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Review

APPLICATIONS OF DNA AMPLIFICATION TECHNIQUES IN VETERINARY DIAGNOSTICS

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ABSTRACT

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An overview of the principles of the polymerase chain reaction, ligase chain reaction, self-sustained sequence replication and Qβ replicase is given. The application of these methods for the diagnosis of veterinary infectious and hereditary diseases as well as for other diagnostic purposes is discussed and comprehensive tables of reported assays are provided. Specific areas where these DNA-based amplification methods provide substantial advantages over traditional approaches are also highlighted. With regard to PCR-based assays for the detection of viral pathogens, this article is an update of a previous review by Belák and Ballagi-Pordány (1993).

Keywords: diagnosis, LCR, ligase chain reaction, PCR, polymerase chain reaction, Qβ replicase, self-sustained sequence replication, 3SR

Abbreviations: 3SR, self-sustained sequence replication; BHV, bovine herpesvirus; BLAD, bovine leukocyte adhesion deficiency; cDNA, complementary DNA; CFU, colony-forming units; EHV, equine herpesvirus; ELISA, enzyme-linked immunosorbent assay; FHV, feline herpesvirus; LCR, ligase chain reaction; PCR, polymerase chain reaction; PRV, pseudorabies virus; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; RT, reverse transcriptase; rRNA, ribosomal RNA; wt, wild-type

INTRODUCTION

The polymerase chain reaction (PCR) is an *in vitro* enzymatic method which allows several million-fold amplification of a specific DNA sequence. Since its introduction in 1985, PCR has facilitated the development of a variety of nucleic acid-based detection systems for bacterial, viral and other pathogens, as well as for genetic disorders (Erlich *et al.*, 1991). Owing to its high sensitivity, specificity and speed, PCR offers advantages over conventional diagnostic methods. While a variety of PCR-based assays have been described in the literature for the detection of infectious agents affecting man and animals, their application is not yet routine, even in large diagnostic laboratories. Problems with contamination, as well as cost- and time-intensive post-PCR detection methods, currently hamper its widespread use.

However, despite its current limitations, PCR already finds relatively broad use in the routine diagnosis of hereditary diseases in animals, such as bovine leukocyte adhesion deficiency (BLAD) and porcine malignant hyperthermia syndrome (see Shuster *et al.*, 1992).

Other recently developed DNA amplification methods, such as the ligase chain reaction (LCR), self-sustained sequence replication (3SR) and Qβ replicase amplification have so far found only limited applications, but show promise for specific diagnostic applications. While PCR and LCR rely on temperature cycling and therefore require some investment in the appropriate equipment, 3SR and Qβ replicase amplification are isothermal methods of DNA amplification, so making potential 'field' use feasible.

This review gives a short overview of the principles of the different DNA amplification techniques, with emphasis on current technical developments which will facilitate their use in veterinary diagnostics in the near future. Examples of diagnostic tests which show promise for more widespread application are also given.

PRINCIPLES OF NUCLEIC ACID AMPLIFICATION METHODS

The DNA amplification methods will be described briefly. More detailed reviews on the principles and technical details of the different methods can be found in Barany (1991), Erlich and colleagues (1991), Fahy and colleagues (1991), Wolcott (1992), Abramson and Meyers (1993) and Wiedmann and colleagues (1994b).

Polymerase chain reaction

PCR is defined by repetitive cycles, each consisting of three steps performed at different temperatures. In the first step, the double-stranded target DNA is denatured at high temperatures, resulting in single-stranded molecules. Two oligonucleotide primers each hybridize to their respective complementary DNA strands in the second step (annealing step), thus defining a region of the target DNA. In the last step, the 3′ ends of these bound primers are extended by a thermostable DNA polymerase. During each cycle, the complementary DNA strands are copied by the sequential elongation of the two primers (see Figure 1). Newly synthesized DNA molecules can serve as templates in the next cycle, thus resulting in exponential amplification. A variety of parameters can affect the reaction kinetics and the success of amplification (for details see Xu and Larzul, 1991).

The specificity of the amplification depends on the primer design. Mispriming can be minimized by using a 'hot-start', whereby an essential PCR component (usually *Taq* polymerase) is added only after the annealing temperature is reached (Chou *et al.*, 1992).

The most common source of false positive results is carry-over contamination from previous PCRs (Kwok and Higushi, 1989). A variety of methods have been developed to minimize carry-over contamination, which currently seems to be one of the major problems for the application of PCR in a routine diagnostic setting (Kwok, 1990).

Amplification of RNA sequences, e.g. RNA viruses or mRNAs, requires

transcription into cDNA using reverse transcriptase (RT). In order to avoid additional manipulations and to diminish the risk of contamination, a single-tube RT-PCR format has been developed (Sellner *et al.*, 1992). Some thermostable DNA polymerases possess RT activity, so making a single-tube RT-PCR more feasible, allowing reduced manipulation time and minimizing carry-over contamination. Moreover, since reverse transcription is carried out at higher temperatures, longer cDNA molecules can be made, especially from RNAs with extensive secondary structures (Myers *et al.*, 1994).

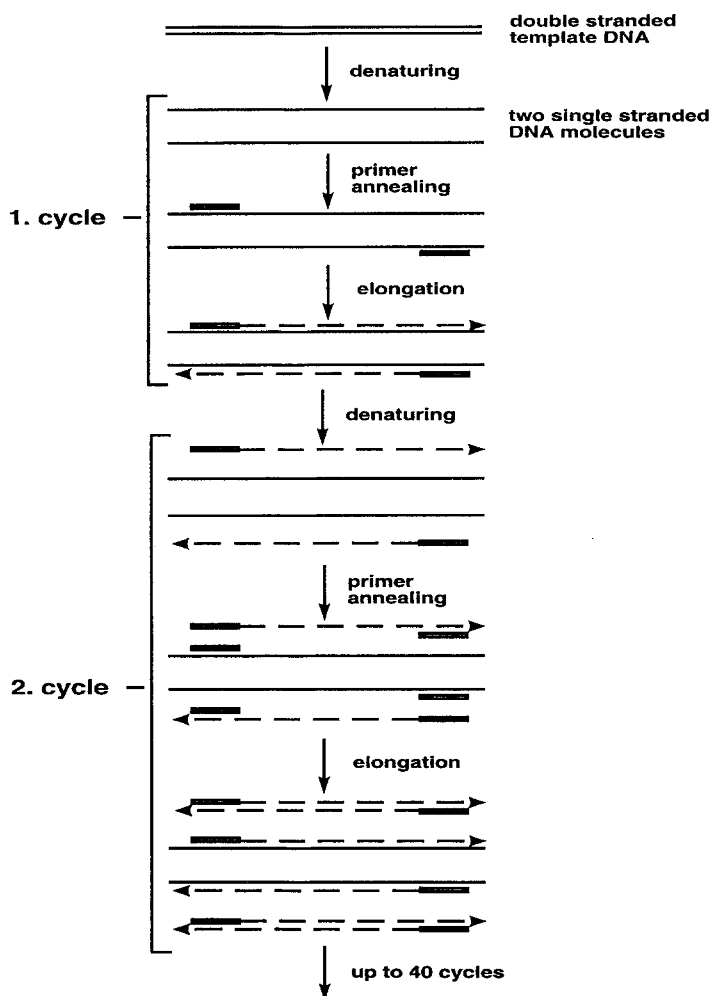

Figure 1. A schematic representation of the polymerase chain reaction. Template DNA is shown by solid lines, newly synthesized DNA strands are shown by broken lines, and primers are indicated by boxes. One cycle, consisting of the three steps of denaturing, primer annealing and elongation, is indicated by brackets. For details see the section in the text on the polymerase chain reaction

