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Review

Detection of Animal Pathogens by Using the Polymerase Chain Reaction (PCR)

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SUMMARY
The polymerase chain reaction (PCR) is a nucleic acid-based technique that enables the rapid and sensitive detection of specific micro-organisms. Although this technique is widely used in veterinary research, it has not yet found applications in routine microbiological analysis of veterinary clinical samples. However, advances in sample preparation together with the increasing availability of specific gene sequences will probably lead to the more widespread diagnostic use of PCR in the future. PCR is likely to have a strong impact in the epidemiology, treatment and prevention of animal infectious diseases.

KEYWORDS: PCR; diagnosis; virus; bacteria; parasites.

INTRODUCTION
The development of polymerase chain reaction (PCR) has revolutionized the field of molecular biology. The technique consists basically of the enzymatic synthesis of millions of copies of a target DNA sequence (Saiki et al., 1985). Using a thermostable DNA polymerase (Saiki et al., 1988), and a succession of cycles that includes denaturation of the template DNA, hybridization of specific DNA primers to the template and extension of the primers, it is possible to generate multiple copies of the target region enzymatically. Thus, PCR provides a method for obtaining large quantities of specific DNA sequences from small amounts of DNA, including degraded DNA samples. The technology has been extensively reviewed (see for example, Ehrlich, 1989; Innis et al., 1990; Griffin & Griffin, 1994). Although PCR is widely used in an increasing number of applications, those in the area of microbiology and diagnosis of infectious diseases have undergone outstanding advances in recent years.

PCR IN VETERINARY MICROBIOLOGY
Traditionally, strategies for identifying most microbial pathogens involve isolation on selective agar media or cell cultures, and the use of phenotypical tests but these techniques are usually slow and laborious. The important cost that animal infectious diseases can have on national economies has therefore stimulated the search for faster, more sensitive and more specific methods to identify microbial pathogens. Many useful nucleic acid probes and immunological assays have been developed for diagnostic purposes, but these techniques also have some deficiencies (Jones & Bej, 1994). The emergence of PCR, however, offers the potential to improve the laboratory-based diagnosis of pathogens (Mahbubani & Bej, 1994). Although PCR has some shortcomings, such as the problems caused by contaminants and inhibitors or the lack of suitable sequences for designing specific primers, the outstanding research effort focused on this technique, together with the remarkable development of molecular biology have minimized the deficiencies and allowed its increased general use as a diagnostic tool.

VIRUSES
Ruminants
Foot-and-mouth disease virus (FMDV). FMDV is
one of the most dangerous viruses of ruminants. Its speed of spread and ability to change its antigenic identity makes FMDV very threatening to the beef and dairy industries of many countries. Fast and accurate detection of FMD outbreaks is needed to limit spread of the disease. The virus consists of 60 copies of each of the four proteins VP1 to 4, of which VP1 is the main protein determining antigenic identity. PCR systems for detecting FMDV have been developed by different laboratories (Meyer et al., 1991; Laor et al., 1991, 1992). There are also reports on the use of PCR to determine FMDV serotype (Rodríguez et al., 1992; Stram et al., 1993, 1995).

Rinderpest (RPV). Animal diseases greatly influence animal production and trade. Diagnosis should enable fast implementation of control measures to minimize losses. This is particularly important in the case of highly-contagious pathogens such as RPV and peste des petit ruminants (PPRV) viruses. They are, at present, confined to developing countries where they remain a constant threat to livestock. RPV may infect all artiodactyls, with cattle and buffaloes being the most susceptible species. While PPRV specifically causes disease in small ruminants. Field diagnosis of classical rinderpest, with its many indicative clinical signs, is easy but those signs are not always clearly seen, particularly in countries where the disease is endemic (Diallo et al., 1995). Moreover, some mild strains can fail to produce clinical signs unless the infected animals are stressed. The situation is more complicated in small ruminants because they can be infected with RPV and/or PPRV, and the disease produced by both viruses is similar. The laboratory tests commonly used are either expensive (animal inoculation), slow (virus isolation or neutralization) or insensitive (agar gel immunodiffusion). PCR can drastically improve the diagnosis. Chamberlain et al. (1993) grouped different isolates of RPV combining PCR with sequencing studies. Using the same procedure, Barrett et al. (1993) identified two different RPV strains in the same clinical sample and Warmwayi et al. (1995) showed that there was co-circulation of two different lineages of RPV in Nigeria during the epizootics of the 1980s.

