ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

VETERINARIA 13

Diagnostic Application of the Polymerase Chain Reaction (PCR) in Veterinary Microbiology

Carlos Ros Bascuñana

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

Diagnostic application of the polymerase chain reaction (PCR) in veterinary microbiology

Carlos Ros Bascuñana

Akademisk avhandling som för vinnande av veterinärmedicine doktorsexamen kommer att offentligen försvaras i Ettans föreläsningssal, Klinikcentrum, Ultuna, Uppsala, onsdagen den 11 juni 1997, kl 9.15.

Abstract

The practical applicability of the polymerase chain reaction (PCR) in veterinary diagnostics was evaluated in the present studies. Various PCR-based detection procedures were developed in order to improve the diagnosis of several infectious diseases of importance in veterinary medicine.

A PCR system was developed for the detection and identification of *Mycoplasma capricolum* subsp. *capripneumoniae*, which causes a respiratory disease in goats, called contagious caprine pleuropneumonia (CCPP). A specific PCR was developed for the detection of *Mycoplasma bovis*, which causes mastitis, arthritis and respiratory disease in cows, and of *Mycoplasma agalactiae*, which is responsible for mastitis in goats and sheep. In this study the PCR proved to be an expeditious way to detect *Mycoplasma bovis* in nasal swabs of infected animals. *Mycobacterium bovis* and *Mycobacterium avium-intracellulare*, which are the most important agents of animal mycobacteriosis, were detected and identified by a PCR/REA assay. The system was applied to fresh and formalin-fixed, paraffin-embedded tissue specimens (PET). An internal control of amplification was constructed to recognize the false negative results.

During the Aujeszky's disease eradication programme established in Sweden, several peculiar cases emerged in which only a single animal was found seropositive on certain farms. These animals were called 'single reactors' (SR). Apart from the serological result, the SR animals had no other signs of infection. The PCR technology was applied to ascertain the infectious status of the SR animals. While virus isolation proved negative, three different nested PCR assays showed that Aujeszky's disease virus sequences were present in these particular, apparently uninfected individuals.

The technology of the PCR was also used to improve the diagnosis of the closely related caliciviruses rabbit hemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV). Reverse transcription-PCR (RT-PCR) was applied to amplify a segment of the VP60 gene from a large number of RHDV and EBHSV isolates. The sequence data obtained from the PCR products were used to infer phylogenetic relationships. The results clearly separated RHDV and EBHSV as two distinct members of the *Caliciviridae* family. The sequence data were also used to develop two specific and consistent RT-PCR assays by locating the primers in highly conserved sequence motifs. The diagnostic value of the assays was evaluated in a large number of fresh and fixed specimens from various geographic regions and years of collection. The phylogenetic information and the specific RT-PCR systems obtained established a basis for the molecular epidemiology of the rabbit caliciviruses.

Key words: PCR, diagnostic, bacteria, virus, animal, sequencing, phylogeny, REA.

Distribution:
Swedish University of Agricultural Sciences
Department of Veterinary Microbiology
S-751 23 Uppsala, Sweden

Uppsala 1997
ISSN-1401-6257S-751
ISBN 91-576-5247-3

Diagnostic Application of the Polymerase Chain Reaction (PCR) in Veterinary Microbiology

Carlos Ros Bascuñana
Department of Veterinary Microbiology
and
Department of Virology, National Veterinary Institute
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 1997

Acta Universitatis Agriculturae Sueciae
Veterinaria 13

ISSN 1401-6257
ISBN 91-576-5247-3

Tryck: SLU Service/Repro, Uppsala 1997

A mis padres

Abstract

Ros Bascuñana, C. 1997. *Diagnostic application of the polymerase chain reaction (PCR) in veterinary microbiology.* Doctor's dissertation.
ISSN 1401-6257, ISBN 91-576-5247-3.

The practical applicability of the polymerase chain reaction (PCR) in veterinary diagnostics was evaluated in the present studies. Various PCR-based detection procedures were developed in order to improve the diagnosis of several infectious diseases of importance in veterinary medicine.

A PCR system was developed for the detection and identification of *Mycoplasma capricolum* subsp. *capripneumoniae*, which causes a respiratory disease in goats, called contagious caprine pleuropneumonia (CCPP). A specific PCR was developed for the detection of *Mycoplasma bovis*, which causes mastitis, arthritis and respiratory disease in cows, and of *Mycoplasma agalactiae*, which is responsible for mastitis in goats and sheep. In this study the PCR proved to be an expeditious way to detect *Mycoplasma bovis* in nasal swabs of infected animals. *Mycobacterium bovis* and *Mycobacterium avium-intracellulare*, which are the most important agents of animal mycobacteriosis, were detected and identified by a PCR/REA assay. The system was applied to fresh and formalin-fixed, paraffin-embedded tissue specimens (PET). An internal control of amplification was constructed to recognize the false negative results.

During the Aujeszky's disease eradication programme established in Sweden, several peculiar cases emerged in which only a single animal was found seropositive on certain farms. These animals were called 'single reactors' (SR). Apart from the serological result, the SR animals had no other signs of infection. The PCR technology was applied to ascertain the infectious status of the SR animals. While virus isolation proved negative, three different nested PCR assays showed that Aujeszky's disease virus sequences were present in these particular, apparently uninfected individuals.

The technology of the PCR was also used to improve the diagnosis of the closely related caliciviruses rabbit hemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV). Reverse transcription-PCR (RT-PCR) was applied to amplify a segment of the VP60 gene from a large number of RHDV and EBHSV isolates. The sequence data obtained from the PCR products were used to infer phylogenetic relationships. The results clearly separated RHDV and EBHSV as two distinct members of the *Caliciviridae* family. The sequence data were also used to develop two specific and consistent RT-PCR assays by locating the primers in highly conserved sequence motifs. The diagnostic value of the assays was evaluated in a large number of fresh and fixed specimens from various geographic regions and years of collection. The phylogenetic information and the specific RT-PCR systems obtained established a basis for the molecular epidemiology of the rabbit caliciviruses.

Key words: PCR, diagnostic, bacteria, virus, animal, sequencing, phylogeny, REA.

Author's address: Carlos Ros Bascuñana, Department of Virology, National Veterinary Institute, Box 585, S-751 23 UPPSALA, Sweden

Contents

Appendix

Papers I-VI

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Ros Bascuñana C., Mattsson J.G., Bölske G. and Johansson K.-E. 1994. Characterization of the 16S rRNA genes from *Mycoplasma* sp. strain F38 and development of an identification system based on PCR. *Journal of Bacteriology* **176**, 2577-2586.

II. Chavez Gonzalez Y.R., Ros Bascuñana C., Bölske G., Mattsson J.G., Fernandez Molina C. and Johansson K.-E. 1995. In vitro amplification of the 16S rRNA genes from *Mycoplasma bovis* and *Mycoplasma agalactiae* by PCR.*Veterinary Microbiology* **47**, 183-190.

III. Ros Bascuñana C. and Belák K. 1996. Detection and identification of Mycobacteria in formalin-fixed, paraffin-embedded tissues by nested PCR and restriction enzyme analysis. *Journal of Clinical Microbiology* **34**, 2351-2355.

IV. Ros Bascuñana C., Björnerot L., Ballagi-Pordány A., Robertsson J.-Å. and Belák S. 1997. Detection of pseudorabies virus genomic sequences in apparently uninfected 'single reactor' pigs. *Veterinary Microbiology*, In press.

V. Nowotny N., Ros Bascuñana C., Ballagi-Pordány A., Gavier-Widen D., Uhlén M. and Belák S. 1997. Phylogenetic analysis of rabbit hemorrhagic disease virus and European brown hare syndrome virus isolates by comparison of capsid protein gene sequences. *Archives of Virology* **142**, 657-673.

VI. Ros Bascuñana C., Nowotny N. and Belák S. 1997. Detection and differentiation of rabbit hemorrhagic disease and European brown hare syndrome viruses by amplification of VP60 genomic sequences from fresh and fixed specimens. Submitted for publication.

Abbreviations

A	adenine
AD	Aujeszky's disease
BHV-1	bovine herpesvirus type 1
bp	base pair
BSA	bovine serum albumin
C	cytosine
CCPP	contagious caprine pleuropneumonia
cDNA	complementary DNA
CFU	colony forming unit
DMSO	dimethyl sulfoxide
dNTP	2'-deoxynucleoside-5'-triphosphate
EBHSV	European brown hare syndrome virus
EHV-1	equine herpesvirus type 1
ELISA	enzyme-linked immunosorbent assay
G	guanine
kb	1.000 bases
LC	large colony type
LLT	large latency transcript
NP-40	nonidet P-40
PCR	polymerase chain reaction
PEG	polyethylene glycol
PET	paraffin embedded tissue
PHYLIP	phylogeny inference package
PrV	pseudorabies virus
REA	restriction enzyme analysis
RHDV	rabbit hemorrhagic disease virus
rrnA operon	the ribosomal RNA operon A
rrnB operon	the ribosomal RNA operon B
RT	reverse transcriptase or reverse transcription
SC	small colony type
SDS	sodium (lauryl) sulphate
SR	single reactor
T	thymine
Tm	melting temperature
TMAC	tetramethylammonium chloride
UNG	uracil *N*-glycosylase

Introduction

The rapid detection of pathogens by sensitive, specific and simple techniques is indispensable for the effective control of infectious diseases. Diagnostic microbiology has traditionally relied on direct detection methods such as microscopic examination of clinical specimens and cultivation of the infectious agent in an *in vitro* propagation system. Isolation of the microorganisms from the infected tissue is still regarded as the 'gold standard' for diagnosis. However, these methods have serious limitations. For example, microscopy has generally poor sensitivity and specificity and cultivation is time-consuming. Some microorganisms need several weeks to grow, while there are other pathogens for which cultivation is not possible. In some cases, e.g., enteric or respiratory specimens, the competing normal microbial flora hampers the isolation of the pathogenic agent. These limitations of the conventional direct methods have stimulated the search for new and more practical diagnostic procedures.