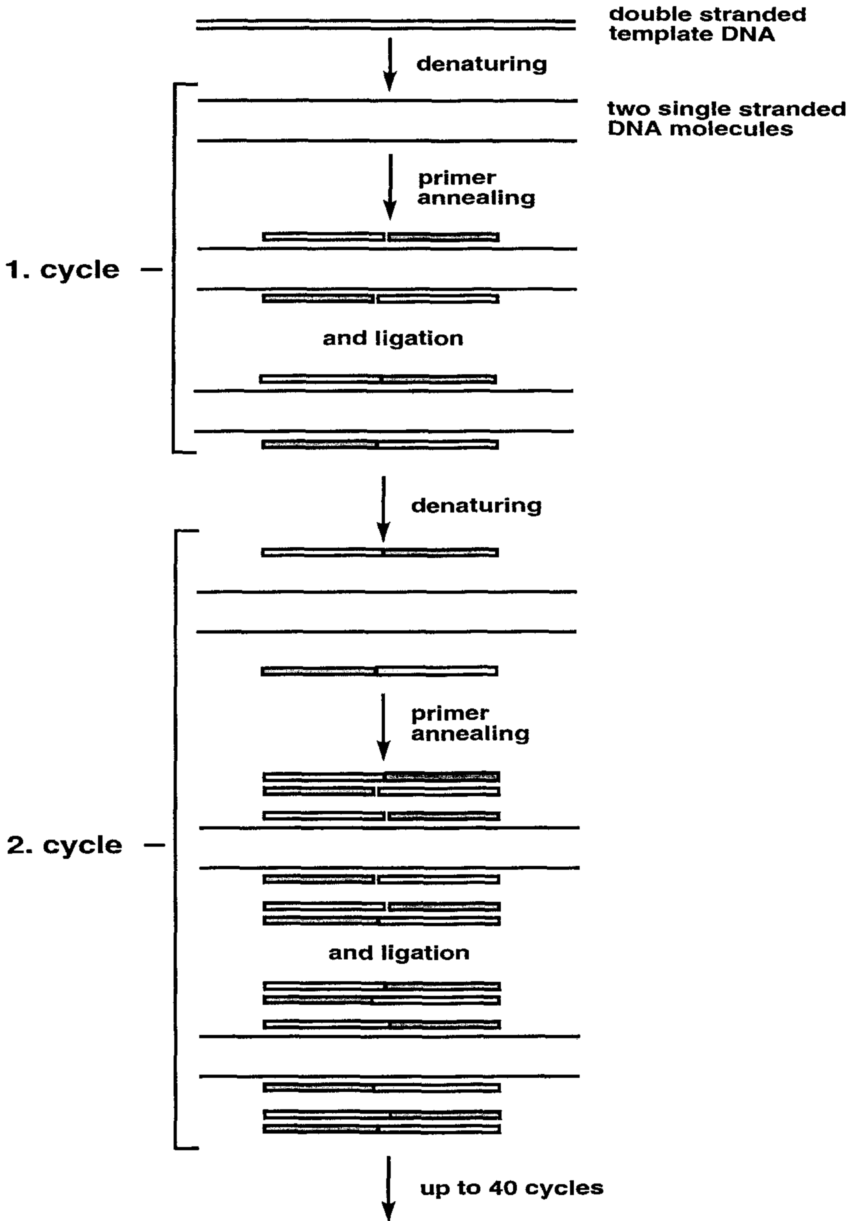

Figure 2. A schematic representation of the ligase chain reaction (adapted from Barany, 1991). Template DNA is shown by solid lines and the four primers are represented by hatched boxes. The site of the discriminating nucleotide is shown as a gap between one primer pair at the annealing step. Each primer pair can only be ligated when the overlapping 3' end of the light hatched primers contains the matching complementary nucleotide to the template. One cycle, consisting of a denaturing, a primer annealing and a ligation step, is indicated by brackets. For details see the section in the text on the ligase chain reaction

PCR amplification products, termed amplicons, can be detected in several ways. Verification of the amplicon size by agarose gel electrophoresis using a molecular weight standard is applied in most cases. The PCR amplicon can also be digested with restriction endonucleases to confirm internal restriction enzyme sites. PCR in combination with restriction fragment length polymorphism (RFLP) is often used to type a group of pathogens or to define different alleles in the detection of hereditary diseases. A more time-consuming method is Southern blotting in which, after gel electrophoresis and blotting on a membrane, the PCR product is hybridized with a specific probe to verify homology.

Ligase chain reaction

The principle of LCR is based in part upon the ligation of two adjacent oligonucleotide primers, which uniquely hybridize to one strand of the target DNA (see Figure 2). The junction of the two primers is usually positioned such that the nucleotide at the 3' end of the upstream primer coincides with a known single base pair difference in the target sequence. This single base pair difference may define two different alleles, species or other phenotypic differences. If the target nucleotide at that site complements the nucleotide at the 3' end of the upstream primer, the two adjoining primers can be covalently joined by the ligase. The unique feature of LCR is a second pair of primers, almost entirely complementary to the first pair, which are designed with the nucleotide at the 3' end of the upstream primer, denoting the sequence difference. In a cycling reaction, using a thermostable DNA ligase, both ligated products can then serve as templates for the next reaction cycle, leading to an exponential amplification, analogous to PCR. If there is a mismatch at the primer junction, this structure will not be recognized by the thermostable ligase and the primers will not be ligated. The absence of the ligated product therefore indicates at least a single base pair change in the target sequence (Barany, 1991; Wiedmann *et al.*, 1994b). LCR is often utilized in conjunction with primary PCR amplification. Such a PCR-coupled LCR combines the sensitivity of PCR with the specificity of LCR for detection of each possible single base pair change.

Self-sustained sequence replication (3SR) and Qβ replicase amplification

3SR allows an exponential amplification of either RNA or DNA molecules, which are defined by two specific DNA primers. These primers are complementary to the target at their 3' ends and incorporate a promoter recognized by T7 RNA polymerase at the 5' end. Amplification is performed at a constant temperature and, where RNA is the template, a RT first produces a complementary DNA strand. This newly synthesized strand will be copied again by the RT, resulting in two newly synthesized DNA strands with a T7 promoter sequence at the 5' end. Therefore, T7 RNA polymerase can initiate synthesis of multiple copies (10–1000 per cycle) of complementary RNA strands. These RNA molecules can then be reverse transcribed to produce more DNA molecules. Only in an RNA–DNA hybrid will the RNA strand then be degraded by a third enzyme, RNaseH. The DNA strands are therefore free to initiate more RNA synthesis, which ensures continuation of the amplification (Fahy *et al.*, 1991). A schematic diagram of this process is shown in Figure 3.