Bovine viral diarrhoea virus (BVDV). BVDV is another important pathogen of cattle, causing considerable economic losses throughout the world. Three syndromes caused by BVDV have been described: an acute gastroenteritis with severe diarrhoea, mucosal disease, and chronic infections of several weeks' duration in calves up to 1-year-old. Persistently infected animals are the main source of infection to herdmates because they continually shed large quantities of virus in body secretions and excretions. Due to the obvious impact of BVDV infections, screening of animals must be carefully made and current methods of detection, such as virus isolation or immunoassays, either lack optimal sensitivity or rapidity for consistent and large scale testing in animal specimens (Radwan et al., 1995). PCR, however, can readily detect BVDV (Belak & Ballagi-Pordany, 1991; Brock, 1991; Hertig et al., 1991; Ward & Misra, 1991; Hoft van Iddekinge et al., 1992; Gruber et al., 1994), and PCR analysis of bulk tank milk samples has provided a rapid and sensitive method to screen herds for the presence of the virus. Sensitive studies using reference strains of BVDV from persistently infected carriers have shown that reverse transcription (RT)-PCR has greater sensitivity than other tests, including enzyme-linked immunosorbent assay (ELISA) (Horner et al., 1995); unfortunately, cost currently makes this technique unsuitable for large-scale testing but it should be valuable as a confirmatory test in cases where ELISA results are in the ‘suspicious range’ or where the viral titre is low, such as in batches of foetal bovine serum. Additionally, it is possible to discriminate among different BVDV strains using PCR (Tajima et al., 1995) and PCR-restriction fragment length polymorphism (RFLP) tests have demonstrated that 13 BVDV isolates from ruminants on four different farms in Sweden were herd-specific rather than species-specific, and that the virus is readily transmitted between cattle and sheep (Paton et al., 1995).

Bluetongue (BT). BT is an arthropod-borne viral infection of ruminants caused by bluetongue virus (BTV). Clinically, BT varies depending on factors such as population density and competence of the Culicoides sp. vector, the distribution of susceptible hosts and the virulence of the different serotypes of BTV. Among ruminants, only sheep are clinically affected while cattle are usually asymptomatic reservoirs. PCR-based procedures have been developed for the diagnosis of BTV (Gould et al., 1989; Dangler et al., 1990; Wade-Evans et al., 1991; McColl & Gould, 1991; Akita et al., 1993; Parsonson et al., 1994). Whole blood seems to be the most convenient clinical
sample, but fractions of blood have also been successfully used for PCR detection of BTV infection in sheep (McColl & Gould, 1994). In a comparison of methods for isolation of BTV in infected calves, virus was detected in embryonating chicken eggs for 2–8 weeks, whereas PCR detected BTV nucleic acid for 16–20 weeks (MacLachlan et al., 1994). The sensitivity of the technique means that it can be adapted to detected BTV in Culicoides sp. samples (Wilson & Chase, 1993).

Epizootic haemorrhagic disease virus (EHDV). EHDV is an orbivirus related to BTV that causes fatal haemorrhagic disease in domestic and wild ruminants. Clinical signs and pathological changes caused by EHDV and BTV are indistinguishable. Aradaib et al. (1995) compared the value of PCR with virus isolation for the detection of EHDV in clinical samples taken from naturally-infected deer, and concluded that PCR assays for EHDV can provide a diagnostic alternative superior to the current cumbersome and time-consuming virus isolation procedures.

Bovine immunodeficiency virus (BIV). BIV is structurally and genetically related to human immunodeficiency virus (HIV). BIV causes lymphoproliferative changes and enlargement of subcutaneous lymphatic nodules in cattle. Although infection by BIV is widely prevalent in beef and dairy cattle, there is no accurate diagnostic test for the virus. Using PCR, Nash et al. (1995) detected BIV-infected leucocytes in the blood and milk of BIV-seropositive cows. These data confirmed the presence of BIV in milk and highlighted the potential for lactogenic transmission of the virus. Suarez et al. (1995) examined blood samples from BIV-experimentally infected calves by virus isolation, protein immunoblot and nested PCR and showed that the nested PCR test is more sensitive than any other method for the detection of BIV infection in cattle.

Bovine herpesvirus-1 virus (BHV-1). BHV-1 causes infectious bovine rhinotracheitis (IBR), an economically important disease of cattle characterized by acute respiratory infection and reproductive problems such as abortion, infertility, vulvovaginitis and balanoposthitis. Latently infected animals can be reservoirs of BHV-1 in the herd. Virus detection is often requested for the laboratory diagnosis of most cases of respiratory and reproductive problems in cattle. Several reports have described the PCR of different BHV-1 genes from tissue cultures (Vilcek, 1993; Kibenge et al., 1994; Yason et al., 1995) and bovine semen (Wiedman et al., 1993; Xia et al., 1995).

Louping-ill, Turkish sheep encephalitis (TSE) and Spanish sheep encephalitis (SSE). All three viruses belong to the tick-borne encephalitis virus group, within the genus Flavivirus. These viruses produce a similar clinical syndrome, and the histological changes that they produce in the brains of affected sheep are indistinguishable. Moreover, animals from affected flocks have antibodies that cross-react with the other viruses (Gonzalez et al., 1987). Sequencing of PCR products obtained from cDNA of SSE have permitted the location of specific genetic markers for this flavivirus (Marin et al., 1995). PCR has also enabled the construction of recombinant vaccinia virus expressing PrM and E glycoproteins of louping-ill virus (Venugopal et al., 1994).

Caprine arthritis encephalitis (CAE). CAE is a worldwide multisystemic disease of domestic goats, characterized by progressive arthritis, leucoencephalomyelitis and mastitis. Although the virus persists for life, infection of goats with CAE is often subclinical. Isolation of CAE is not attempted routinely as a diagnostic tool but PCR has recently been adapted for the detection of proviral DNA in CAEV-infected cells from clinical specimens (Clavijo & Thorsen, 1995). The technique has a sensitivity which is several orders of magnitude higher than direct hybridization, and may represent an important alternative procedure for identification of persistently infected animals.