Molecular diagnostic techniques for the detection of nucleic acids were first described in the 1970s. Nucleic acid hybridization techniques such as Southern or dot blots were among the first to be applied (Southern 1975). *In situ* hybridization (ISH) was developed soon thereafter. By this technique, the tissue structure is preserved, allowing the identification of the individual cells involved in the infection. Many of these early molecular diagnostic procedures were not widely adopted because they were labour intensive, expensive and in most of them radioactive materials were used. Recent improvements, especially non-isotopic detection methods, have now made these techniques widely available in clinical laboratories.

No other recent discovery in molecular diagnostic techniques has had greater impact than DNA amplification by the polymerase chain reaction (PCR). Essentially, the PCR can be defined as an *in vitro* enzymatic method, by which several million-fold amplification of a specific DNA sequence can be achieved. The great advantage over the hybridization procedures is, that the nucleic acid is amplified by the PCR before detection, while hybridization can only be used to detect the nucleic acid of the microorganism that has accumulated during the infection, which in many cases is minimal (e.g., latent herpesvirus infections). Since the first publications reporting the use of the PCR in the mid-1980s (Saiki et al. 1985; 1986; 1988; Mullis et al. 1986; Mullis and Faloona 1987), the original method has undergone noteworthy improvements and its applications are now numerous, and not only in diagnostics. Today, thousands of publications from different disciplines highlight the importance of this technique. One gains the impression that PCR technology permeates every field of molecular biology, where for example, it is especially popular as an alternative to cloning, because it allows one to

amplify, subclone, express, and mutagenize a sequence available only in a journal or in a computer database. Forensic pathology, evolutionary biology, the human as well as bacterial genome projects, are examples of disciplines where the PCR plays a central role.

The present thesis is focused on the application of the PCR for the diagnosis of infectious diseases in veterinary medicine and is illustrated by several examples.

General concepts of the PCR

PCR was first devised and named by Mullis and colleagues (Mullis and Faloona 1987; Mullis 1990), although the basic principle of *in vitro* DNA amplification was described a decade earlier (Kleepe et al. 1971; Panet and Khorana 1974).

PCR consists of repetitive cycles, each involving three steps performed at different temperatures (Fig. 1). In the first step, the double-stranded target DNA is heat denatured, resulting in single-stranded molecules. Denaturing is followed by the annealing step, in which two oligonucleotide primers, generally between 18 and 30 nucleotides long, specifically hybridize to opposite strands flanking the target region in the DNA to be amplified. The last step is the extension, in which the annealed primers are extended by a DNA polymerase. The newly synthesized strands serve as templates in the next cycle. The cycles are repeated 20 to 45 times, resulting in the exponential accumulation of the target sequences, approximately 2^n, where 'n' is the number of cycles. This formula is not exact because the efficiency of each cycle is not 100% and it depends on many factors, as discussed below.

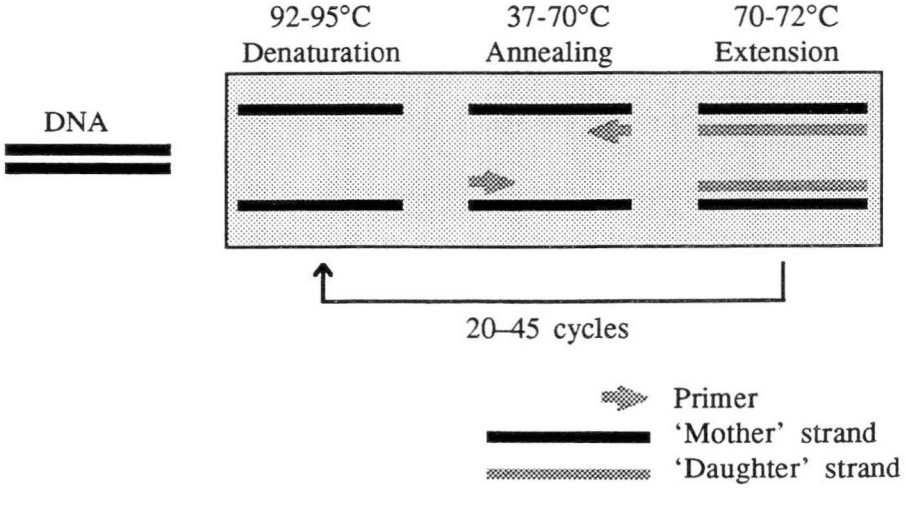

Fig.1. Schematic representation of the PCR process.

During the PCR, the templates must be heat denatured in each cycle at temperatures that inactivate most enzymes. The automation of the technique became therefore possible only after the discovery of thermostable DNA polymerase enzymes, as such obtained from the thermophilic bacterium, *Thermus aquaticus* (*Taq*) (Chien et al. 1976; Saiki et al. 1988). The development of a variety of simple temperature cycling devices (thermo cyclers), has also facilitated the amplification process.

The efficiency of the PCR varies notably from reaction to reaction, depending on the components, quality and concentration of the template, temperature profiles, primers, etc. All these parameters should be optimized for each individual PCR. A proper optimization will result in a highly efficient exponential reaction. However, there is a point at which the amplification is no longer exponential; this point is called 'plateau'. The main causes of the plateau effect are a reduction in the availability of the polymerase and a lower efficiency per cycle of the denaturation and primer annealing. Plateau is inherent to the PCR. Attempts to amplify beyond the plateau will inevitably result in amplification of non-specific sequences (Sardelli 1993).

Compared with single PCR, higher sensitivity can be achieved with nested PCR. By this approach, an aliquot of the PCR product is used as a template for a second amplification reaction with internal primers. Nested PCR has two main advantages over single PCR. It is more sensitive (about 100-fold) and more specific, since four primers are involved in the overall amplification process. The main drawback is, paradoxically, its high sensitivity. Products from the first reaction (carry-over), that are inadvertently introduced in the second reaction, can be amplified, leading to false positive results. For this reason, when using nested PCR, strict precautions must be taken to minimize the false positive results as discussed below.

Not only DNA, but also RNA can serve as a template for PCR. In this case, a preparatory step is needed in which the RNA is first converted into complementary DNA (cDNA) by reverse transcription (RT) (First described by Seeburg et al. 1986, UCLA Symposium, unpublished and Veres et al. 1987). RT-PCR is particularly useful for studying gene expression and for detection of RNA viruses.

At present, the PCR methodology is covered by patents owned by Hoffman-La Roche Inc. and F. Hoffman-La Roche, Ltd. This means that if the PCR is used as a routine diagnostic method, a special licence has to be obtained, where the terms are depending on the application.

PCR Reagents

The reagents of a PCR are readily available from commercial suppliers. In a standard reaction, the reagents are:

- Reaction buffer
- Deoxynucleoside triphosphates (dNTPs)
- Primers
- DNA polymerase
- Target DNA

The most widely used reaction buffer contains Tris buffer, magnesium chloride, potassium chloride and gelatin or BSA. Other additives or enhancers may also be useful for certain applications, for example, non-ionic detergents (Triton X-100, NP-40 or Tween 20), DMSO, PEG, glycerol, TMAC, formamide. The use of such reagents at specific concentrations may be absolutely essential in certain cases, but may not be beneficial in or even inhibit other reactions. For example, we found that 5-10% DMSO was essential to amplify the highly G+C-rich bovine herpesvirus type 1 (BHV-1) DNA. However, 10% glycerol drastically reduced the yield of our PCR developed for the detection of Aujeszky's disease virus (unpublished data).
It is possible to vary the salt concentrations in the buffer to obtain better results, particularly the $MgCl_2$ concentration. We have noticed that in certain reactions the $MgCl_2$ concentration can vary from 0.5 to 5 mM without a visible effect on the yield, while in other cases, slight variations of only 0.5 mM caused a profound effect on the specificity and yield of the reaction. In general, insufficiency of magnesium leads to low yield, while an excess leads to unspecific amplifications. The magnesium concentration and the annealing temperature are the most considered parameters when starting the optimization of a PCR assay.

The dNTPs are the building blocks for DNA synthesis. The variation in their concentration influences the specificity and fidelity of the reaction and it is connected with the $MgCl_2$ concentration. One should use the lowest and well balanced concentration that ensures the optimal amplification of a given target. Usually 20 to 200 µM of each is enough for most applications. A final concentration greater than 50 mM total dNTP inhibits *Taq* DNA polymerase activity (Innis et al. 1990). There are dNTP analogues for certain applications. For example, 7 deaza-dGTP reduces secondary structure in G+C-rich sequences (McConlogue et al. 1988). It is also possible to replace dTTP with dUTP to obtain PCR products which can be digested with uracil *N*-glycosylase (UNG) to minimize contamination (Longo et al. 1990). Modified dNTPs are used for sequencing purposes (Innis et al. 1988)

and dNTPs, with radioactive and non-radioactive markers, are widely used to label PCR products.