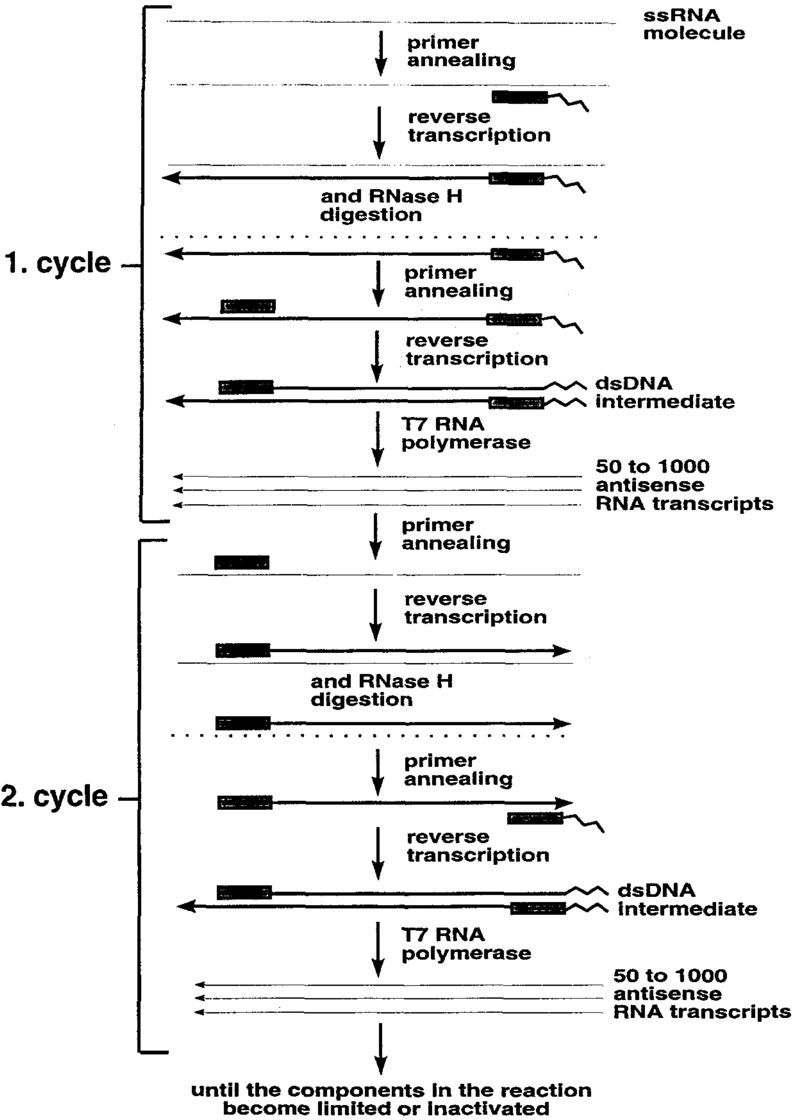

Figure 3. A schematic representation of the self-sustained sequence replication (3SR) procedure, as used for the amplification of an RNA template (thin line). Primer and primer sequences are shown as hatched boxes, the T7 RNA polymerase promotor sequence is shown by a zigzag line. Digested RNA strands are shown by dotted lines and newly synthesized DNA strands are indicated by bold lines. One cycle, consisting of two primer annealing steps, two reverse transcription steps, the RNA digestion, and the synthesis of antisense RNA transcripts by the T7 RNA polymerase, is indicated by brackets. For details see the section in the text on the 3SR procedure

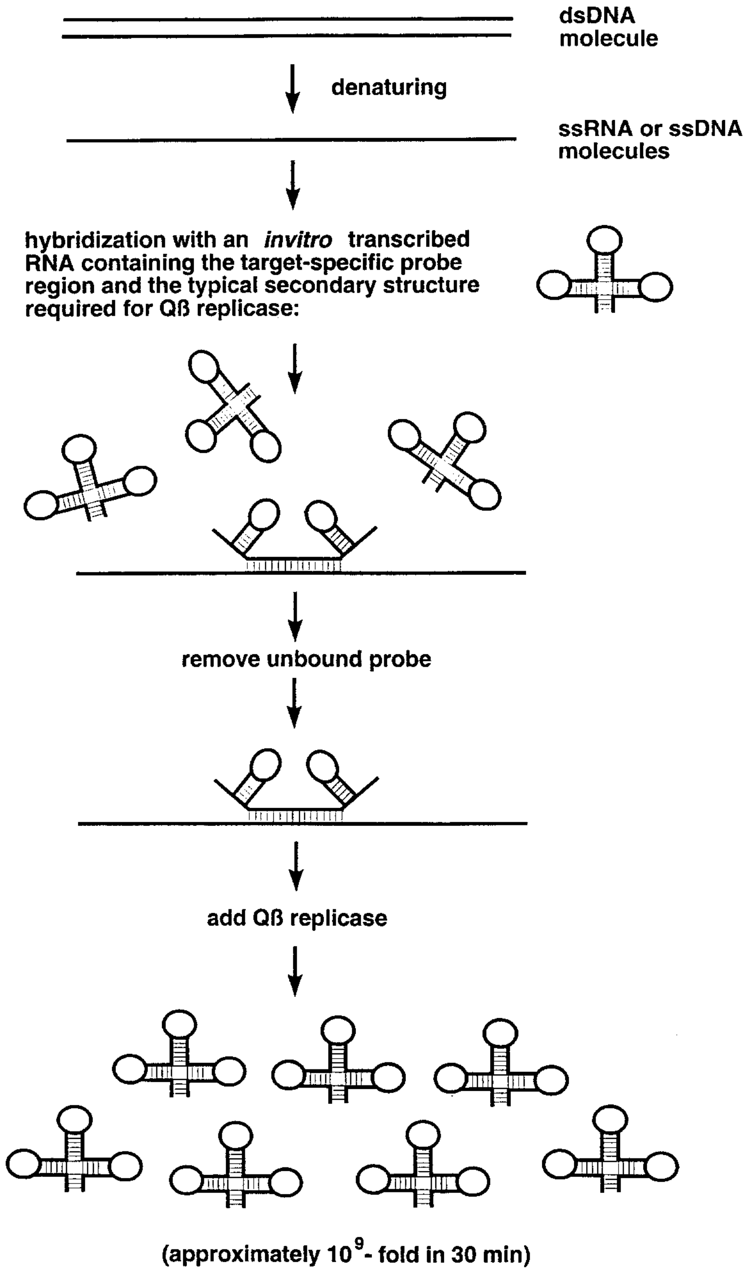

Figure 4. A schematic representation of Qβ replicase-based probe amplfication. A Qβ replicase substrate, which contains the target-specific probe region (shown as a molecule with three stem-loops), specifically hybridizes to the target RNA (bold line). After unbound molecules have been removed, Qβ replicase is added, resulting in amplification of the probe molecule. For details see the section in the text on Qβ replicase amplification

$Q\beta$ replicase amplification utilizes the replicase from $Q\beta$ bacteriophage, an RNA-dependent RNA polymerase, to self-replicate an RNA template designated MDV-1. In this assay, unlike in PCR, a reporter RNA rather than the actual target is amplified. Specificity is achieved by inserting a target-specific probe sequence in MDV-1. After hybridization of the probe-MDV-1 sequence to the target, unbound probe is removed and the remaining probe is amplified after addition of $Q\beta$ replicase (see Figure 4). The resulting amplified probe-MDV-1 sequences can be visualized in ethidium bromide-stained gels or by use of a secondary probe complementary to MDV-1 sequences. An example of a sensitive non-isotopic $Q\beta$ replicase assay for detecting an infectious agent (*Chlamydia trachomatis*) has recently been reported (Shah *et al.*, 1994).

Both 3SR and $Q\beta$ replicase have the advantage of being isothermal, and therefore do not require thermal cyclers. This offers potential for use outside well-equipped laboratories and might make these procedures especially useful for some veterinary applications.

EXAMPLES OF DNA AMPLIFICATION-BASED VETERINARY DIAGNOSTIC TESTS

Viral pathogens

PCR has proved to be a very valuable technique for the detection of many infectious agents, among which viruses form the largest group. Table I lists PCR-based assays for the diagnosis of veterinary viral pathogens. Owing to the very large number of publications which appear every year, only those that have been published since the last review by Belák and Ballagi-Pordány (1993) are included. The herpesviruses have been selected here as a relevant example of how DNA amplification-based assays can be applied to veterinary diagnostic problems.