Other ruminants viruses for which PCR protocols have been successfully developed include bovine leukaemia virus (Naif et al., 1990, 1992; Murtaugh et al., 1991; Ballagi-Pordany et al., 1992; Sherman et al., 1992; Agresti et al., 1993; Kelly et al., 1993), bovine coronavirus (Verbeek & Tijssen, 1990), rotavirus (Xu et al., 1990), and Maedi-visna virus (Staskus et al., 1991; Zanoni et al., 1992).

Pigs

Porcine parvo virus (PPV). The role of PPV in inducing swine reproductive failure characterized by embryo and fetal deaths has been extensively described, often when other clinical signs of disease are lacking. Sources of PPV include contaminated semen, the female reproductive tract or
exposure during gamete/embryo manipulation. Molitor et al. (1991) developed a PCR amplification test for the detection of PPV thereby minimizing the risk of transmission of PPV to seronegatives recipients through embryo transfer (Gradil et al., 1994).

**Swine influenza.** Swine influenza induces high morbidity and low mortality in pig populations throughout the world. Although the disease usually resolves, infected pigs represent a substantial source of economical loss to the producer because of their weight loss and poor weight gain. The results obtained by Schorr et al. (1994) proved that RT-PCR from nasal swabs specimens of pigs is significantly more sensitive than the techniques currently used such as the infectivity assay in embrionating chicken eggs.

**Porcine reproductive and respiratory syndrome (PRRS).** The disease complex known as PRRS has become an economically important health problem throughout Europe and North America. PCR has been used to confirm the presence of PRRS genes in infected monolayers (Katz et al., 1995), thus providing the first steps for the development of a PCR test to analyze PRRS virus in clinical samples.

**Pseudorabies virus (PRV).** PRV is the aetiological agent of a major disease that has substantial economical impact in swine industry. The disease is fatal to young pigs but in adults the infection is less severe, and sometimes clinical signs are not apparent. Pigs surviving PRV infection remain latently infected for life. PCR has become the recommended method for evaluating PRV latency; reports from several laboratories have indicated that neuronal tissues, and especially the trigeminal ganglia, are the most reliable sources for detection of latent PRV genome (Belak et al., 1989; Wheeler & Osorio, 1991; Volz et al., 1992; Brockmeier et al., 1993) but trigeminal ganglion assay can be performed only after death of the affected animal. Tonsil biopsy specimens can be obtained from live animals and used to amplify PRV sequences by PCR (Chung, 1995). PRV has also been detected in the semen of boars (Guérin et al., 1995). The method is simple and allows the detection of around 370 viral DNA sequences per microlitre of sample. PRV infects cells of the lymphatic tissue and white blood cells of a variety of mammals. These cells are also present in sausages, and Schunk & Rziha (1994) established a PCR method specifically to detect PRV in artificially contaminated sausages and showed that PCR was less affected by extreme pH values than tissue culture techniques usually employed to recover the virus.

Other important swine virus that have been detected by PCR include hog cholera virus (Boyce et al., 1991; Liu et al., 1991; Wirz et al., 1993) and African swine fever virus (Steiger et al., 1992).

**Poultry**

Intensive breeding of poultry means that high populations often live in confined spaces. Under such conditions, the entry of a virulent virus can cause high mortality and big economical losses. Rapid diagnostic tests are needed to minimize the consequences of viral outbreaks in these environments. When compared with virus isolation and other classic techniques, PCR is the method of choice for diagnosis of many poultry viruses including Marek's disease virus (Becker et al., 1992, 1993; Silva, 1992; Zhu et al., 1992; Davidson et al., 1994; Zerbes et al., 1994), reticuloendotheliosis virus (Aly et al., 1993; Davidson et al., 1994), avian leucosis virus (van Woensel et al., 1992), infectious bronchitis virus (Andreasen et al., 1991; Lin et al., 1991; Jackwood et al., 1992; Zwaagstra et al., 1992; Kwon et al., 1993a, b), Newcastle disease virus (Jestin & Jestin, 1991), lymphoproliferative disease virus (Sarid et al., 1994) and infectious bursal disease virus (Lee et al., 1992; Wu et al., 1992a, b).

**Horses**

**Equine viral arteritis (EVA).** EVA is a ubiquitous disease present throughout mainland Europe. The variety and severity of clinical signs vary widely from inapparence to abortion and death. A proportion of seropositive stallions shed the causal organism, equine arteritis virus (EAV), in their semen, and play a primary role in its dissemination and perpetuation in the equine population. Therefore, when a stallion is identified as EAV positive, the first priority is to ascertain whether virus is being shed before the animal is allowed to cover mares. PCR is included among the three methods that may be used to establish the presence of virus in the semen (Chirnside & Spaan, 1990; Horserace Betting Levy Board, 1993).

**Equine herpesvirus.** PCR has been successfully
applied to detect EHV 1 and 4 in aborted equine fetuses (Ballagi-Pordany et al., 1990) and in nasopharyngeal swab specimens from horses with respiratory or neurological disease (Sharma et al., 1992; Wagner et al., 1992).

Other equine viral diseases which have been diagnosed by PCR include equine infectious anaemia (O’Rourke et al., 1991) and African horse sickness (Zientara et al., 1993; Stone-Marschat et al., 1994).