The primers are among the most important components for the overall success of a PCR experiment. The usual length of the primer molecules is 18–30 nucleotides. The optimal concentration varies depending on the application; a range between 0.1 and 0.5 μM of each should be adequate. Ideal primers should have least secondary structure and low complementarity to each other. Complementarity at the 3' end induces primer-dimer artifacts, which is an amplification caused when one primer is extended by the polymerase with the other primer or itself as template, resulting in a short, undesired product. The primer dimers disturb the amplification of the target, resulting in a lower yield of the specific product. Primers should be free of polypurine and polypyrimidine stretches or repetitive motifs. Also, the presence of many G or C bases at the 3' terminus should be avoided, as this promotes mispriming. In this way, it is better to select primers that are stable at their 5' end but somewhat 'loose' at their 3' end. The primer pairs should have similar melting temperatures (Tm). Also, the Tm of the amplified product should be sufficiently low to ensure complete denaturation at 92°C. Different formulas exist to calculate the Tm of the primers and of the PCR product, the simplest being those defined by Suggs et al. (1981) for primer Tm and Baldino et al. (1989) for the Tm of the PCR product.

$$\text{Primer } T_m = 2°C(A+T) + 4°C(C+G)$$

$$*\text{PCR product } T_m = 59.9 + 0.41\ (\%G+C) - 675/\text{length}$$

*Under standard conditions with 50 mM KCl

This primer Tm formula is often used because of its simplicity and accuracy. Other more complicated and slightly more accurate formulas are based on thermodynamic parameters (Rychlick et al. 1990).

Primers are usually designed to be exactly complementary to the template. However, in some cases, specific mismatches or 5' adaptors are introduced for applications like the *in vitro* mutagenesis, or to introduce new restriction enzyme sites into a specific region of the genome, or to prepare fragments with customized extreme target sequences. Amplification of a group of related microorganisms, or a new gene related to a known family of genes, is carried out by degenerate primers consisting of a mixture of oligonucleotides of the same length but with certain sequence differences at specific positions, in order to fit a wider range of target sequences. For

example, because of the degenerate nature of the genetic code, a given amino acid may be encoded by one to six different triplets. The selection of amino acid with least degeneracy is preferred. The specific codon usage for translation should be considered. In addition, one should prevent the 3´ end of the primer from coinciding with the last nucleotide of the codon, since it is the most mutable. In fact, degeneracy should be kept to a minimum at the 3' end because mismatched bases are inefficiently extended. Instead of degenerate primers, it has been suggested to use the analogue inosine in primers because it is known to form base pairs (bp) with all of the four bases (Knoth et al. 1988).

In some cases, one or more arbitrary primers are selected to target specific but unknown sites in the genome, many of which are polymorphic. The PCR with such primers results in strain-specific patterns of DNA fragments that can distinguish even closely related strains of a species. These methods are termed randomly amplified polymorphic DNA (RAPD) (Williams et al. 1990), arbitrary primed PCR (AP-PCR) (Welsh and McClelland 1991), and DNA amplification finger printing (DAF) (Caetano-Anollés et al. 1991). The main differences between them are the primer length, the amplification stringency and the method for resolving DNA patterns (Caetano-Anollés 1993).

The positions of the primers determine the size of the PCR product. The ideal size depends on the application. If the target is expected to be damaged, as when amplifying from fixed specimens, one should avoid long PCR products (Greer et al. 1991). In addition, the amplification efficiency of very long PCR products is lower. Short fragments should also be avoided as they can easily be confused with primer dimers. In addition, short fragments greatly limit a further characterization of the PCR product by restriction enzyme analysis (REA) or sequencing. For diagnostic applications a size between 200 and 600 bp should be optimal.

The selection of primers has been markedly simplified by the availability of computer programs that rapidly calculate parameters like Tm, secondary structures and interaction between the primers (Rychlick and Rhoads 1989; Lowe et al. 1990; Hillier and Green 1991; Lucas et al. 1991). The primer selection criterion mentioned above is not necessarily the same among the different softwares and it is, therefore, important to be critical.

The DNA polymerase most widely used in PCR is the thermostable *Taq* DNA polymerase. It replaced the thermolabile polymerase used in the initial PCR procedure (Saiki et al. 1985) and made possible the automation of the reaction. The *Taq* enzyme was first isolated from the bacterium *Thermus aquaticus* from a hot spring in Yellowstone National Park. The enzyme is now available from many commercial suppliers. The recombinant version is

named Amplitaq (Perkin-Elmer, Norwalk, CT, USA) and it lacks the 3' to 5' exonuclease 'proofreading' activity. This means that misincorporation will not be corrected, affecting the fidelity of the reaction. A version of Amplitaq is the Stoffel fragment (Perkin-Elmer) which is more thermostable and exhibits optimal activity over a broader range of magnesium concentrations. The *Taq*, Amplitaq and Amplitaq Stoffel fragment DNA polymerases leave a single 3'-dA nucleotide overhang on their reaction product, which can be utilized when cloning a PCR product. The *Taq* polymerase activity is optimal at 72°C and at pH 7.0–7.5. At this temperature the polymerase incorporates approximately 100 bases per s to the primer. At 95°C the half-life of the enzyme is 40 min. Urea, DMSO, and formamide at a low concentration do not disturb the enzyme activity. Up to 10% of ethanol has no effect on the PCR. SDS, commonly used in combination with proteinase K for nucleic acid preparation for PCR, is a strong inhibitor of the enzyme activity, even at very low concentration. The inhibitory effect of SDS can be neutralized by adding non-ionic detergents such as Tween 20 or NP-40 (Gelfand 1989; Goldenberger et al. 1995). Examples of other inhibitory substances are heparin, phenol, xylene cyanol, bromophenol blue and hemoglobin.

Although *Taq* polymerase is the most widely used enzyme for PCR, other polymerases isolated from various bacterial sources have also been introduced. Vent DNA polymerase, Vent (exo$^-$) and DeepVent are commercially available (Biolabs, Beverly, MA, USA). These enzymes are more thermostable than *Taq* and, except Vent (exo$^-$), they have 3' to 5' exonuclease activity, which provides 5 to 15-fold greater fidelity than *Taq* polymerase. The *Pfu* DNA polymerase enzyme (Stratagene, La Jolla, CA, USA) has also 3' to 5' exonuclease activity. A variant exo$^-$ is the enzyme of choice for cycle sequencing because it incorporates [α-^{35}S]dATP very efficiently. The UlTma DNA polymerase has also 3' to 5' exonuclease activity. The *Tth* DNA polymerase has reverse transcriptase activity in the presence of manganese and at temperatures around 70°C. This enzyme is used for single tube RT-PCR, by chelating the manganese and adding magnesium, or for cDNA synthesis of G+C-rich RNA templates (Meyers et al. 1994).

The template can be either DNA or RNA, the latter after previous cDNA synthesis. There are many different ways in which the template can be prepared for PCR. In general, there is no need to purify the template because the PCR can tolerate the presence of impurities, but on the other hand, care must be taken to minimize the presence of undefined polymerase inhibitors in certain clinical samples. If the material consists of cell suspensions, a simple heat disruption of the cell might be enough. For example, because mycoplasmas have no cell walls, simple boiling of the sample is often sufficient to make their DNA accessible. For dense tissue, however, more

thorough treatment is recommended, such as proteinase K digestion and the standard phenol-chloroform extraction procedure.

Formalin-fixed, paraffin-embedded tissues (PET) are very common test material in clinical laboratories. A problem associated with these specimens is the frequent occurrence of false negative results due to the presence of inhibitory substances. For this reason, it is important to thoroughly purify the sample and/or to include an internal control of the amplification, to identify the false negative results. The extraction procedure with phenol-chloroform, with a previous removal of the paraffin with xylol, can be applied to obtain nucleic acids from PETs (Wright and Manos 1990).

In general, one should not add more than 1 µg of total (target and host) DNA to the reaction, since amounts between 0.05 to 1.0 µg are sufficient to amplify single copy targets. Greater quantities of DNA often have an inhibitory effect on the activity of the polymerase.

Cycling conditions

The selection of times, temperatures and number of cycles depends mostly on the primers and on the target. These parameters should be optimized for each individual PCR. In general, incubation times should be kept low to reduce unspecific amplifications and the time required for the reaction.

It is very helpful, especially when amplifying a low copy number of the target molecules, to use 'hot start'. In this procedure, a component of the reaction, preferably the polymerase, is withheld until the reaction has reached a stringent primer annealing temperature. This method has the advantage of minimizing the non-specific primer extension during the precycling period or during the initial ramp to denaturation. Consequently, the specificity and the yield can both be improved (Chou et al. 1992). A drawback is that the missing reagent has to be transferred manually to the reaction tube, which increases the risk of contamination. This problem was eliminated by adding a wax to the reaction tubes which releases the reagents at high temperatures. Recently, a modified *Taq* polymerase has been introduced (AmpliTaq Gold, Perkin-Elmer). This enzyme is inactive at set-up temperatures and is activated after incubation at 94°C for 10 min. It therefore provides the specificity of the 'hot start' without adding any extra reagent or opening the reaction tube.