Animal herpesviruses are known to cause severe losses in livestock. While the acute form of the diseases can easily be diagnosed by virus isolation, there is at present no direct method for detecting the latent state of infection. During latency, infectious virus cannot be isolated because only the herpesviral DNA persists within the cells and no viable virus is produced. In contrast to human herpesviruses, latency-associated transcripts have not been found in any of the animal herpesviruses, apart from pigs infected with pseudorabies virus (PRV; Cheung, 1989). However, using PCR, the trigeminal ganglion has been shown to be one location for latent equine herpesvirus type 1 (EHV-1; Slater *et al.*, 1994), for PRV in pigs (Belák *et al.*, 1989) and for feline herpesvirus (FHV) in cats (Reubel *et al.*, 1993). The virus, however, can be reactivated by superinfection or immunosuppression. It is therefore of epidemiological importance to identify carriers in order to vaccinate or to cull them.

TABLE I
DNA amplification-based assays for veterinary viral pathogens (listed in alphabetical order)

Organism	Assay/target gene	Reference
African horse sickness virus	RT-PCR/major core protein gene (VP3); S8 gene	Sakamoto *et al.* (1994); Stone-Marschat *et al.* (1994)
	RT-PCR/VP7 gene	Zientara *et al.* (1993,1994)
African swine fever virus	PCR/p72 gene	Carrillo *et al.* (1994)
Avian leukosis virus	PCR/endogenous viral elements (ev15 repeat)	Benkel and Smith (1993)
Avian reticuloendotheliosis virus (REV)	PCR/long terminal repeat	Aly *et al.* (1993)
Bluetongue virus (BTV)	RT-PCR/VP3 gene	Akita *et al.* (1993) MacLachlan *et al.* (1994)
	RT-PCR/VP1 gene	Wilson (1994);
	Nested RT-PCR/NS1 protein gene	Katz *et al.* (1993a)
	Nested and multiplex RT-PCR/NS1 and VP2 genes	Wilson and Chase (1993)
Borna disease virus	Nested RT-PCR	Zimmermann *et al.* (1994)
Bovine herpesvirus type 1	PCR/gC gene	van Engelenburg *et al.* (1993)
	PCR/gpIV gene	Wiedmann *et al.* (1993b)
	PCR/gI gene	Vilcek *et al.* (1994b)
Bovine immunodeficiency virus (BIV)	PCR/gag and pol genes	Nadin-Davis *et al.* (1993b)
Bovine leukaemia virus (BLV)	PCR/env gene	Mirsky *et al.* (1993); Agresti *et al.* (1993); Eaves *et al.* (1994)
	RT-PCR/pol gene	Poon *et al.* (1993)
	PCR/gag gene	Kelly *et al.* (1993)
	Nested PCR/gp51 gene	Klintevall *et al.* (1994)
Bovine respiratory syncytial virus (BRSV)	PCR/F fusion protein gene	Vilcek *et al.* (1994a); Oberst *et al.* (1993a,b)
Bovine rotavirus	RT-PCR/VP4 and VP7 genes	Isegawa *et al.* (1993); Parwani *et al.* (1993); Suzuki *et al.* (1993); Brüssow *et al.* (1994)
Bovine viral diarrhoea virus (BVDV)	RT-PCR/p125 gene	Gruber *et al.* (1993)
	Nested RT-PCRs/3' end	Alansari *et al.* (1993)
	RT-PCR/3' end and 5' end	Schmitt *et al.* (1994)
	RT-PCR-RFLP/3' end 5' end, p54 and p80 genes	Vilcek *et al.* (1994c)
	RT-PCR/5' end	Easton *et al.* (1994)
Canine distemper virus	RT-PCR/nucleocapsid gene	Mee *et al.* (1993)

TABLE I (cont)

Organism	Assay/target gene	Reference
Canine parvovirus	PCR/capsid protein gene PCR/VP2 gene	Mochizuki *et al.* (1993) Truyen *et al.* (1994)
Caprine arthritis encephalitis virus (CAEV)	PCR/gag gene	Rimstad *et al.* (1993)
	PCR/gag and pol genes	Reddy, P.G. *et al.* (1993); Barlough *et al.* (1994)
Chicken infectious anaemia virus (CIAV)	PCR/two DNA fragments uncharacterized	Soine *et al.* (1993)
	PCR/DNA fragment uncharacterized	Tham and Stanislawek (1992)
Eastern equine encephalomyelitis virus (EEEV)	RT-PCR/capsid gene	Vodkin *et al.* (1993)
Epizootica haemorrhagic disease (EHD) virus	RT-PCR/NS1 gene	Wilson (1994)
Equine arteritis virus	RT-PCR/ORF1b, 3, 4 and 7	St. Laurent *et al.* (1994)
Equine herpesvirus types 1 and 4	PCR/glycoprotein gB gene Nested PCR/gB gene Multiple PCR/gB gene	Wagner *et al.* (1992) Borchers and Slater (1993) Kirisawa *et al.* (1993)
type 4	PCR/gC gene	Gilkerson *et al.* (1994)
Equine influenza virus A	RT-PCR/matrix gene (segment 7)	Donofrio *et al.* (1994)
Feline coronavirus	RT-PCR/S (spike protein) gene	Li and Scott (1994)
Feline herpesvirus type 1	PCR/thymidine kinase gene	Reubel *et al.* (1993)
Feline immunodeficiency virus (FIV)	PCR/gag and pol genes PCR/gag gene	Greene *et al.* (1993) Lawson *et al.* (1993); Momoi *et al.* (1993)
Feline leukaemia virus (FeLV)	RT-PCR/env gene	Papenhausen & Overbaugh (1993)
	PCR/long terminal repeats	Jackson *et al.* (1993)
Fish lymphocystis disease virus	PCR/major capsid protein gene	Schnitzler and Darai (1993)
Foot and mouth disease virus	RT-PCR/VP1 gene	Saiz *et al.* (1993); Amaral-Doel *et al.* (1993)
	RT-PCR/capsid protein gene RT-PCR/polymerase gene	Höfner *et al.* (1993) Prato-Murphy *et al.* (1994)
Hog cholera virus (HCV)	RT-PCR and multiplex RT-PCR Nested RT-PCR/5'-genomic terminus and major envelope protein gene	Wirz *et al.* (1993) Katz *et al.* (1993b)
	RT-PCR/p45-p75 boundary of p120 gene	Harding *et al.* (1994)

TABLE I (cont)