Dogs

Rabies. Rabies is still one of the most life-threatening zoonosis in some regions of the world. Obviously, fast and accurate detection of infected animals is of vital importance. Research results have shown that PCR can play a remarkable role in the rapid, sensitive and specific detection of the rabies virus (Ermine et al., 1990; Sacramento et al., 1991; Shankar et al., 1991; Kamolvarin et al., 1993; McColl et al., 1993) and the technique should spread among the reference laboratories located in regions at risk.

Canine parovirus (CPV). CPV is the causative agent of haemorrhagic enteritis and myocarditis, and at present is one of the most common pathogenic viruses causing diarrhoea in dogs. CPV is not easily inactivated with the usual disinfectants, and can survive more than 3 months once a hospital or kennel is contaminated, often leading to secondary infections. As a result, it is important to have a rapid, specific and sensitive method to distinguish infected from uninfected dogs. PCR assays based on VP1 and VP2 genes have been used to detect CPV in paraffin-embedded tissues (Truyen et al., 1994; Uwatoko et al., 1995) and in faeces of diarrhoeic dogs (Hirasawa et al., 1994). Additionally, PCR-RFLP analysis is a practical and reliable method for differentiating wild- and vaccine-type CPVs (Hirasawa et al., 1995; Senda et al., 1995).

Canine distemper virus (CDV). CDV induces a multifocal demyelinating disease in the central nervous system of dogs, in which virus persistence plays a key role. PCR has been an essential research tool to study the virus’s nucleocapsid protein, and to provide a molecular basis for the observed differences in virus release and spread between attenuated and virulent CDV (Stettler & Zurbriggen, 1995).

Cats

Feline infectious peritonitis virus (FIPV). FIPV causes a severe, often fatal disease in domestic and wild cats. Despite considerable research, no routine diagnostic method is available. Detection of FIPV by nested PCR has been attempted (Egberink et al., 1995) but the authors concluded that the value of PCR for the identification of sick animals and asymptomatic carriers needed to be further studied. In their work a positive PCR in healthy animals failed to provide an absolutely definitive diagnosis of FIP; equally, a negative PCR result from a sick animal did not completely exclude FIP.

Better results have been achieved in the PCR detection of active and latent feline herpesvirus 1 (Nunberg et al., 1989; Reubel et al., 1993) and feline immunodeficiency virus (Rimstad & Ueland, 1992).

Marine mammals

Morbillivirus infections in marine mammals were first reported in 1988, and are known to be distributed among a wide spectrum of seals and cetaceans in the Atlantic ocean and the Mediterranean sea. RT-PCR has revealed that there were no obvious links between the morbillivirus outbreak in marine seals in Northern Europe in 1988 and that which occurred in freshwater seals in Lake Baikal in 1987 (Visser et al., 1990; Barrett et al., 1992). Direct sequencing of PCR products that included the haemagglutinin protein gene of the Lake Baikal seals isolate (PDV-2) revealed that it was closely related to two isolates of CDV from Germany but different from CDV vaccines currently used in the Lake Baikal region (Mamaev et al., 1995).

BACTERIA

Staphylococcus

Staphylococcal mastitis is an important problem in dairy farms. Several staphylococci, mainly Staphylococcus aureus strains, cause acute and chronic mastitis, and can lead to gangrenous mastitis. Human handling of the udder or the milking machine is a potential source of staphylococci, and contaminated milk can be the cause of foodborne intoxication in man. Rapid detection of
staphylococci, including those killed by heat treat-
ment, in suspected food could prevent foodborne
staphylococcal gastroenteritis, and differentiation
of *S. aureus* strains has been achieved by DNA
amplification fingerprinting (Saurnier et al., 1993;
Van Belkum et al., 1993).

**Listeria monocytogenes**

Although *Listeria monocytogenes* infection may
produce clinical syndromes of abortion and neo-
natal septicemia, encephalitis is most common in
adult animals. The clinical diagnosis of listeric
encephalitis in ruminants is difficult because of
the existence of a broad spectrum of central ner-
vous system diseases with similar clinical symp-
toms. In addition, listeria can only rarely be cul-
tured from the cerebrospinal fluid (CSF) of
affected animals. Because PCR is able to detect
low numbers of bacteria, it may be a tool for
increasing the sensitivity of listeria detection in
CSF of ruminants (Peters et al., 1995). It is also
important to detect asymptomatic carriers because
of the zoonotic nature of the infection. During
the last decade several outbreaks and single cases
of human listeriosis have demonstrated that the
disease is often transmitted by contaminated food.
Jaton et al. (1992) developed a sensitive nested
PCR assay for the detection of *L. monocytogenes* in
human CSF. Additionally, PCR has confirmed its
usefulness to detect specific strains in the epidemi-
ological investigations of listeriosis (Ericsson et al.,
1995).

**Anthrax**

Anthrax is a fatal infection of humans and live-
stock that is caused by the Gram-positive, endos-
pore-forming bacterium *Bacillus anthracis*. Human
are infected primarily through contact
with products derived from contaminated ani-
mals. There is a growing need for methods to
detect *B. anthracis* spores and vegetative cells, not
only to prevent large-scale livestock destruction,
but also to protect humans that may come into
contact with them. PCR amplification of some *B.
anthracis* genes has already been reported (Carl et
al., 1992; Turnbull et al., 1992; Hutson et al., 1993;
Johns et al., 1994; Reif et al., 1994), allowing the
detection of even a single spore of *B. anthracis*
(Reif et al., 1994). Henderson et al. (1994) exam-
ined the variation among isolates of *B. anthracis*
using restriction patterns and PCR and found that
the *B. anthracis* profiles were unique when
compared with those of closely related species,
including *B. cereus, B. thuringiensis* and *B. mycoides.*
Their results showed that isolates of *B. anthracis*
are almost completely homogeneous and distinct
from other members of the *B. cereus* group.