The annealing temperature has to be optimized experimentally. The Tm of the primers gives a starting point. Too high annealing will result in a lower yield or even complete failure, while too low annealing leads to unspecific amplification.

The extension time depends on the length of the region to be amplified. The *Taq* polymerase enzyme incorporates more than 70 bases per s under standard conditions. However, one should take into consideration the time required for the reaction mixture to reach the desired temperature. Usually, a 1 min extension should be enough for amplifications of up to 1 kb. One can even obtain good amplification without the extension step, by two-temperature PCR. In this procedure, annealing and extension are combined, which is particularly recommended when the annealing is performed at high temperatures, close to the optimum of the *Taq* polymerase. Up to 10 min of extension is often included in the final cycle to ensure that all the molecules are fully extended.

The optimal number of cycles depends on the amount of the initial template. Usually, especially in diagnosis, this amount cannot be predicted and the number of cycles should be optimized experimentally, and is normally in the range 20–45 cycles. When the number of cycles is increased beyond the optimal, one can often observe PCR products of different sizes due to unspecific amplifications (Bell and DeMarini 1991).

Detection and identification of the PCR products

The systems used to detect amplified DNA can be divided into methods that detect any synthesized DNA and those that detect specifically the target under study.

The simplest and most widely used non-specific procedure to detect amplified DNA is electrophoresis with agarose gel (0.8 to 4%) and ethidium bromide staining (Sambrook et al. 1989). This method is inexpensive but has a detection threshold of around 10-20 ng of amplified DNA, which is under the optimal. In addition, non-specific amplifications complicate the interpretation of the results. The sensitivity can be increased to 0.1 pg of DNA by using labeled dNTPs (biotin, digoxigenin, ^{32}P), although specificity is still a problem because non-specific amplification are also detected. For diagnostic purposes, therefore, it is recommendable to not only detect but also identify with a reliable method the specific target.

Two commonly used techniques that detect and identify the amplified DNA are slot blot and Southern blot hybridization. By these procedures, the PCR products are transferred onto membranes and are hybridized to oligonucleotide probes that are complementary to an internal part of the target. The difference between slot and Southern blot is that in the latter, the PCR products are transferred onto the membrane after electrophoresis, which provides an additional discrimination by the specific size. The probes can be either radiolabeled or labeled with non-radioactive markers. Although highly sensitive, radiolabeling has the problems associated with the handling

of radioactive material. Non-radioactive labeling, e.g. biotin, digoxigenin and fluorescein is widely used.

Chemiluminescence, the emission of light from a special class of chemical reaction, has been introduced as a reporter system for DNA detection. It has the advantages of long shelf-life, high sensitivity and no background light signals generated, since the light is created in a dark chemical reaction. A modification of this procedure is electrochemiluminescence, in which the excited state products are generated via an electrochemical reaction. For example, a probe with a electrochemiluminescent label can be hybridized to biotinylated PCR products. Magnetic beads are used to purify the biotinylated hybrid, which is stimulated with an electric field and produces light (DiCesare et al. 1993).

ELISA-based detection is an optimal alternative to gel-based methods, as ELISA assays are standardized and familiar in most laboratories. In some procedures, biotinylated (Lehtovaara et al. 1993; Naif et al. 1992; Shindo et al. 1994) or digoxigenated (Chang et al. 1994; Chenal and Griffais 1994) primers are used to label the PCR products. In other techniques, the unlabeled PCR product is detected by means of anti-DNA monoclonal antibodies (Mantero et al. 1991) or biotinylated and fluoresceinated DNA probes (Katz et al. 1993).

A novel 'real time' quantitative PCR method has recently been developed (Heid et al. 1996) on the basis of the 5' nuclease assay (Holland et al. 1991). By this approach, the accumulation of PCR product is measured with a fluorogenic probe (e.g., TaqMan Probe). This technique does not require post-PCR manipulation, thus reducing the time of diagnosis and the risk of contamination.

Application of the PCR in veterinary microbiology

An important application of PCR technology is the detection and identification of pathogens. Its chief advantage is that the PCR can be used to detect a specific single DNA sequence from the complex background of the animal tissue. As a PCR can be completed in a few hours (there are already special reaction tubes, capillaries and PCR machines that have reduced this time to a few minutes), it is particularly interesting for the detection of pathogens that do not grow *in vitro* or which are difficult and slow to culture.

The first publications on PCR in veterinary diagnostics appeared in the late 1980s (Belák et al. 1989; Nunberg et al. 1989; van Eys et al. 1989). Since then, a large number of PCR assays have been developed for the detection of

many animal pathogens (reviews by Belák and Ballagi-Pordány 1993; Belák et al. 1994; Pfeffer et al. 1995).

Relevant improvements for diagnostics

Important modifications of the original procedure have greatly facilitated the adaptation of PCR to diagnostics. Examples of improvements which have been successfully applied in diagnostics are: detection of RNA targets by RT-PCR, nested PCR, multiplex PCR, and *in situ* PCR.

By RT-PCR, RNA viruses and RNA related to infection can be detected, e.g., latency associated transcripts during latent herpesvirus infection.

For a successful RT-PCR, the quality of the RNA template is very important. Measures should be taken to prevent the introduction of RNase enzymes. The most widely used method to isolate good quality RNA from cell or tissue samples is the guanidine isothiocyanate/acid-phenol method (Chomczynski and Sacchi 1987), and its modification, the TriZOL reagent (Gibco BRL, MD, USA) (Chomczynski 1993). Guanidine isothiocyanate-based lysis method (Chirgwin et al. 1979), and its modifications (Han et al. 1987; Sambrook et al. 1989), are also used. For micro-scale isolation of RNA for PCR, the GlassMAX system has been described (Simms 1993).

Various reverse transcriptase enzymes can be used to synthesize cDNA. Those obtained from Moloney murine leukemia virus (MMLV RT) and avian myeloblastosis virus (AMV RT), are widely used. These enzymes do not tolerate temperatures above 42°C, which is non-stringent for most primers and not enough to avoid secondary structures in G+C-rich RNA templates. Higher temperatures (50°C) can be employed with two MMLV RT-RNase-H$^-$ mutants, Superscript and Superscript II. A thermostable DNA polymerase, derived from *Thermus thermophilus* (*Tth* polymerase), has reverse transcriptase activity in the presence of Mn^{2+}. The thermophilic nature of this enzyme allows RT at 70°C (Meyers and Gelfand 1991). By using Bicine buffer, *Tth* polymerase has been used in a single-tube RT-PCR (Meyers et al. 1994).

In diagnostics, when using RT to convert viral RNA into cDNA, two different kinds of primers are recommended: non-specific priming with random hexamers, and specific priming with the reverse primer. The latter approach has the advantage that exclusively the specific cDNA is produced. For special applications, such as cDNA synthesis from latency associated transcripts, an oligo(dT) primer can be used for specific selection of poly(A) mRNA.

Nested PCR has proved very useful in the field of diagnostics because it is more sensitive and more specific than single PCR. The increased sensitivity is especially interesting when amplifying a low copy number of targets or when certain factors, such as inhibitors of polymerase activity, reduce the sensitivity of PCR. Transfer of the first PCR product to a new reaction mixture leads to beneficial dilution of the possible inhibitor present in the original sample. We found for example that nested PCR was better than single PCR when a simple DNA preparation procedure, e.g. Chelex (Biorad), was applied for BHV-1 detection in semen. However, when a more thorough procedure, e.g. phenol-chloroform extraction, was used, the single and the nested systems were both optimal and gave the same results (unpublished data). An additional advantage of nested PCR is its greater specificity, by virtue of the fact that, instead of two, four primers are needed to hybridize to the target in order to obtain amplification. On certain occasions, it is not possible to select two internal primers as the outer primers are very close to each other and/or the sequence outside the outer primers is very limited or has not been determined. In such cases a semi-nested PCR may be the best method of choice, in which the second PCR is performed with an internal primer only and one primer from the first round. One has to consider that nested PCR requires opening of the reaction tube in order to transfer the first-round PCR product to the second-round PCR. This increases the possibility of contamination due to carry-overs. Moreover, contamination precautions protocols such as the UNG treatment mentioned above, cannot be used, because the first PCR product is the template for the second amplification. A single-tube nested PCR has been described (Wolff et al. 1995), which may solve the problems associated with the use of nested PCR in diagnostics. The advantage is a lesser risk of contamination, but this method is technically more complex (Podzorski and Persing 1995).

Multiplex PCR (M-PCR) is a technique for the simultaneous detection of more than one target sequence in a single reaction, with two or more primers. All the primers must have a similar Tm, in the range ±5°C. The sizes of the PCR products should also be similar, in the range 200–500 bp (Bej et al. 1990; Atlas and Bej 1993). The application of M-PCR in veterinary diagnostics permitted the simultaneous detection of pathogens in a single test or the co-amplification of internal controls (Persing et al. 1993). For specimens such as the respiratory, enteric or genital tract secretions, from which several pathogens can be recovered, this technique offers potential cost savings (Wilton and Cousins 1992; Wirz et al. 1993; Reubel et al. 1995).