Organism	Assay/target gene	Reference
Infectious bronchitis virus (IBV)	PCR-RFLP/S1-glycoprotein gene	Kwon *et al.* (1993a,b)
Infectious bursal disease virus	RT-PCR/VP2 gene RT-PCR-RFLP/genomic segment B	Lin *et al.* (1993) Kibenge and Qian (1994)
Infectious haematopoietic virus (IHNV)	RT-PCR/N-gene	Arakawa *et al.* (1990)
Infectious laryngotracheitis virus	PCR/thymidine kinase gene	Scholz *et al.* (1994)
Infectious pancreatic necrosis virus (IPNV)	RT-PCR/VP2 gene	Lopez-Lastra *et al.* (1994)
Marek's disease virus	PCR/thymidine kinase gene PCR/132 bp repeat	Rong-Fu *et al.* (1993) Becker *et al.* (1993)
Newcastle disease virus (NDV)	RT-PCR/F0 fusion protein gene	Collins *et al.* (1993c)
Orthopoxviruses	PCR-RFLP/ATI gene PCR-coupled LCR/ATI gene	Meyer *et al.* (1994) Pfeffer *et al.* (1994)
Ovine herpesvirus type 2	PCR/tegument protein gene	Baxter *et al.* (1993); Wiyono *et al.* (1994)
Ovine lentivirus (maedi visna virus)	PCR/pol gene and long terminal repeat	Brodie *et al.* (1993, 1994)
Porcine parvovirus	PCR/VP2 gene	Gradil *et al.* (1994a)
Porcine reproductive and respiratory syndrome virus (Lelystad virus)	PCR/nucleocapsid protein gene (ORF 7)	Suarez *et al.* (1994); Mardassi *et al.* (1994)
Pseudorabies virus (Aujeszky's disease virus)	PCR/gI and gp50 genes PCR/gp63 gene PCR/gp50, gI and gp63 genes Nested PCR/gI and thymidine kinase genes	Schang and Osorio (1993) Banks (1993) Hasebe *et al.* (1993) Glass *et al.* (1994)
Rabies virus	Nested RT-PCR/nucleocapsid gene RT-PCR-RFLP/nucleocapsid gene	Kamolvarin *et al.* (1993) Nadin-Davis *et al.* (1993a); Kulonen and Boldina (1993)
Striped jack nervous necrosis virus (SJNNV)	RT-PCR/RNA2	Nishizawa *et al.* (1994)
Swine influenza virus (H1)	RT-PCR/haemagglutinin gene	Noble *et al.* (1993)
Transmissible gastroenteritis virus (TGEV)	RT-PCR/S, Orf 3a-3b genes	Britton *et al.* (1993)
Vesicular stomatitis virus (VSV)	RT-PCR/phosphoprotein gene Hemi-nested RT-PCR/L gene	Rodriguez *et al.* (1993) Höfner *et al.* (1994)

During campaigns to eradicate animal herpesviruses, genetically altered modified live vaccines (so-called 'marker vaccines') have been employed to combat and displace wild-type (wt) viruses. Vaccine strains of PRV have been generated that lack one of the four non-essential glycoproteins, i.e. gp63 (Petrovski *et al.*, 1986), gI (Quint *et al.*, 1987), gIII (Kit *et al.*, 1987), and gX (Marchioli *et al.*, 1987). ELISAs with monoclonal antibodies, which distinguish between wt and vaccine virus, were used for screening wt PRV carriers, but gave some false positive results (Annelli *et al.*, 1991). Consequently, a panel of PCR-based assays has been developed to replace these ELISAs and to minimize false positives (see Belák and Ballagi-Pordány, 1993; and Table I). Recently, a BHV-1 gE deletion mutant has been developed as a potential modified live vaccine (Kaashoek *et al.*, 1994). PCR would be suitable to screen for wt BHV-1 carriers in BHV-1 eradication programmes. A deletion mutant of EHV-1 (strain RacH) is widely used as a vaccine strain in Europe. Owing to the lack of monoclonal antibodies which discriminate between the vaccine and wt EHV-1 strains, RacH-induced abortions of vaccinated mares could not be excluded. To allow specific screening for the vaccine strain, a PCR assay which discriminates RacH from EHV-1 and EHV-4 field strains has been developed (Osterrieder *et al.*, 1994).

PCR has been successfully applied to the detection of EHV-1 and EHV-4 in nasal swabs (Sharma *et al.*, 1992; Gilkerson *et al.*, 1994) and in aborted fetuses, replacing time-consuming virus isolation and immunofluorescence detection methods (Hardt *et al.*, 1992; Borchers and Slater, 1993; Kirisawa *et al.*, 1993; Osterrieder *et al.*, 1994). PCR assays have also been developed to detect bovine herpesvirus type 1 (BHV-1; e.g. Vilcek, 1993), type 3 (BHV-3, formerly named BHV-4; e.g. Naeem *et al.*, 1991) and ovine herpesvirus type 2 (OHV-2; Wiyono *et al.*, 1994). Direct detection by PCR of BHV-1 from nasal swabs from cattle showing respiratory tract diseases has also been described (van Engelenburg *et al.*, 1993; Vilcek *et al.*, 1994b).

Direct diagnosis of herpesviruses by virus isolation from some biological materials, such as semen, has proved to be difficult owing to cytotoxic components which interfere with tissue culture tests. Nevertheless, to control the transmission and spread of BHV-1 through semen, practicable PCR protocols have been developed for amplification of BHV-1 sequences (van Engelenburg *et al.*, 1993; Wiedmann *et al.*, 1993b).

Methods for isolating DNA or RNA from fixed tissues have been improved (Kallio *et al.*, 1991; Koopmans *et al.*, 1993). While viral proteins are usually no longer immunologically detectable and viable virus cannot be isolated from formalin-fixed material, PCR often allows a retrospective diagnosis of viral infections using such pathological specimens (Rimstad and Evensen, 1993; Osterrieder *et al.*, 1994).

Bacterial and fungal pathogens

Overviews of PCR and other DNA amplification-based assays for detecting veterinary bacterial and fungal pathogens are given in Tables II and III, respectively. In general, references have only been included in these tables if they describe specific veterinary applications. Only in cases where no veterinary assays have been described for a given pathogen are one or two other examples included.

TABLE II
DNA amplification-based assays for veterinary bacterial pathogens

Organism	Assay/target gene	Reference
Actinobacillus pleuropneumoniae *A. lignieresii*	PCR/1.5 kb DNA fragment	Sirois *et al.* (1991)
Aeromonas salmonicida	PCR/vapA gene	Gustafson *et al.* (1992)
	PCR/DNA fragment, uncharacterized	Hiney *et al.* (1992)
Bordetella avium	PCR/DNA fragment, uncharacterized	Savelkoul *et al.* (1993)
Bordetella bronchoseptica	PCR-RFLP/pertussis toxin gene	Reizenstein *et al.* (1993)
Borrelia burgdorferi	PCR/*ospA* gene	Malloy *et al.* (1990)
Borrelia corriaceae	PCR/55-60 kDa protein gene	Zingg and LeFebvre (1994)
Brucella spp.	PCR/OMP gene	Kulakov *et al.* (1992)
Campylobacter jejuni	PCR/*flaA* gene	Oyofo *et al.* (1992); Wegmüller *et al.* (1993)
Clostridium perfringens	PCR/epsilon toxin gene	Havard *et al.* (1992)
Coxiella burnettii	PCR/QpH1, QpRS plasmid, 16S rRNA gene, CbbE gene	Willems *et al.* (1993, 1994)
Chlamydia psittaci	PCR/16S rRNA gene	Pollard *et al.* (1989); Thiele *et al.* (1992)
	PCR/MOMP gene	Domeika *et al.* (1994)
Dichelobacter nodusus	PCR/16S rRNA gene	La Fontaine *et al.* (1993)
Ehrlichia canis	PCR/16S rRNA gene	Iqbal *et al.* (1994) Iqbal and Rikihisa (1994)
Ehrlichia risticii	PCR/DNA fragment, uncharacterized	Biswas *et al.* (1991, 1994)
Erysipelothrix rhusiopathiae	PCR/16S rRNA gene	Makino *et al.* (1994)
Escherichia coli	PCR/enterotoxin STIa, LTI; verotoxins VTI+2	Woodward *et al.* (1992)
	PCR/verotoxin VT2	Gradil *et al.* (1994b)
Leptospira spp.[a]	PCR/16S rRNA gene	Merien *et al.* (1992)
	PCR/DNA fragment, uncharacterized	van Eys *et al.* (1989); Gerritsen *et al.* (1991)
	PCR/repetitive element	Woodward *et al.* (1991);
	PCR/repetitive element	Savio *et al.* (1994)
	PCR/DNA fragment, uncharacterized	Kee *et al.* (1994)
Listeria monocytogenes	PCR/*iap* gene	Jaton *et al.* (1992)
	PCR/*hly* gene	Bsat and Batt (1993); Wiedmann *et al.* (1994a)
Mycobacterium paratuberculosis	PCR/IS900	Collins *et al.* (1993a,b)
	PCR/IS900	Sanderson *et al.* (1992); Challans *et al.* (1994)