**Clostridium botulinum**

Botulism is a severe foodborne disease caused
by *Clostridium botulinum* and is characterized by
generalized flaccid paralysis. Botulinal neuro-
toxins, produced by seven distinct serological
types of *C. botulinum* are among the most potent
biological substances known and neurotoxins A,
B, C, D, E and F have all been implicated as causes
of human and/or animal disease. The mouse
bioassay is the established method for the detect-
tion of neurotoxin but alternatives to the use of
animals for diagnostic purpose are ethically desir-
able and should be encouraged. Some immunol-
ogical methods have been proposed but the use of
DNA-based techniques has not been extensively
explored. However, some authors have confirmed
that PCR has a great potential for the identifi-
cation of botulism neurotoxin-producing strains
(Szabo et al., 1992, 1993; Fach et al., 1995), and
clearly demonstrated that PCR methods should be
used for the development of highly sensitive and
specific assays for organisms harbouring botulism-
neurotoxin genes.

**Clostridium perfringens**

*Clostridium perfringens* enterotoxin genes have
also been detected in stools without isolation of
the organism (Saito et al., 1992; Fach et al., 1993).
Although the isolates were from human food-
poisoning outbreaks or sporadic diarrhoeal cases,
*C. perfringens* is also a well-known animal patho-
gen, being the aetiological agent of haemorrhagic
and necrotic enteritis. Thus, the application of
PCR should be desirable and appropriate in veter-
inary laboratories. A PCR assay has in fact been
developed recently for the rapid detection of
genes encoding *C. perfringens* enterotoxins (Buogo
et al., 1995), and successfully applied in samples of
small and large intestine from infected piglets.

**Escherichia coli**

Enterotoxigenic *Escherichia coli* (ETEC) is a
major cause of diarrhoea in neonatal and post-
weaned calves, lambs and piglets. Several fimbrial
adhesins and enterotoxins are recognized as the
virulence factors of ETEC. The sequencing of the
enterotoxins and fimbrial genes have made possi-
ble the application of nucleic acid-based methods
for their detection (Harel et al., 1991; Woodward et al., 1992). These methods have the advantage that they are readily applicable to a large number of isolates, in contrast to classic methods such as agglutination, infant mouse, ligated swine intestine and cell culture assays. PCR results obtained in Sweden by Kennan et al. (1995) showed that the gene for the major subunit of F107 fimbria was present on approximately half of the strains not expressing K88, K99, 987P and F41 fimbria isolated from piglets older than 1 week with diarrhoea. This suggested that F107 fimbria are of major importance among ETEC strains causing post-weaning diarrhoea. Ojeniyi et al. (1994) applied two different genotyping methods, colony hybridization and PCR, to detect enterotoxin, verocytotoxin and fimbrial genes in 708 E. coli strains from piglets with diarrhoea, and the results were compared with those obtained by phenotypic methods. The correlation between the genotypic and phenotypic results was 97.7–100%. Detection of fimbrial and enterotoxin genes detected more pathogenic strains than the serotyping using a set of rabbit OK antisera. Using such techniques, the verocytotoxin and the fimbrial F107 genes were found to be more frequent in post-weaning than in neonatal E. coli strains and genotypic tests are becoming valuable tools in the identification of pathogenic E. coli.

Together with staphylococcal mastitis, coliform mastitis is a major problem in dairy farms. Identification of E. coli strains from cows with clinical mastitis can be accomplished by PCR amplification using repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences. Such procedure has revealed that E. coli strains isolated from repeated episodes of clinical mastitis in the same cow have similar genotypes (Lipman et al., 1995).

In Western countries, enterohaemorrhagic E. coli (EHEC), especially serotype O157:H7, have become a major concern for human health. EHEC strains produce verocytotoxins, and have been identified as causative agents of human diarrhoea, haemorrhagic colitis (HC), haemolytic-uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Cattle seem to be the most important reservoir of EHEC, and although EHEC can produce haemorrhagic colitis in calves, many healthy animals are carriers. The high levels of EHEC carriage among young animals is of concern as meat may be a significant source of transmission from bovines to humans. Because verocytotoxin genes can be detected by PCR (Smith et al., 1988; Tyler et al., 1991), this technique has become useful to determine the prevalence and clinical significance of EHEC isolated from cattle herds with and without calf diarrhoea. Burnens et al. (1995) found a 20% level of EHEC carriage among cows, but it was reassuring that no EHEC were detected in milk samples.