An important improvement has been PCR *in situ* hybridization, in which the high sensitivity of PCR is combined with the cell-localizing ability of *in situ* hybridization (Nuovo 1994). First time described on cell suspensions (Haase 1990), was later improved and applied on tissue sections (Nuovo 1991). In a modified procedure called *in situ* PCR, labeled nucleotides are used to avoid

the hybridization step. These techniques have three major advantages; the high sensitivity, the enormous amount of information provided relative to histological distribution of the target and the lack of the contamination problems associated to solution-phase PCR. However, this methodology is still in its infancy and problems with regard to background signal and inconsistency of results are common.

General considerations for PCR design in diagnostics

When one decides to apply the PCR for the diagnosis of an infectious disease, the first question is whether to select an already described system or to develop a new one. The obvious advantage of using an established system is the time and cost saving. However, it is difficult to find a PCR system that satisfies all the demands of a specific application. Moreover, many of the assays described are not evaluated with regard to reliability as a routine diagnostic method. If it is decided to develop a new assay, one should consider that the selection of the target sequence, the construction of primers, the template preparation, the PCR conditions and the method for detection of the PCR product, must be optimized for each newly developed system.

A starting point is the selection of the target sequence. It is preferable to select a target that has several copies throughout the genome, which will improve the sensitivity. Avoiding highly G+C-rich regions will facilitate primer selection and minimize secondary structures in both primer and template. The target should be conserved among the different strains of the species under study. It is possible to widen the range of detection by selecting a region which is conserved in a group of microorganisms. In the case of bacteria, the 16S rRNA genes are valuable targets for PCR because they are present in several copies in the genome and are fairly stable within a single bacterial species. As 16S rRNA genes contain well defined regions of variable conservation (Gray et al. 1984), species-specific primers can be selected from evolutionarily variable regions, while group-specific primers can be selected from semi-conserved and conserved regions. Because there are several thousand copies of rRNA molecules in a growing bacterium, the sensitivity of the PCR can be improved by RT-PCR (van Kuppeveld et al. 1992). An additional advantage is that the number of 16S rRNA gene sequences, deposited in databases, are rapidly increasing (Olsen et al. 1993) and provide useful data to infer phylogenetic relationships.

Considering the above points, the selection of an appropriate amplification target must begin with a search in the sequence databases (EMBL data bank or GenBank). Ideally, the sequences from different strains of the intended pathogen should be aligned with other sequences from related microorganisms. By these means, highly specific primers can be selected.

There are computer programs, available from several commercial sources, that perform multiple alignments and provide consensus sequences. This information is used to select the diagnostic primers, which should follow the considerations mentioned above. It is not always possible to select fully complementary primers. In fact, mismatches can be tolerated providing that they are not many and that they are not near to the 3' end of the primer. The fact that few differences within the primer–template complex can be tolerated is of capital importance in diagnostics, because sequence differences may occur even between closely related strains of the same species, particularly among RNA viruses. Studies have been conducted to investigate the effects of the kind and position of mismatches in relation to the amplification efficiency. Kwok et al. (1990) observed that single internal mismatches had no significant effect, while those at the 3' end had variable effects, being the less tolerated the A:G, G:A and C:C (primer:template) mismatches. On the contrary, a mismatched T at the 3' end allowed an efficient amplification. Variations in the reaction components and annealing temperatures directly affect the amplification by mismatched primers, which is used in diagnostics to help or to avoid amplifications. For example, by decreasing the concentration of dNTPs, shortening the annealing time and raising the annealing temperature, mismatches are less tolerated.

PCR in the diagnosis of infectious diseases: possibilities and limitations

The application of PCR in the diagnosis of microbial pathogens has its biggest potential in the sensitivity and specificity, since single DNA sequences in a high background of cellular DNA can be detected by PCR. Within one to two days, a microbial pathogen can be detected and characterized directly from the animal tissue.

With regard to speed, cost and reliability, the PCR is often superior to standard techniques. For example, there are non-cultivable organisms, for which PCR offers the best alternative of detection, e.g., adenoviruses or caliciviruses. There are also slow growing or fastidious microorganisms, such as mycobacteria, mycoplasma, borrelia, chlamydia or non-cytopathic viruses such as respiratory syncytial virus, mucosal disease virus, coronavirus, etc. The detection of such agents may take several weeks, while by PCR, diagnosis can be obtained within one day.

PCR can be used to detect non-viable organisms, e.g., viruses inactivated due to an inadequate sample conservation, or non-viable bacteria in specimens from animals treated with antibacterial substances. In such cases, PCR may still yield positive results, while cultural methods would be negative. The ability of PCR to detect non-viable organisms can be advantageous to reduce the risks for personnel when studying human infectious pathogens such as

mycobacteria, rabies virus, influenza virus, etc. In this sense, the sample suspected to contain a pathogen capable of causing zoonotic infections, can be heat-inactivated before opening the tube. The capability of PCR to detect non-viable organisms can also be a drawback, because a positive PCR result does not give any information on the real infectious status.

PCR is also very useful for retrospective diagnosis of infections from fixed tissues, where proteins may not be immunologically detectable and viable microorganisms cannot be isolated.

Culture from difficult samples, like semen, is hampered by the natural cytotoxic components. PCR however, has proved a reliable method to detect BHV-1 in semen (van Engelenburg et al. 1993). For other samples such as enteric or respiratory specimens, where the competing normal microbial flora hamper the isolation of the microbial pathogen, PCR is a good alternative (Uwatoko et al. 1995; Cohen et al. 1996).

PCR has also been proved optimal for differentiation of wild-type agents from vaccine strains; for example, *Mycoplasma gallisepticum* F-vaccine strain (Nascimento et al. 1993). Genetically modified live vaccines, called marker vaccines, that lack specific sequences of the genome, are commonly used to combat wild-type viruses. Marker vaccines have been generated for example from pseudorabies virus (PrV) (Mettenleiter et al. 1994; Peeters et al. 1994; Stegeman et al. 1994) and from BHV-1 (Kaashoek et al. 1994). A PCR that can be used to discriminate between wild equine herpesvirus type-1 (EHV-1) and a deleted mutant vaccine strain has also been described (Osterrieder et al. 1994). Although distinction between vaccine and wild-type can be done by serology, PCR offers a rapid and reliable alternative.

Another important diagnostic application of PCR is the detection of latent infections, such as those established by herpesvirus. While acute herpesvirus infections can be readily diagnosed by standard methods, the diagnosis is difficult during latency, when infectious viruses cannot be recovered and only viral DNA –and in certain cases, latency associated transcripts– persist in certain tissues such as the trigeminal ganglia. PCR has been used successfully to detect latent herpesviral infections, for example PrV (Belák et al. 1989), feline herpesvirus (FHV) (Reubel et al. 1993), and EHV-1 (Slater et al. 1994).

Despite all these advantages, and in contrast to ELISA, few PCR kits are commercially available. In fact, PCR is currently only available in well equipped laboratories, and it is far from being utilized by veterinary practitioners in the field, although not impossible (Barker 1994). The lack of commercial kits contrasts with the enormous interest that PCR has created in the scientific community. It seems that there are several reasons for this

situation. Development of new assays and maintenance of a PCR laboratory require knowledge, time and heavy economic investment, especially in the beginning. This is probably the case for any other diagnostic method, but in the case of PCR, it might be accentuated by the lack of standardized protocols from the sample preparation to the detection of the final amplification product. This confusion has been demonstrated in several studies involving a large number of laboratories that tested control samples in a blind fashion (Defer et al. 1992; Zaaijer et al. 1993). The results emphasize the clear need for international PCR standardization efforts for which a number of organizations are already involved (Bockstahler 1994).

The most important goals of the standardization efforts are to minimize false positive results due to contamination and false negative results caused by inhibitors. Indeed, contamination in PCR is one of the major drawbacks that restricts its use in routine diagnosis. The fact that the same reaction is repeated in the same laboratory over long periods of time is especially problematical when the reaction has the extraordinary sensitivity of the PCR. The most common source of contamination is by far carry-overs from previous reactions. Other sources might be the positive controls and other samples tested.

There are numerous procedures described for minimizing the contamination (Kwok 1990; Sarkar and Sommer 1990; Belák and Ballagi-Pordány 1993; Yap et al. 1994). Punctilious general precautions must be followed at all times. Among them are, physical separation of pre- and post-PCR procedures, use of laminar flow hoods, laboratory clothes for each dedicated area, frequent change of gloves, micropipettes with aerosol-resistant tips, etc. In addition, there are a number of specific preventive methods, e.g., enzymatic or chemical digestion, ultraviolet irradiation and UNG treatment. Negative controls, are essential for the detection of false positive results, and ideally they should cover the entire process from sample collection. Positive controls are needed to assess the efficiency of the reaction. This can be best performed with internal controls of the amplification, in which a 'mimic' molecule is co-amplified with the target (Ballagi-Pordány and Belák 1996). The 'mimic' is essentially an *in vitro* constructed DNA molecule, larger in size than the specific target and with primer complementary sequences at both ends. When it is added to the PCR at a low concentration, the mimic molecule is co-amplified with the specific target, provided that the reaction has been efficient. Mimics are also used to quantify the initial amount of template (Nash et al. 1995).

Following meticulous laboratory precautions, PCR contamination can be kept to a minimum acceptable level. In addition, the new technologies for PCR product detection without the need to open the tube, the so-called 'real

time' PCR (Livak et al. 1995; Heid et al. 1996; Gibson et al. 1996), will facilitate complete automation of the amplification procedure.