TABLE II (cont)

Organism	Assay/target gene	Reference
Mycoplasma sp. strain F38 (contagious caprine pleuropneumonia)	PCR/16S rRNA gene	Ros Bascunana *et al.* (1994)
Mycoplasma bovis	PCR/DNA fragment, uncharacterized	Hotzel *et al.* (1993)
Mycoplasma gallisepticum	PCR/DNA fragment, uncharacterized PCR/16S rRNA gene	Nascimento *et al.* (1991); Kempf *et al.* (1993, 1994b)
Mycoplasma hyorhinis	PCR/16S rRNA gene	Dussurget and Roulland (1994); Rawadi *et al.* (1993)
Mycoplasma iowae	PCR/16S rRNA gene	Kempf *et al.* (1994a)
Mycoplasma mycoides subsp. *mycoides*	PCR/Cap21 sequence and DNA fragment, uncharacterized	Dedieu *et al.*(1994)
Mycoplasma synoviae	PCR/16S rRNA gene PCR/DNA fragment, uncharacterized	Lauerman *et al.* (1993) Zhao and Yamamoto (1993)
Pseudomonas mallei, *Pseudomonas pseudomallei*	PCR/23S rRNA gene	Lew and Desmarchelier (1994)
Pasteurella multocida	PCR/*toxA* gene	Nagai *et al.* (1994)
Renibacterium salmoninarum	PCR/p57 gene PCR/DNA fragment, uncharacterized RT-PCR/16S rRNA gene	Brown *et al.* (1994) Leon *et al.* (1994) Magnusson *et al.* (1994)
Salmonella spp.	PCR/DNA fragment, uncharacterized PCR/*invA*, *invE* genes PCR/*spvR* gene PCR/DNA fragment, uncharacterized	Cohen *et al.* (1994a,b,c) Stone *et al.* (1994) Mahon and Lax (1993) Nguyen *et al.* (1994)
Serpulina hyodysenteriae	PCR/DNA fragment, uncharacterized	Elder *et al.* (1994)
Staphylococcus aureus	PCR/*gyrA* gene PCR/*nuc* gene	Zambardi *et al.* (1994) Brakstad *et al.* (1992)
Streptococcus uberis *S. parauberis*	PCR/23S rRNA gene	Harland *et al.* (1993)
Taylorella equigenitalis	PCR/16S rRNA gene	Bleumink-Pluym *et al.* (1994)

[a]All the PCR assays listed detect one or more pathogenic *Leptospira* spp., some of them are specific for a certain serovar; for more details on the specificity of the particular PCR refer to the respective reference

Detection systems for pathogenic bacteria and fungi based on PCR or other DNA amplification techniques usually depend upon the availability of well characterized, genus- or species-specific target sequences. This strategy is easily applied to well-documented bacterial and fungal pathogens, where the sequence of one or more genes is known (e.g. *Listeria monocytogenes*, *Salmonella* spp.). However, for many

animal pathogens there is not sufficient information available for the design of species-specific PCR primers. The 16S rRNA gene, encoding part of the prokaryotic rRNA, consists of both highly conserved and variable regions. The latter regions usually contain at least single base pair differences that are species-specific or longer stretches of genus-specific differences. A general method for PCR amplification and sequencing of this gene has been described by Weisburg and colleagues (1991). Other methods, such as direct sequencing of the 16S rRNA using reverse transcriptase, are also available, although this technique does not always seem to be sufficiently accurate (Collins *et al.*, 1991). After determination of the 16S rRNA gene nucleotide sequence for the organism of interest, species- or genus-specific sequences can be determined by alignment with sequences present in the 16S rRNA database and, where necessary, by sequencing the 16S rRNA for closely related species. Currently, 16S rRNA sequence data are available for more than 1500 bacterial species (Olsen *et al.*, 1994).

TABLE III
DNA amplification-based assays for veterinary fungal pathogens

Organism	Assay/target gene	Reference
Aspergillus spp.	PCR/18S rRNA gene	Melchers *et al.* (1994)
Aspergillus fumigatus	PCR/alkaline protease gene	Tang *et al.* (1993)
	PCR/26S RNA, intergenic spacer region genes	Spreadbury *et al.* (1993)
	PCR/*Asp*f1 gene	Reddy, L.V. *et al.* (1993)
Candida albicans	PCR/EO3 (uncharacterized DNA fragment)	Miyakawa *et al.* (1992)
	PCR/rRNA	Holmes *et al.* (1994)
Cryptococcus neoformans	PCR/5.8S rRNA and internal transcribed spacer genes	Mitchell *et al.* (1994)

While the design of genus-specific PCR primers based on 16S rRNA sequences is relatively easy (see Bleumink-Pluym *et al.*, 1994; Iqbal *et al.*, 1994), the development of species-specific assays is usually more difficult. In many cases, closely related species within the same genus differ only by a single base pair difference in one of the variable regions of the 16S rRNA. Restriction fragment length polymorphism (RFLP) of a PCR amplicon offers one possibility for discriminating these single base pair differences, but only if a suitable restriction site is present. PCR-RFLP is, however, a fairly cumbersome technique which does not lend itself to automation. PCR assays for discriminating single base pair differences have also been described, but such an allele-specific PCR often does not allow reliable discrimination of any single base pair difference (Kwok *et al.*, 1990). A different approach to achieving specific detection of a bacterial pathogen based on species-specific 16S rRNA sequences has been reported by Wiedmann and colleagues (1992). After initial sequencing of the 16S rRNA genes of various *L. monocytogenes*, and of the closely related non-pathogenic bacterium *L. innocua*, consistent single base pair differences specific for *L. monocytogenes* were located (Czajka *et al.*, 1993). These sequences were used to

design LCR primers able to specifically identify *L. monocytogenes*. To improve the sensitivity of this LCR, a set of flanking PCR primers was employed to initially amplify the segment containing the specific single base pair difference (Wiedmann *et al.*, 1992). This PCR-coupled LCR was shown to be highly specific for *L. monocytogenes* and was able to detect a minimum of 10 colony-forming units using a non-isotopic detection method (Wiedmann *et al.*, 1993a).

DNA amplification-based systems can be used simply to detect a bacterial species, as well as to obtain information about the characteristics of these bacteria. For example, in human medical diagnostics, PCR probes have been developed to detect *Staphylococcus aureus* using 16S rRNA or *gyr*A primers and to assess methicillin resistance (Geha *et al.*, 1994; Zambardi *et al.*, 1994). These probes can be employed in a multiplex PCR to identify methicillin resistant and sensitive *S. aureus* strains in a single PCR reaction.

The differentiation of vaccine strains from wild-type strains of the same species can also be achieved using PCR. An example is the detection of the *Mycoplasma gallisepticum* F-vaccine strain using primers for fMGF-1 (Nascimento *et al.*, 1993).