Salmonella

Enteric disease caused by infection with Salmonella is an important cause of morbidity in animals. S. enteritidis in particular is associated with human food-borne illness resulting from the consumption of contaminated poultry eggs or meat. Salmonellas are generally identified by microbiological culture of faeces, tissue or body fluids. Although ELISAs may be used to identify salmonellas, full identification still requires culture. Amplification of salmonella genes offers a specific and direct means of detection (Rahn et al., 1992; Widjojoatmodjo et al., 1992; Aabo et al., 1993; Cohen et al., 1993; Way et al., 1993; Nguyen et al., 1994; Wood et al., 1994). Booster PCR methods for the genus-specific detection of salmonellas in equine and chicken faeces have been developed (Cohen et al., 1994a, b) with detection possible within 10–12 h from the time of submission of samples. Although booster PCR is highly sensitive, its cost is about twice that of a simple PCR reaction. Cohen et al. (1995) described an alternative method using enrichment followed by a simple PCR reaction that enabled Salmonella to be detected in faeces within 24 h of submission of samples. A quantitative method using a known quantity of competitor DNA to quantify the numbers of salmonellas in chicken faeces has also been developed (Mahon & Lax, 1995), but some problems with inhibitory substances have been reported. Comparison of PCR and microbiological cultures for the detection of salmonellas in drag-swabs from poultry houses have revealed that PCR is significantly more sensitive than culture for environmental monitoring (Cohen et al., 1994c).

Yersinia enterocolitica

Y. enterocolitica also causes food-borne human gastroenteritis, with pigs implicated as the major reservoir for the pathogenic serotypes O:3, O:8 and O:9. Detection of Y. enterocolitica often includes enrichment and biochemical confirmation but the whole process can take up to 3
weeks. PCR can be successfully used for recognition of pathogenic Y. enterocolitica (Kapperud et al., 1993; Koeppel et al., 1993; Rasmussen et al., 1994), and the best results are achieved if the bacteria are concentrated by immunomagnetic separation (IMS) before PCR. This approach has been used to detect Y. enterocolitica O:3 in faecal samples and tonsil swabs from pigs (Rasmussen et al., 1995) and the authors concluded that IMS-PCR was a reliable method when used on pre-enriched medium, enabling the detection of positive samples which are not recognized by traditional methods.

*Helicobacter pylori*

*H. pylori* is a microaerophilic, Gram-negative spiral organism that has received great attention for its association with human gastritis, peptic ulcers and even gastric cancer. Other species of the genus have been isolated from the gastric mucosa of animals and mostly associated with gastritis of the host. Because it has been suggested that some strains of *Helicobacter canis* are capable of zoonotic transmission, sensitive methods for their detection are needed, and PCR has already been shown to be useful (Stanley et al., 1993).

*Brucella*

Bacteria of the genus *Brucella* are well-known as intracellular pathogens that cause animal and human infections. Rapid and sensitive PCR detection of brucellas with or without extraction of DNA has been accomplished (Fekete et al., 1990a, 1990b; Ouahran et al., 1993).

*Mycobacterium*

*Mycobacterium bovis*, the causative agent of tuberculosis in cattle, is a member of the tuberculosis complex, a group of related species that includes *Mycobacterium tuberculosis*, the major cause of human tuberculosis. Histological examinations enable rapid decisions to be made on suspect carcasses during meat inspection. However, agents other than *M. bovis* can induce similar lesions, and additionally, the microscopic detection of acid-fast organisms can only detect bacteria in great concentrations. Laboratory culture of *M. bovis* is sensitive but requires viable bacteria, and the growth of this organism may take 6–8 weeks. Species identification procedures extend the reporting time even further. Tests based on PCR have been shown to be very promising for mycobacterial detection in clinical samples (Cousins et al., 1991; Buck et al., 1992; Yule et al., 1994; Wards et al., 1995).

*Mycobacterium paratuberculosis* causes Johne's disease, a commonly diagnosed disease of sheep, goats and other ruminants. The organisms can be detected by PCR from intestinal and lymph node tissue of infected animals (Ridge et al., 1995).

*Ovine foot rot*

Ovine foot rot is a highly contagious, economically serious disease of sheep with worldwide distribution, especially in temperate farming areas. Although foot rot results from a mixed bacterial infection, *Dichelobacter nodosus* has been shown to be the essential pathogen for the initiation and establishment of the disease. Clinical diagnostic methods currently available for foot rot are subjective and lack precision. Consequently, there is a demand for rapid and precise tests to differentiate virulent strains. The use of PCR based on specific regions of 16S rRNA constitutes a competent assay for foot rot (La Fontaine et al., 1993). PCR assays employing virulent- and benign-specific primers are capable of specific and sensitive differentiation of strains causing virulent, intermediate or benign foot rot (Liu & Webber, 1995).

*Leptospirosis*

Leptospirosis is probably one of the world’s most widespread zoonoses. Rapid diagnosis of leptospirosis is important in view of the need for adequate early treatment. Clinically, it is sufficient to know whether or not a patient is infected with pathogenic leptospires but, epidemiologically, it would be of considerable value if the causative leptospira can be identified at the strain level. Serology does not contribute to early diagnosis as antibodies become detectable on approximately the seventh day of infection. Conventional methods to detect leptospires in blood are either unreliable or too slow to give early results. PCR is a promising tool for early detection of leptospires in blood, urine or CSF in the period between the first appearance of clinical symptoms and the time when antibodies become detectable (Van Eys et al., 1989; Gerritsen et al., 1991; Hookey, 1992; Merien et al., 1992; Gravekamp et al., 1993).