Today, sequence data from microorganisms are increasing considerably and correspondingly our knowledge of its biological significance. These advances are mostly assisted by PCR technology. Such amount of sequence data deposited in databases are very useful for diagnostic PCR in two ways:

- The possibility to specifically control the spectrum of detection by locating the primers in specific genomic regions. In this way, discrimination would be possible with regard to important features such as pathogenicity or antibiotic resistance, eg., methicillin resistance in *Staphylococcus aureus*, using primers specific for this gene.
- The rapid genetic characterization of microbial agents that are involved in a given outbreak. By running PCR or RT-PCR, microbial nucleic acid sequences are amplified from the samples of diseased animals or from culture. The nucleotide sequences of the amplified regions are rapidly determined and compared with those available in databases. Rapid and absolutely precise identification of the microorganisms can be achieved at the genetic level, which facilitates the immediate application of epizootiological control measures.

These aspects will provide novel and valuable future perspectives to the application of the PCR in the diagnosis of infectious diseases.

Aims of the study

The general goal of the work presented in this thesis was the application of PCR to improve the diagnosis of several relevant infectious diseases in veterinary medicine.

- Development of PCR assays for the detection and identification of important animal pathogens. These microorganisms included *Mycoplasma capricolum* subsp. *capripneumoniae* (formerly known as *Mycoplasma* sp. strain F38), which causes a respiratory disease in goats, called contagious caprine pleuropneumonia (CCPP) (I). *Mycoplasma bovis*, a pathogen causing mastitis, arthritis and respiratory disease in cows and the related *Mycoplasma agalactiae*, which is responsible of mastitis in goats and sheep (II). *Mycobacterium bovis* and *M. avium-intracellulare*, which are the most important agents of animal mycobacteriosis (III).

- Evaluation of PCR as a diagnostic tool in the Aujeszky's disease eradication programme (IV).

- Development of RT-PCR for detection, differentiation and molecular epidemiology of rabbit hemorrhagic disease and European brown hare syndrome viruses (V, VI).

Present investigations and discussion

Development of diagnostic PCR assays

Mycoplasma capricolum subsp. *capripneumoniae* (I)

Mycoplasma is the trivial name for the group of bacteria which is included in the class *Mollicutes*, which means 'soft skin'. This name refers to the lack of the rigid cell wall which is typical of other bacteria. More than 120 species have been described, many of them being important pathogens of humans, animals and plants (Maniloff et al. 1992). In addition, they often contaminate cell cultures (McGarrity et al. 1985). Considered the smallest and simplest self-replicating organisms (Razin 1987), the mollicutes are also characterized for the low G+C content in their genomes and their special nutritional requirements, due to the reduced genome size (0.6–1.8 Mbp). The most common diseases caused in animals are pneumonia, arthritis and mastitis.

The newly named *Mycoplasma capricolum* subsp. *capripneumoniae* (*M. capripneumoniae*) (Leach et al. 1993; Tully et al. 1993), formerly known as *Mycoplasma* sp. strain F38 (term used in paper I), is the causative agent of a goat respiratory disease called contagious caprine pleuropneumonia (CCPP). The disease is distributed chiefly in Asian and African countries and it is recognized as the major infectious problem for goat breeding in those parts of the world (Picavet 1991).

M. capripneumoniae is a member of the '*Mycoplasma mycoides* cluster', which also includes *M. capricolum* subsp. *capricolum* (*M. capricolum*), *M. mycoides* subsp. *mycoides* (large- and small-colony type; LC and SC, respectively), *M. mycoides* subsp *capri* and *Mycoplasma* sp. strain PG50. All these species are important animal pathogens. They are closely related, showing serological cross-reactions and similar biochemical features (Cottew et al. 1987; Ernø 1987; Jones 1992). Consequently, it is difficult to correctly identify *M. capripneumoniae*. In addition, CCPP lacks pathognomonic characteristics, and symptoms can be shared by or can mimic other mycoplasmoses, such as those caused by *M. mycoides* subsp. *mycoides* (LC) and *M. mycoides* subsp *capri* (Jones 1992). Accordingly, the diagnosis of CCPP can be easily misinterpreted. The similar characteristics shared by the members of the *M. mycoides* cluster not only make their correct identification difficult, but also their taxonomic classification. Due to these important diagnostic limitations of the conventional methods, a novel method by which is possible to detect and distinguish *M. capripneumoniae* from the remaining members of the *M. mycoides* cluster is greatly needed.

In the present study, the complete nucleotide sequence of the 16S rRNA gene from the *rrnB* operon from *M. capripneumoniae* was determined. This sequence was used to develop an identification system based on the PCR and restriction enzyme analysis (REA) of the amplicons. In addition, the sequence data served to clarify the taxonomic status of *M. capripneumoniae* within the *M. mycoides* cluster.

The phylogeny of *M. capripneumoniae*

The 16S rRNA sequence of *M. capripneumoniae* was aligned with known sequences from other members of the *M. mycoides* cluster. Phylogenetic analysis was performed by using the PHYLIP computer program package (Felsenstein 1989). The parsimony method was chosen, because there are few nucleotide substitutions between the different species. Three equally parsimonious trees were generated in which *M. capricolum*, *M. capripneumoniae* and *Mycoplasma* sp. strain PG50 formed a group with an ambiguous branch topology. Bootstrapping analysis of the data showed a monophyly of a *M. capricolum*, *M. capripneumoniae*, *Mycoplasma* sp. strain PG50 and *M. mycoides* subsp. *mycoides* (LC) clade, since 100 of 100 bootstraps supported this monophyly. However, bootstrapping analysis did not resolve the node ambiguity between *M. capricolum*, *Mycoplasma capripneumoniae* and *Mycoplasma* sp. strain PG50. A cause of this ambiguity is the relatively few nucleotide differences in the 16S rRNA genes within the *M. mycoides* cluster. This problem suggests the need to obtain highly accurate and complete sequences from the 16S rRNA genes. In addition, both operons should be sequenced, since polymorphisms between the two operons do exist. In a recent work, the taxonomic status of *M. capripneumoniae* has been further investigated by sequencing both operons from ten representative strains of the species and subspecies within the *M. mycoides* cluster (Pettersson et al. 1996). Polymorphisms between the two operons were observed in all the strains. In that work it was suggested to include *M. capripneumoniae* in the same group as *M. capricolum* and *Mycoplasma* sp. strain PG50.

Detection and identification of *M. capripneumoniae* based on PCR and REA

Due to the few nucleotide differences between the 16S rRNA genes of the *rrnB* operon from *M. capripneumoniae* and the corresponding sequences from the other members of the *M. mycoides* cluster, the development of a specific PCR system for the detection of *M. capripneumoniae* was not possible. Instead, primers specific for all the members of the group were constructed. The PCR proved to be specific for the *M. mycoides* cluster. In sensitivity studies as few as 5–10 mycoplasmas were detected. In order to identify *M. capripneumoniae*, a REA of the amplicons was performed. A *Pst*I site in the 16S rRNA genes, common to all the members of the *M.*

mycoides group, was not present in *M. capripneumoniae*. After digestion of the 548 bp amplicon with *Pst*I, two bands of 420 and 128 bp were generated in all the mycoplasmas, except for *M. capripneumoniae* which, unexpectedly, was also digested, but generating three bands of 548, 420 and 128 bp. In order to explain this result, a part of the *rrnA* operon of *M. capripneumoniae* was sequenced, revealing a *Pst*I site homologous to the site of the other members of the *M. mycoides* cluster. This unique *Pst*I site, present only in the *rrnA* of *M. capripneumoniae*, but present in both operons in the rest of the members of the *M. mycoides* cluster, explained the unexpected band pattern and provided a suitable identification system for *M. capripneumoniae*. The fact that all the specific amplicons generated by this PCR are digested by *Pst*I, regardless of origin, is advantageous, since inhibition of the activity of the enzyme can be easily detected.

Evaluation of the PCR/REA system for the detection and identification of *M. capripneumoniae*

The reliability of the system was evaluated on i) several *M. capripneumoniae*-like strains; ii) mycoplasmas and bacteria of importance in pneumopathies of goats; iii) mycoplasmas that cross-react in laboratory tests with *M. capripneumoniae*; iv) clinical specimens from an experimentally infected goat. The results showed that in all cases, only those mycoplasmas belonging to the *M. mycoides* cluster were amplified. After REA, the expected three bands were only generated from the amplicons of *M. capripneumoniae*.

In a recent work, the PCR/REA system was further evaluated with respect to specificity and diagnostic efficacy for the detection and identification of *M. capripneumoniae* in various clinical samples (Bölske et al. 1996). The identification system was verified on 55 strains from different geographic areas, among them 15 strains of *M. capripneumoniae*. In that work, it was concluded that this system seems to be a reliable tool for the rapid and convenient identification of *M. capripneumoniae*.

***Mycoplasma bovis* and *Mycoplasma agalactiae* (II)**

Mycoplasma bovis is known to cause mastitis, arthritis and diseases of the respiratory tract in cattle (Boughton 1979; Ter Laak et al. 1992). *M. agalactiae* is the etiological agent of contagious agalactia in goats and sheep (Lambert 1987; DaMassa et al. 1992). Both species are closely related. The 16S rRNA sequences differ only in 8 nucleotide positions. The *in vitro* cultivation and immunological techniques are the basis of detection and identification. However, the former technique is time-consuming and sometimes hampered by other bacterial strains and the latter is, in some cases, not sensitive enough. Hybridization techniques have been developed

for the detection and identification of *M. bovis* (Mattsson et al. 1991; McCully and Brock 1992; Tola et al. 1994). This technique proved adequate in sensitivity for certain diagnostic applications, however animals with subclinical or chronic disease only harbour a few microorganisms. Samples from such animals have to be propagated in growth medium before using the hybridization technique. When such a high sensitivity is needed, PCR is the method of choice.