Suitable target sequences for the detection of fungal pathogens are often more difficult to define than those for bacterial pathogens. The characterization of virulence genes in fungi is more demanding, since tools to analyse their genetics are less advanced and their genetic structure is more complex. However, for fungi, cloning and sequencing of the rRNA genes (5S, 5.8S, 18S and 23S), their internal transcribed spacers and non-transcribed spacers can be achieved using PCR primers targeting conserved regions. This genetic information can then be used to achieve a genus- or species-specific detection system analogous to the system described above for the detection of specific 16S rRNA genes in bacteria (see Check, 1994).

The detection of bacterial and fungal pathogens using DNA amplification-based techniques leads to results which have to be interpreted differently from those obtained using cultural methods. PCR and other similar techniques detect DNA from the targeted organism, whether this organism is alive or not. This is of particular concern with regard to the direct detection of microorganisms from environmental samples, where organisms killed by disinfectants might still give positive results by PCR. The detection of non-viable bacteria might, however, offer an advantage over cultural methods if animals treated with antibacterial or antifungal substances are to be tested. In such cases, DNA amplification-based methods might allow diagnosis 'after the fact', where cultural methods would give negative results.

Parasites

The application of DNA amplification methods for diagnosing parasitic infections is still limited, for various reasons. Diagnosis of ectoparasitic diseases can often be achieved by clinical examination of the animal and, in most cases, the parasite can be identified macroscopically or microscopically. Therefore, there is no substantial need for PCR or other DNA amplification methods as a diagnostic tool for ectoparasites. However, in two areas amplification methods are applicable to ectoparasites. PCR has been applied to detect veterinary pathogens carried by these parasites. Examples include the detection of *Borrelia burgdorferi*, the agent of Lyme disease, in *Ixodes* ticks

TABLE IV
DNA amplification-based assays for veterinary parasites

Organism	Assay/target gene	Reference
Anaplasma marginale	PCR/DNA fragment, uncharacterized	Figueroa *et al.* (1993)
Basbesia bigemina	PCR/DNA fragment, uncharacterized PCR/DNA fragment, uncharacterized	Figueroa *et al.* (1992) Figueroa *et al.* (1993)
Babesia bovis	PCR/merozoite surface protein gene PCR/DNA fragment, uncharacterized PCR/apocytochrome *b* gene	Figueroa *et al.* (1994) Figueroa *et al.* (1993) Fahrimal *et al.* (1992)
Cowdria ruminantium	PCR/pCS20 (uncharacterized DNA fragment)	Mahan *et al.* (1992)
Cryptosporidium parvum	PCR/DNA fragment, uncharacterized PCR-RFLP/18S rRNA gene	Webster *et al.* (1993) Awad-El-Kariem *et al.* (1994)
Echinococcus spp.	PCR-RFLP/internal transcribed spacer 1 or rDNA	Bowles and McManus (1993)
Echinococcus granulosus	PCR/conserved gene segment	Bowles *et al.* (1992)
Echinococcus multilocularis	PCR/U1 snRNA gene	Bretagne *et al.* (1993)
Eimeria spp.	RAPD/whole DNA	MacPherson & Gajadhar (1993)
Eimeria tenella	PCR/5S rRNA gene	Stucki *et al.* (1993)
Haemonchus contortus	PCR/β-tubulin gene PCR/small subunit ribosomal DNA	Roos and Grant (1993) Zarlenga *et al.* (1994)
Haemonchus placei	PCR/small subunit ribosomal DNA	Zarlenga *et al.* (1994)
Leishmania spp.	PCR/small subunit rRNA gene	van Eys *et al.* (1992)
Neospora caninum	PCR-RFLP/small subunit rRNA gene	Brindley *et al.* (1993)
Onchocerca ochengi (*O. volvulus*)	PCR/0-150 repeat sequence	Zimmerman *et al.* (1993)
Taenia saginata	PCR/DNA fragment, uncharacterized	Gottstein *et al.* (1991)
Theileria annulata	PCR/small rRNA gene	De Kok *et al.* (1993)
Theileria parva	PCR/repetitive sequence, sporozoite antigen gene	Bishop *et al.* (1992,1993)
Theileria sergenti (*T. orientalis*)	PCR/intraerythrocytic piroplasm surface protein	Tanaka *et al.* (1993)
Toxoplasma gondii	PCR/rRNA gene PCR-RFLP/*SAG*1, *SAG*2 and *ROP*1 locus PCR/B1 and S48 genes	Guay *et al.* (1993) Howe and Sibley (1994) Wastling *et al.* (1993)

TABLE IV (cont)

Organism	Assay/target gene	Reference
Trichinella spp.	RAPD/whole DNA	Dupouy-Camet *et al.* (1994)
	PCR/53 kDa antigene and 1.6 kb repetitive sequence	Dick *et al.* (1992); Soulé *et al.* (1993)
Trypanosoma spp.	MVR-PCR/whole DNA	Majiwa *et al.* (1994); Masiga *et al.* (1992)
	RAPD/whole DNA	Steindel *et al.* (1993)
Trichomonas fetus	PCR/DNA fragment, uncharacterized	Ho *et al.* (1994)
Trichostrongylus colubriformis	PCR/β-tubulin gene	Roos and Grant (1993)

(Persing, 1991) and of arthropod-borne viruses, which are transmitted by mosquitoes, ticks and other blood-feeding arthropods (Ward *et al.*, 1990; Vodkin *et al.*, 1993, 1994). PCR allows easy screening of large numbers of samples and is therefore helpful for detecting infected vectors in epidemiological surveys. Another application for PCR-based techniques is the determination of phylogenetic relationships among species of parasites, which enables reassessment of their current taxonomic classification (Brindley *et al.*, 1993). Owing to the complexity of these organisms and the lack of sufficient sequence information, modified PCR techniques have been applied for DNA fingerprint analysis in parasites. These include amplification of minisatellite repeats (MVR-PCR; for review see Arnot *et al.*, 1994), random amplified polymorphic DNA (RAPD; MacPherson and Gajadhar, 1993; Dupouy-Camet *et al.*, 1994) or the amplified polymorphic-PCR (AP-PCR; McClelland and Welsh, 1994).

PCR-based fingerprinting techniques have also been applied to helminths and protozoan parasites (see Table IV). An increasing number of purely diagnostic applications of PCR techniques have been described, mostly for protozoan parasites (Wilson, 1991; van Eys *et al.*, 1992; Webster *et al.*, 1993; Awad-El-Kariem *et al.*, 1994; Figueroa *et al.*, 1994; Howe and Sibley, 1994; Majiwa *et al.*, 1994). Because of the intracellular localization of most protozoans, accurate identification of the infective agent is difficult by conventional methods. Molecular approaches, such as DNA hybridization assays, have been applied, but they are sometimes not sufficiently sensitive (MacPherson and Gajadhar, 1993). Conversely, the sensitivity of a diagnostic test is not an important issue for a wide variety of diarrhoea-causing protozoans, because they are usually shed in high numbers. Rather, rapid and convenient identification of different *Coccidia* spp. often proves difficult because unequivocal morphological markers are missing (Brindley *et al.*, 1993). The identification of more virulence genes and of specific DNA markers for all kinds of pathogenic parasites will probably facilitate the development of further DNA-based assays for veterinary parasites in the near future. This has been done for human parasites, e.g. *Plasmodia*, *Trypanosoma* and *Leishmania*. Nevertheless, application of such assays will only be important for routine diagnostic purposes where conventional methods cannot provide either the necessary sensitivity or a quick and reliable identification of the species.

