to a labelled probe. No cross-reaction with twelve other viruses (including enterovirus) was demonstrated (47).

Hog cholera virus

Total RNA isolated from hog cholera virus-infected tissue was reverse transcribed and the resulting complementary DNA was amplified with a sensitivity of 10,000 TCID$_{50}$. Sensitivity increased 1,000-fold when the PCR product was reamplified with a set of nested primers (40).

Infectious avian laryngotracheitis virus

The infectious laryngotracheitis virus (IALV) homologue of the herpes simplex virus glycoprotein B (gB) gene was identified by PCR amplification of genomic IALV DNA (59).

Infectious bronchitis virus

A PCR technique was developed which permitted the typing of avian infectious bronchitis virus isolates which caused nephritis (39).

Influenza virus

In order to determine the persistence of influenza virus in ducks after oral infection, PCR analysis of the haemagglutinin gene was used, showing no detectable RNA in spleen samples (82).

Maedi-visna virus

By using a multiple primer set which generates DNA segments with overlapping cohesive termini, maedi-visna virus can be amplified, retained, and detected in infected cells with greater sensitivity (by more than two orders of magnitude) than using existing methods (26). A maedi-visna-like virus (designated EV-1) isolated from sheep showing symptoms of arthritis and pneumonia was analysed by PCR in order to demonstrate the degree of homology with known maedi-visna virus strains (65).
Malignant catarrhal fever virus (Alcelaphine herpesvirus-1)

Two pairs of thirty nucleotide primers were selected, corresponding to the nucleotide sequence of the genomic DNA of alcelaphine herpesvirus-1 (AHV-1) (the causative virus of bovine malignant catarrhal fever). The primers were synthesised and successfully used for PCR on crude cell lysates infected with AHV-1 (31). A fragment of AHV-1 DNA was subcloned into pVC18 and sequenced. The subclone hybridised strongly to AHV-1 DNA, weakly to AHV-2 DNA and not at all to DNA from bovine herpesvirus (BHV)-1, BHV-2 and BHV-4. A two-stage (nested) PCR diagnostic test was devised, using a portion of the subcloned AHV-1 DNA sequence. Five AHV-1 and AHV-2 isolates were detected identically and specifically by PCR, while the same procedure failed to detect BHV-1, BHV-2 and BHV-4 viruses. By this technique, levels of AHV-1 as low as 0.01 TCID$_{50}$ were detected (34).

Pseudorabies virus (Aujeszky virus)

Genomic sequences of Aujeszky virus (pseudorabies virus: PRV) were amplified by PCR from cells of infected cultures, nasal cells and organs from acutely-diseased pigs, as well as from organs of latently-infected pigs (5). When used directly on disrupted cells with twenty-five cycles, results were obtained in 1 h which were equal in specificity and sensitivity to conventional methods (32). Various aspects of the latency of PRV in swine were investigated using in vitro nucleic acid amplification methods based on PCR. Primers flanking the 156 bp region of the PRV gpII gene were annealed to purified PRV DNA, as well as DNA isolated from trigeminal ganglia of swine latently infected with PRV. Following amplification, 100 fg of PRV DNA were visualised on stained gels and 1 fg (equivalent to six viral genome copies) was detected when amplification was combined with blot hybridisation (41). When blot hybridisation of PCR-amplified DNA (gX and gII genes) was used, latent virus was detected in seven of eight samples (44). A PCR protocol which specifically amplifies sequences coding for the glycoprotein gp50 (a 217 bp sequence between 433 and 651 of the gp50 gene) was able to consistently detect PRV in tissues of latently-infected swine. Results were obtained in 24 h by sampling tonsil tissues to investigate latent PRV infection (85). Latent viral DNA was detected by PCR in trigeminal ganglia of all of ten pigs which were necropsied 81 or more days after being infected intranasally with a thymidine kinase-negative vaccine strain of PRV (80).