The purpose of this study was to develop a PCR system for the detection of *M. bovis* and *M. agalactiae* and to evaluate the applicability of the *M. bovis*–PCR system to analyze clinical specimens. Due to the extensive similarity in the 16S rRNA sequences from both mycoplasmas, the selection of fully specific primers was not possible. Instead, a common forward primer and a reverse primer, differing in two nucleotides, one situated at the 3'-end, were constructed. By this approach, a semi-specific system was obtained in which the cross-amplifications were reduced approximately 100-fold. No other closely related bovine or caprine mycoplasmas were amplified, the only exception being *M*ycoplasma sp. strain A1343 of the caprine group 7, which was positive in both systems. This strain is very closely related to *M. bovis*, as shown by protein mapping in two-dimensional polyacrylamide gel electrophoresis (Rodwell 1982) or by biochemical reactions. In fact, it was suggested in 1982 by the Working Team for Caprine and Ovine mycoplasmas of the International Organization for Mycoplasmology, that strain A1343 should be classified as a variant strain of *M. bovis*, which is in agreement with our results.

The detection limits for *M. bovis*–PCR and *M. agalactiae*–PCR systems were 4×10^2 and 2×10^2 CFU/ml, respectively. The detection limits in the respective heterologous PCR systems were approximately 100-fold higher, due to the mismatched bases in the reverse primer.

The *M. bovis*–PCR was also used to analyze clinical samples, consisting of nasal swabs from a herd previously affected by an outbreak of respiratory disease caused by *M. bovis*. In this study the PCR proved to be a expeditious method for the detection and identification of *M. bovis* in nasal swabs from infected animals.

The fact that, although of lower efficiency, there is cross-amplification between the two PCR systems, can be disregarded for diagnostic applications, as *M. agalactiae* has never been isolated from cattle and *M. bovis* only occasionally from goats (DaMassa 1992). Therefore, these species can be considered as host-specific. The sensitivities observed in both PCR assays are adequate for most diagnostic purposes and can probably be used to detect mycoplasmas in animals with subclinical or chronic disease.

Mycobacterium bovis and *M. avium* (III)

Tuberculosis is a chronic granulomatous infection caused by *Mycobacterium* spp. Cattle tuberculosis has been eradicated from industrial countries, but cases still occur, mainly from wild animals, game farms and zoos. *Mycobacterium bovis* and *M. avium-intracellulare* are the two most relevant mycobacteria in veterinary medicine. The conventional detection of mycobacteria in clinical samples is based on culturing. This method has serious drawbacks. Its sensitivity is not optimal and it is not possible to distinguish between closely related species (Bates 1979). Moreover, since mycobacteria are slow-growing microorganisms, diagnosis based on culturing can take several weeks. Low sensitivity and specificity are other problems associated with methods like direct microscopy or immunological tests (Yeager et al. 1967; Morris and Ivanyi 1985). Differentiation of mycobacteria at the species level can be done by methods such as the high-performance liquid chromatography, but this method has also low sensitivity. Nucleic acid probes have been developed to specifically identify many important mycobacterial species (reviewed by Kohne 1989), but these tests are also deficient with regard to sensitivity, as at least 10^5 organisms are necessary to give consistent results.

Considering the importance of these pathogens and the diagnostic limitations described above, a highly sensitive nested PCR assay was developed. In order to distinguish the amplicons of *M. bovis* and *M. avium-intracellulare*, a subsequent REA of the PCR products was applied.

In order to generate sequence information for the selection of general primers, a region of the 65 kDa surface antigen of mycobacteria was amplified, cloned and sequenced from ten mycobacterial strains. The sequence data revealed highly conserved regions. These regions were used to select general primers in order to amplify all the *Mycobacteria* spp. To determine the specificity of the PCR, various cultures of mycobacteria and of other bacteria were analyzed. The specific PCR product was obtained from all the mycobacteria tested. None of the non-mycobacterial species was amplified. In sensitivity studies with serial tenfold dilutions of purified *M. bovis* DNA, the nested PCR assay could be used to detect as little as 5 to 10 fg of purified mycobacterial DNA or approximately 1 to 2 genome copies, assuming a genome size of 2.0×10^9 Da for *M. bovis* (Baess 1984).

Amplification from formalin-fixed, paraffin-embedded tissue (PET)

The assay has been adapted to PET specimens, which is one of the most common test material in the routine laboratory. Of the various procedures of mycobacterial DNA preparation from PETs, proteinase K digestion was the most consistent, in our experience. The presence of inhibitors of PCR in PET

specimens frequently led to false negative results. It has been reported that extensive purification procedures, such as DNA extraction with organic solvents, may not be sufficient to completely remove such inhibitors (Greer et al. 1991; An and Fleming 1991). Instead of complex DNA purification procedures, we used an internal control of amplification, called 'mimic' (as described above). Our results indicated, that the 'mimic' provided a simple and rapid means to detect cases of false negative reactions and allowed the use of a simple and rapid DNA preparation for PCR.

Identification system for *M. bovis* and *M. avium-intracellulare*

Direct digestion of the 424 bp amplicons with the restriction endonuclease *Sau*96 I allowed the identification of *M. bovis* and *M. avium-intracellulare* amplicons in agarose gels. Digestion of *M. bovis* amplicons produced two visible bands of 285-bp and 118-bp, while digestion of *M. avium-intracellulare* amplicons produced three visible bands: 168-bp, 117-bp and 108-bp. The amount of PCR product used did not affect the digestion efficiency. The digestion of the amplicons from other mycobacteria tested showed specific restriction patterns. However, reliance on REA of PCR products for identification of a large number of mycobacterial species in the routine diagnostic laboratory is hampered by the presence of nucleotide differences among strains of the same species. In addition, the identification of very similar restriction patterns is difficult. The accurate identification of very similar restriction patterns would require special equipment, computer programs, internal standards of the migration, and specially trained personnel (Plikaytis et al. 1992; Totsch et al. 1995).

The consistency of the results obtained with a large number of clinical isolates, confirms the present system as being a new option for detecting and identifying mycobacteria in clinical samples.

PCR as a diagnostic tool in eradication programs

Aujeszky´s disease eradication program and the 'SR' animals

Aujeszky´s disease (AD; pseudorabies) causes serious economic losses in the pig industry all over the world. The etiological agent of the disease is pseudorabies virus (PrV). Following a primary replication, PrV can establish latent infection in swine. Under certain stressing factors, latent infection may become reactivated, which results in re-shedding and transmission of the virus to susceptible animals (Davies and Beran 1980).

In the course of the program established in Sweden for the eradication of AD, a gB ELISA was used as screening test. Altogether, 480.000 pigs on 8.900 farms were tested and approximately 1.300 cases were observed with

only one single animal reacting positively on the farm. These animals were called 'single reactors' (SR). Interestingly, most of the SR animals (99%) reacted negatively in the gE ELISA. Symptoms of AD had not been reported in the SR pigs and virus transmission to animals in contact was never observed (Robertsson, personal communication).
Immunohistochemistry (peroxidase–antiperoxidase method) did not demonstrate PrV in the tissues of the SR animals. Virus neutralization (VN) test detected PrV specific antibodies in the sera of a few SR animals (Björnerot et al., manuscript in preparation).

PCR to investigate the single reactor phenomenon (IV)

Three nested PCR assays were developed in order to investigate the presence of PrV in organs of 73 SR animals from the field and in 39 pigs farrowed by SR sows. By PCR, viral gB, gE and gD gene sequences (formerly gII, gI and gp50, respectively) were detected in the tissues of trigeminal ganglia, olfactory bulb, tonsils and brain from approximately 20% of the SR from the field. By the same PCR assays, viral genomic sequences were not detected from the progeny of SR sows.

In order to asses the possibility of virus reactivation, 20 SR animals from the field were experimentally immunosuppressed. The PCR results of this group were similar to those obtained with the SR from the field but the number of positive results was up to 50%. Virus could not be isolated from the SR pigs in any case.

The nucleotide sequences of the amplicons revealed 98% to 100% homology with the corresponding sequences of PrV.

The presence of the large latency transcript (LLT), was investigated by a nested reverse transcription PCR (RT-PCR) in 20 SR animals, 10 of them previously positive by DNA PCR. The LLT was not detected in any SR animal.

In view of the present observations and the facts that i) PrV vaccination is not applied in Sweden; ii) the SR animals occur not only in the South, but also in Northern Scandinavia, which has no history of PrV infection; iii) viral reactivation was not observed under natural conditions or after experimental immunosuppression, it is concluded that the SR phenomenon should hardly be considered as a typical PrV latency. The present findings show that certain herpesviral genomic sequences exist in apparently uninfected individuals.