*Borrelia*

The genus *Borrelia* contains several human and animal pathogens. The aetiological agent of Lyme disease is *Borrelia burgdorferi*, which is primarily transmitted by *Ixodes* ticks. Several authors have
successfully employed PCR for diagnosis of Lyme disease (Rosa & Schwan, 1989; Marconi & Garon, 1992; Kawabata et al., 1993). It is well-known that ticks feed on deer species, and using PCR, Kimura et al. (1995) demonstrated the presence of *B. burgdorferi* in the skin of naturally infected wild sika deer, thus confirming the potential of deer as a source of transmission. PCR data also support the notion that birds are partly responsible for the heterogeneous distribution of Lyme disease *Borrelia* spirochetes in Europe (Olsén et al., 1995). Zingg and LeFebvre (1994) have developed a high-sensitive PCR assay for *Borrelia coriaceae* that does not cross-react with any other closely related spirochetes.

*Mycoplasma*

*Mycoplasmas* are known to produce a wide spectrum of animal diseases. Cattle infected with *Mycoplasma mycoides* subsp. *mycoides* infection can either remain apparently healthy or develop contagious bovine pleuropneumonia (CBPP), a disease characterized by respiratory problems. *Post mortem* findings should be followed by bacteriological culture of the organism from affected tissue which can take up to 2 weeks to complete. The serological detection of antibodies is highly specific but asymptomatic animals in the early stages of infection and chronically-infected animals may not have detectable levels of antibodies. Bashiruddin et al. (1994) described the use of PCR to detect specific DNA in clinical material and isolates from outbreaks of CBPP in cattle and buffaloes in Italy. These data showed that PCR can identify the aetiological agent within 2 days of extraction of clinical material, and the specificity of the PCR test to distinguish *M. mycoides* from other subspecies was confirmed.

*Mycoplasma hyopneumoniae* has been identified as the causative agent of mycoplasmal pneumonia in pigs. Because an effective vaccine is not currently available, efforts to control the disease have focused on the elimination of sick animals. Unfortunately, efforts have been hampered by difficulties in differentiating *M. hyopneumoniae* from cross-reacting *Mycoplasma flocculare* and *Mycoplasma hyorhinis*. Stemke et al. (1994) developed a method for differentiation of those three species on the basis of amplification of a 16S rRNA gene sequence. PCR methodology for detection of *Mycoplasma gallisepticum* have also been reported (Nascimento et al., 1991; Kempf et al., 1993, 1994). The organism is the cause of chronic respiratory disease in chickens which results in reduced egg production and significant downgrading of carcasses at slaughter.

*Chlamydia psittaci*

*Chlamydia psittaci* includes a heterogeneous group of mammalian and avian isolates but, at present, there is no generally accepted and accessible method for typing these. The major outer-membrane protein (MOMP) is the most important antigen at the cell surface of chlamydia. Recently, PCR-RFLP analysis of the MOMP encoding gene has been used for typing of *C. psittaci* strains (Denamur et al., 1991; Kaltenboeck et al., 1992; Sayada et al., 1994).

*Coxiella burnetii*

*Coxiella burnetii*, a zoonotic organism, is the aetiological agent of Q fever. In humans, Q fever occurs as a influenza-like illness, pneumonia, granulomatous hepatitis or chronic endocarditis. In animals, coxiella can reach high concentrations in the female reproductive system and infection can be followed by abortion or infertility. Although the infection of cattle is usually latent, *C. burnetii* may be shed via milk by infected cows for one or several lactation periods. The organism can survive, in low numbers, for a long time in dairy products made from non-pasteurized milk of infected cows and detection in milk requires a high-sensitive method. A PCR approach with primers based on repetitive transposon-like sequences have been established for the highly sensitive and specific detection of *C. burnetii* in cow’s milk (Willems et al., 1994).

**PARASITES**

*Leishmania*

Leishmaniasis is a group of infestations of the viscera, skin and mucous membranes caused by protozoa of the genus *Leishmania*. Multicopy 16S rRNA has been the basis of some PCR assays that specifically detects *Leishmania* sp. (Guevara et al., 1992; Van Eys et al., 1992). Kinetoplast DNA (kDNA) is a target of interest because both maxi- and minicircles are present in each cell in multiple copies. However, it has proved to be difficult to select species-specific kDNA sequences for diagnosis by PCR (Smyth et al., 1992; López et al., 1993), and it is important to investigate only small regions of minicircles to find species-specific
sequences conserved among strains of the same species. PCR has been used to detect leishmanias in conjunctival biopsies (Roze, 1995), showing that a number of cases of ocular inflammation can be attributed to this parasite.

Trypanosoma

In some tropical countries, the protozoan parasites of the genus *Trypanosoma* are responsible for life-threatening diseases in animals and humans, and PCR is now being used to evaluate the vectorial ability of *Glossina longipalpis* in Western Africa (Solano et al., 1994; Weiss, 1995).

Toxoplasma

The cyst-forming apicomplexan parasite *Toxoplasma gondii* infects a broad spectrum of vertebrates. Domestic and feral cats are the definitive hosts but humans and other animal species can be infested by ingestion of oocysts or tissue cysts. Overwhelming infestations, especially in immunosuppressed individuals, may be fatal. Application of PCR can quickly and accurately detect *T. gondii* in a variety of clinical specimens including formalin-fixed and paraffin embedded tissues (MacPherson & Gajadhar, 1993; Wastling et al., 1993; Hyman et al., 1995).