Rabies virus

PCR was used to study the primary multiplication site of a recombinant vaccinia virus (VV) expressing the rabies virus G glycoprotein VVTGgRAB, in comparison with the parenteral VV Copenhagen strain, after oral administration to foxes. VVTGgRAB was detected in the tonsils of foxes tested after 24 h and 48 h, in the buccal mucosa of foxes tested after 24 h and 48 h and in the soft palate of one of two foxes tested after 24 h and one of three foxes after 48 h. Foxes were inoculated with virus isolated from fox tonsils 24 h after oral administration (with or without cell-culture amplification) to perform back passages. No virus could be subsequently isolated in either case. The innocuity of VVTGgRAB was also demonstrated when foxes were inoculated with passaged virus (75). PCR was also used to study the pathogenesis of rabies virus in a mouse model by inoculation of the maseter muscle, showing that virus replication occurred on the trigeminal ganglia at early stages, while at later stages (96 h post-infection) positive sense RNA was present in large amounts in the maseter muscle (71).
**Bacteria**

**Brucella abortus**

A specific and sensitive PCR test was developed which was capable of detecting 0.1 pg of *Brucella abortus* DNA (less than 100 brucella cells) by the use of primers which allowed the amplification of a 635 bp fragment of a 43 kDa outer membrane protein gene from *B. abortus* strain 19 (21).

**Escherichia coli**

A PCR has been developed to detect *Escherichia coli* with sensitivity and specificity equal to conventional methods in the following substances: water (4), cheese (46), pig stools (29) and minced meat (84). Similarly sensitive and specific detection of *E. coli* has been achieved using oligonucleotide primers containing inosine for enzymatic amplification of different alleles of the gene coding for heat-stable toxin type I of enterotoxigenic *E. coli* (13). Amplification of the mal B operon of *E. coli* AIIE strains yielded a specific DNA fragment. The minimum detection limit was ten bacteria and the PCR systems were validated by testing twenty-seven *E. coli* strains of known enterotoxigenic properties (13).

**Leptospira spp.**

A PCR was developed to detect leptospira. Primers were used to amplify a 631 bp section of the 5′ region of 16S ribosomal DNA. (28). When applied to the detection of leptospira in urine samples from cows, as few as 5-10 leptospira per ml of urine were detected (23).

**Listeria monocytogenes**

Five oligonucleotides were used as primers in the PCR for the amplification of specific sequences from DNA of *Listeria monocytogenes*. This technique was used for detection of listeria in food products (7). Amplification of the alpha and beta haemolysin gene was used for detection of *L. monocytogenes* DNA in cooked sausages and milk, with a detection limit of ten bacteria per 10 ml of milk in 48 h (22). Variable results were observed when PCR was used on cheese samples (84). With the aid of PCR amplification, it was determined that the absence of a gene coding for phosphatidylinositol is associated with the pathogenesis of *L. monocytogenes* (12).

**Mycobacterium paratuberculosis**

A 278 bp DNA probe (PCR 278) fragment was obtained by PCR amplification of the 5′ region of IS900, an insertion element contained in the genome of *M. paratuberculosis*. When used in conjunction with PCR, this probe could detect as little as 10 fg (equivalent to two genomes) of *M. paratuberculosis* starting material (48).

**Mycobacterium tuberculosis**

Two oligonucleotides were synthesised and used as primers for PCR amplification. A 396 bp fragment was specifically amplified from the *Mycobacterium tuberculosis* genome. No amplification was observed from any of ten different *Mycobacterium* strains, including those belonging to the *M. tuberculosis* complex. The PCR product was detected by dot blot hybridisation, even when as little as 10 fg of purified *M. tuberculosis* DNA was used. A good correlation with isolation methods was established (17). Using a 123 bp segment of IS6110, which is repeated in the *M. tuberculosis* chromosome, a sensitive and specific assay system was devised for detection within 48 h of
M. tuberculosis on sputum samples (19). PCR was also found to be the most sensitive test for the diagnosis of tuberculous meningitis (70).

Salmonella spp.

Specific primers and a magnetic immuno method (magnetic particles coated with monoclonal antibodies) were used to amplify a 163 bp genomic fragment of Salmonella typhimurium. By this method, a PCR amplification of 25 Salmonella strains but not of 19 other Enterobacteriaceae was achieved. A sensitivity of 100 PFU was attained (88).

Other pathogens

Mycoplasma spp.

Primers designed on the basis of the sequences of the 16S ribosomal DNA genes of Mycoplasma pneumoniae and M. genitalis enabled the development of a specific and sensitive in vitro amplification assay system, which detected as few as 100 M. pneumoniae cells and 1,000 M. genitalis cells (66).

Toxoplasma spp.

Tissue samples from aborted foetuses were examined for the presence of Toxoplasma by PCR amplification of the p30 gene of Toxoplasma. All samples were shown to be infected (86).

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REFERENCES