Hereditary diseases

Over the last few years, a variety of hereditary diseases in animals have been traced back to the respective genes and mutations responsible for the biochemical defects and clinical syndromes. PCR facilitated the use of this information for rapid diagnostic assays which allow the screening of large numbers of animals. The majority of hereditary diseases are caused by single base pair mutations, which in many cases result in the loss or acquisition of a restriction enzyme recognition site. Most assays for the detection of these single base pair mutations are based on primary PCR amplification of a region containing the polymorphic site, followed by a restriction digest of the PCR product with a suitable restriction enzyme, such as PCR-RFLP. Current assays for BLAD, citrullinaemia and hyperkalaemic periodic paralysis provide examples of this type of assay (see Table V). For some hereditary diseases, routine diagnosis now involves DNA sequencing after primary PCR amplification of mRNA or of the afflicted exon (Zheng *et al.*, 1994). This approach is usually applied where no specific single base pair change can be linked to a hereditary condition or where no change in a restriction site coincides with a given mutation (Zheng *et al.*, 1994).

While PCR-RFLP provides a good diagnostic system for detecting carriers of many disease alleles, this system has two major disadvantages. First, not all potential single base pair mutations lead to a change in a restriction site. Second, although screening of large numbers of animals can be performed by PCR-RFLP, the technique is very cumbersome and time-consuming and cannot easily be integrated into an automated format. Recently, a non-isotopic LCR assay has been described for the detection of BLAD which overcomes these disadvantages (Batt *et al.*, 1994).

A list of DNA amplification-based assays for the detection of hereditary diseases is given in Table V.

Other diagnostic applications

The application of DNA amplification methods is not limited to the diagnosis of infectious or hereditary diseases. This section gives an overview of other applications in veterinary diagnostics, excluding purely research-oriented areas, such as the detection of mRNA for cytokines or other immunomediators.

PCR or PCR-RFLP analysis is often used in animal breeding to determine the genotype of animals for specific production traits, e.g. the genotype for certain milk proteins. Examples include typing for β-lactoglobulin alleles in sheep (Schlee *et al.*, 1993) or for α-S1-casein, β-casein, κ-casein, β-lactoglobulin or α-lactalbumin alleles in cattle (Pinder *et al.*, 1991; Schlieben *et al.*, 1991; Sulimova *et al.*, 1991; David and Deutch, 1992; Rottmann and Schlee, 1992; Schlee and Rottmann, 1992; Wilkins and Kuys, 1992). Another example of a PCR application in animal breeding is an assay for the sex-linked late-feathering gene in chickens (Iraqi and Smith, 1994).

Only the advent of PCR allowed preimplantation sex determination in embryo transfer. Usually, male sex is determined using primers specific for the sex-determining region on the Y chromosome (SRY). Such PCR assays have been described for cattle, goats, sheep, pigs and a variety of other animals (Miller, 1991; Bredbacka and Peippo, 1992; Kageyama *et al.*, 1992; Rao and Totey, 1992; Utsumi *et*

TABLE V
DNA amplification-based assays for the detection of animal hereditary diseases

Hereditary disease	Assay	Reference
Bovine leukocyte adhesion deficiency (BLAD)	PCR-RFLP PCR-coupled LCR	Shuster *et al.* (1992) Batt *et al.* (1994)
Canine mucopolysaccharidosis I	PCR-RFLP	Menon *et al.* (1992)
Canine rod–cone dysplasia type 1 (rcd1)	PCR-RFLP and PCR double stranded conformational polymorphism	Ray *et al.* (1994,1995)
Canine X-chromosome-linked hereditary nephritis	PCR DNA-sequencing	Zheng *et al.* (1994)
Citrullinaemia	PCR-RFLP	Dennis *et al.* (1989)
Congenital hypothyroidism	PCR-RFLP	Schwerin *et al.* (1994)
Deficiency of uridine monophosphate synthetase (DUMPS)	PCR-RFLP	Schwenger *et al.* (1993)
Hyperkalaemic periodic paralysis	PCR-RFLP	Rudolph *et al.* (1992)
Malignant hyperthermic syndrome (MHS)		Brening & Brem (1992); Brem & Brening (1993); Otsu *et al.* (1992)

al., 1992; Horvat *et al.*, 1993; Saitoh and Totsukawa, 1993; Kawarasaki *et al.*, 1994). Recently, a multiplex PCR has been described which can be used to screen bovine preimplanted embryos for sex and four genetic diseases (Schwerin *et al.*, 1994). PCR has also been used to prescreen microinjected bovine embryos for the presence of a transgene construct (Horvat *et al.*, 1993). Another application of PCR with male sex-specific primers is the determination of bovine chimerism in female calves co-twin to a male (so-called 'freemartins') (Grobet *et al.*, 1992; Lipkin *et al.*, 1993).

Forensic veterinary applications of DNA amplification-based systems include the species determination of meat and carcasses. Species-specific primers have been described for sheep, goats and cattle (Chikuni *et al.*, 1994; Wagner *et al.*, 1994). Species-specific primers for the amplification of regions containing a variable number of tandem repeats have been used to determine parentage in most domestic animal species (Buitkamp *et al.*, 1994).

CONCLUSIONS AND OUTLOOK

Nucleic acid-based technology will have a large impact on the diagnosis and monitoring of many animal diseases. High demands have been placed on DNA amplification techniques. With regard to speed, reliability and cost, these techniques,

and in particular PCR, are often superior to conventional diagnostic methods. However, in contrast to ELISA systems, there are only a few PCR kits for human or veterinary pathogens commercially available.

A patent covering the PCR process and patents for specific products, such as purified thermostable enzymes, are held by Hoffman-LaRoche. This means that the user of the PCR process needs to be properly licensed. For research and development applications, PCR can be performed if licensed enzymes and licensed instruments are used. In contrast, when a PCR assay is to be routinely used in commercial veterinary diagnostics, an end-user 'service license' has to be acquired. The contract with Hoffman-LaRoche includes a royalty obligation and enables the licensee to perform any kind of veterinary diagnostic testing. The royalty rate varies between 5% and 15%, depending on the application (A. Junosza-Jankowski, PCR Licensing Manager, Roche Diagnostic Systems, personal communication, 1995).

In veterinary diagnostics, the application of PCR is currently restricted to fully equipped diagnostic laboratories. Its implementation by veterinary practitioners under field conditions is not straightforward, but nor is it insurmountable (Barker, 1994). Recent developments allow the detection of amplification products by a system which uses microtitre plates. Labelling of the primers with digoxigenin, biotin or different fluorogens has been successfully applied in LCR and PCR (e.g. Wiedmann *et al.*, 1993a). While the ligand group is captured by an immobilized 'receptor' (e.g. biotin–streptavidin), the reporter group on the other end of the amplicon can be used for the detection. Other approaches use microtitre plates coated with a probe to capture the PCR product, which is then detected by a second probe. Very recently, a new approach for the detection and quantification of PCR products has become commercially available. Taqman™ (Perkin Elmer/Applied Biosystems Division) uses a probe which is located between the PCR primers. This probe is labelled with a fluorescent reporter and quencher dye. The fluorescence emitted by the reporter dye is normally quenched by the quencher dye but increases upon hydrolysis of the probe, an event that only occurs during amplification of the target DNA. This system has the potential to allow detection and quantification of PCR products in a microtitre plate in less than 10 minutes (Lee *et al.*, 1993; Anonymous, 1994).

We believe that DNA amplification-based methods will replace conventional methods in some fields of diagnostics, such as hereditary diseases where the dysfunctional genes are known and viral diseases of persistently infected animals. In other areas, DNA amplification methods will be used to complement conventional diagnostics.

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