Cryptosporidium

Cryptosporidiosis is now recognized as an important cause of human and animal diarrhoea. PCR amplification combined with chemiluminescence can specifically detect *Cryptosporidium parvum* DNA present in fixed paraffin-embedded tissues (Laxer et al., 1991, 1992). Species and strain differentiation of domestic fowl coccidia of the genus *Eimeria* has also been achieved by PCR (ProCuinier et al., 1993).

Echinococcus

Echinococcosis is a disease caused by larval stages of different cestode species of the genus *Echinococcus*, especially *Echinococcus granulosus* and *Echinococcus multilocularis*. These species are widely prevalent and may cause severe disease in animals and humans. A PCR study including several independent *E. multilocularis* isolates and various other cestodes revealed that the PCR product was obtained from genomic DNA of all *E. multilocularis* isolates but not from DNA of other cestode species (Gottstein & Mowatt, 1991). The sensitivity of the *E. granulosus* PCR was evaluated experimentally and approached 2.5 pg of template DNA, which corresponds to the DNA content of a single echinococcus egg (Rishi & McManus, 1987). A random amplified polymorphic DNA (RAPD) method has permitted a detailed genetic analysis of Swiss and Spanish isolates of *E. granulosus* (Siles-Lucas et al., 1994). The application of PCR to detect echinococci can allow the identification of biopsy material obtained from liver lesions of unknown aetiology and the demonstration of adult-stage parasite tissue or eggs in samples derived from faeces, small intestines or anal swabs of definitive carnivore hosts (Gottstein, 1992).

Taenia

Tapeworms of the genus *Taenia* can cause human and animal taeniasis and cysticercosis. Although the eggs from *Taenia solium* and *Taenia saginata* cannot be differentiated morphologically, a 500 bp sequence that hybridize specifically to a single-copy gene sequence of *T. solium* and not to *T. saginata* DNA may be available in the future for rapid PCR differentiation (Rishi & McManus, 1988).

Dictyocaulus

Lungworms are common parasites of ruminants, and to a lesser extent, horses. In cattle, they cause considerable economic losses due to weight loss and deaths. RAPD-PCR has proved to be a valuable tool to examine genome differences among *Dictyocaulus* species from cattle, sheep and fallow deer (Epe et al., 1995).

Trichinella

The nematode *Trichinella spiralis* can infect nearly all meat-eating animals. Trichinellosis is transmitted within two cycles that can interact; a sylvatic cycle in wild animals and a domestic cycle in pigs which is the major source of human infestation. Two different sets of primers have been developed specifically to discriminate domestic from sylvatic isolates (Dupouy-Camet et al., 1991; Dick et al., 1992). PCR has been able to detect, in situ, a single excysted larva, as well as a single encysted larva, in infected mouse muscle following boiling (Dick et al., 1992). RAPD-PCR has also been useful for the identification of *Trichinella* species (Bandi et al., 1993; Dupouy-Camet et al., 1993).

CONCLUSION

PCR has already played an important role in studies of the epidemiology, taxonomy and patho-
genesis of micro-organism infections in animals but is not yet used routinely for the diagnosis of any animal infectious disease. In fact, PCR has become a routine tool only in research laboratories. However, infectious diseases will remain among the major areas for application of PCR detection and genotyping, offering the potential to analyse most micro-organisms of veterinary importance by a single technique. Although many systems have been developed, few have proceeded towards field trials or large-scale clinical evaluation, and PCR application to the routine analysis of biological samples is still a major diagnostic challenge. Most of the assays to detect micro-organisms have high sensitivity with purified DNA samples, but advances in sample preparation and detection of amplified products under field or clinical laboratory conditions are needed in order to achieve high sensitivity with animal specimens.

Diagnosis of viral diseases should be a major target for PCR application because laboratory tests for identification of viruses are either slow, expensive or insensitive. The technique has found large-scale application for the routine detection of human pathogens such as HIV and hepatitis viruses. Among animal viral diseases, pseudorabies, equine viral arteritis, bovine leukaemia and bovine viral diarrhoea are good candidates for early development. The approach should also be focused on viral diseases that have a deep socio-economic impact in endemic regions, such as African Swine Fever or rinderpest. Eradication programmes must include the diagnosis of sick animals, asymptomatic carriers and vectors, and often involve the rapid screening of a large number of samples for which PCR would be very useful.

In relation to bacterial diseases, PCR can be used for the rapid detection of those pathogens whose in vitro cultivation is difficult, time-consuming or unavailable. RFLP patterns using PCR-amplified DNA is an excellent method for bacterial typing and has already been used for the identification of the bacterial strains involved in human foodborne outbreaks (Hill, 1996). Parasitic infestations will probably be the last field of veterinary clinical diagnosis to incorporate PCR techniques, partly because of the relative scarcity of important parasitic diseases in the main countries where PCR research is being developed (Weiss, 1995).

In conclusion, PCR will most likely become the standard diagnostic test in situations where either the micro-organism level is low, differentiation between morphologically identical organisms is required, or whether the immune response to the infection is uninformative. As happened with the progressive introduction of enzyme-linked immunosorbent assays (ELISA) as routine diagnostic tools, the existence of a strong demand for improved diagnosis methods will surely lead, in the next decades, to the development of PCR-based test kits suitable for field application.

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