PCR and molecular epidemiology

Diagnosis of rabbit caliciviruses

Rabbit hemorrhagic disease (RHD) is a rather new, highly fatal infection of both wild and domestic rabbits. European brown hare syndrome (EBHS) affects wild and farmed hares. The two diseases are accompanied by similar clinical symptoms as well as similar pathological and histopathological changes (Fuchs and Weissenböck 1992). The two viruses are antigenically closely related (Capucci et al. 1991; Steineck and Nowotny 1993), and morphologically mutually indistinguishable (Lavazza and Vecchi 1989).

Hemagglutination (HA) test (Nowotny et al. 1990; 1991) and ELISA (Capucci et al. 1991) are the usual methods for laboratory diagnosis of RHD and EBHS, but these procedures frequently yield cross-reactions between the two viruses. In addition, certain virus variants show little or no HA activity. Virus isolation is hampered by the lack of an appropriate cell culture system. Detection of viral RNA by RT-PCR has been described recently for RHDV (Guittre et al. 1995). This method, however, was established on the basis of the sequence data from a single isolate; moreover, its specificity was not tested with the genetically related EBHSV.

The aim of the present study was to use PCR technology to generate sequence information on a large number of RHDV and EBHSV isolates from diverse geographic areas and years of isolation. These sequences would then be used to better define the relationship of RHDV and EBHSV, between each other and within the *Caliciviridae* family and to improve the diagnosis by developing a highly specific RT-PCR that can be used to detect and distinguish RHDV from EBHSV in fresh and in fixed specimens.

RT-PCR for phylogenetic studies of RHDV and EBHSV (V)

A 398 bp fragment of the viral VP60 gene was directly amplified by RT-PCR from liver and spleen specimens from 39 clinical cases of RHD and 17 of EBHS, collected between 1981 and 1995 in 17 countries. The amplicons were sequenced by automated solid–phase DNA sequencing with streptavidin–coated magnetic beads (Hultman et al 1991).

Nucleotide sequence comparison revealed that RHDV and EBHSV are clearly separated. The nucleotide differences between the isolates were mainly transitions, occurring all over the selected region, without deletions or insertions. The homology rates between RHDV and EBHSV ranged between 52.6% and 60.0%. The homology within the groups was much higher, 89.4% to 100% for the RHDV, and 89.4% to 100% for the EBHSV. The sequences were used to construct phylogenetic trees by using the computer

program PHYLIP package (Felsenstein, 1993), and the neighbour-joining method (Saitou and Nei, 1987). The parsimony method was also applied (Swofford, 1991). Three main branches were identified in the phylogenetic tree of the RHDV isolates, resembling the epizootiological data. Due to insufficient epizootiological information on hares, the EBHSV dendrogram did not show such well defined branches. In addition, the deduced amino acid sequences of the two most distantly related isolates of the RHDV and EBHSV groups were aligned with the corresponding sequences of other members of the *Caliciviridae* family, i.e. feline calicivirus, San Miguel sea lion virus, Southampton virus, and Norwalk virus, as described by Wirblich et al. (1994). The results showed that RHDV and EBHSV are closely related but differ significantly from other caliciviruses (Fig. 2).

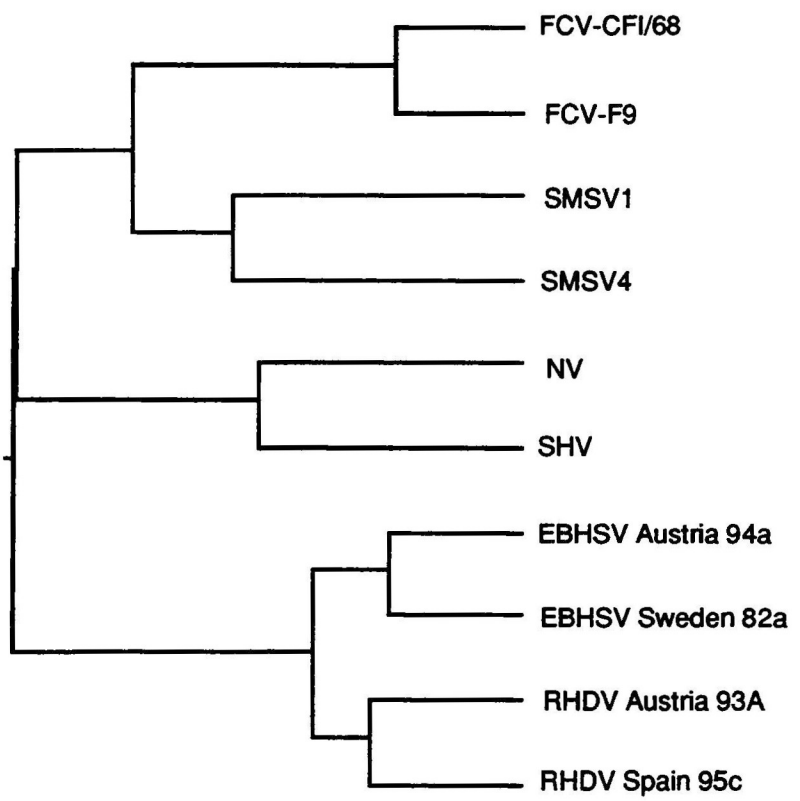

Fig.2. Phylogenetic tree based on a partial nucleotide sequence of the capsid protein gene of two distant isolates of RHDV and EBHSV, respectively and other caliciviruses. FCV, feline calicivirus strain F9 and CFI/68; SMSV, San Miguel sea lion virus serotypes 1 and 4; NV, Norwalk virus; and SHV, Southampton virus.

Consequently, the sequence analysis of several samples presented in this study, indicates a marked difference between RHDV and EBHSV and supports the classification of the two viruses as two distinct members of the *Caliciviridae* family.

RT-PCR for detection and characterization of RHDV and EBHSV (VI)

Two specific RT-PCR assays were developed to detect and differentiate RHDV and EBHSV. The primers were designed to amplify exclusively the respective virus and to detect genetically distant variants. To achieve this specificity, primers were selected from highly conserved regions of the VP60 genes of RHDV and EBHSV, according to our previous sequence data which showed a large number of mismatches with the heterologous virus. In sensitivity studies, as few as 10 copies of cloned viral genomic fragments were detected in each PCR assay, and no cross-amplification was observed between the two viruses. The diagnostic value of the assays was tested on clinical material consisting of fresh, as well as formalin-fixed, paraffin-embedded (PET) liver and spleen specimens from various geographic regions and years of collection. Altogether, 77 fresh and 22 fixed liver and spleen specimens were tested from rabbits and hares. In addition, PET samples obtained between 1974 and 1983 from four Austrian rabbits which showed histologically moderate liver lesions resembling those of a mild RHD, were also included.

The results showed that both RT-PCR assays are consistent and reliable for the detection and differentiation of RHDV and EBHSV from both fresh and fixed specimens.

The RT-PCR assays, presented in this study, in combination with rapid characterization of the PCR products by sequencing, are useful methods to classify newly identified RHDV and EBHSV, and to estimate the phylogenetic relationship to previously sequenced RHDV and EBHSV specimens. The rapid and accurate genetic identification of the virus participating in a given outbreak is known as 'molecular epidemiology'. The information obtained by this method facilitates the immediate application of epizootiological control measures to effectively cut the routes of virus-spread. For example, in the present studies, a EBHSV was detected and sequenced from a Swedish PET specimen collected in 1977. This virus proved to be distinct, but closely related to Swedish EBHSV from the early 1980s. In the literature, EBHSV infection was not reported before 1980. Thus, our studies demonstrated that EBHSV was circulating in the hare populations of Europe already during the 1970s. In similar retrospective studies, RHDV was not detected in PET specimens collected between 1974 and 1983 from Austrian rabbits, showing liver lesions resembling those of a mild RHD. Considering that RHD has been found in Europe after 1986

(Mitro and Krauss 1993), these specimens were examined in order to try to identify a supposed apathogenic virus, from which the pathogenic RHDV may have arisen. According to the negative results, the liver lesions observed in these four rabbits are probably of non-RHDV etiology. These observations are in agreement with the theory that clinical EBHS was present in the hare populations of Europe much earlier than clinical RHD in rabbits.

Concluding remarks

In the present studies the application of the PCR technology was evaluated for the diagnosis of infectious diseases. Several bacteria and viruses of clinical importance were used as models. Compared to conventional diagnostic methods, the PCR provided higher specificity, sensitivity and rapidity.

Despite of the improvements, PCR is still not always accepted as a routine diagnostic technique. Important reasons are the lack of standardized protocols and the cost of establishing a fully equipped PCR laboratory. Other problems are the time and knowledge required to design new PCR assays, as well as to interpret and solve 'unexpected' results. These problems will certainly be solved in the near future. For example, standardization is feasible and the time, knowledge and investment required will be no longer an obstacle as laboratories become more proficient in PCR technology.

References

An, S.F. and Fleming, K.A. 1991. Removal of inhibitor(s) of the polymerase chain reaction from formalin fixed, paraffin wax embedded tissues. J. Clin. Pathol., 44: 924-927.

Atlas, R.M. and Bej, A.K. 1993. Polymerase Chain Reaction, in Methods for General and Molecular Bacteriology (Gerhardt, P., Ed.), pp. 418. ASM press, Washington, D.C.

Baess, I. 1984. Determination and re-examination of genome sizes and base ratios on deoxyribonucleic acid from mycobacteria. Acta Path. Microbiol. Immuno. Scand. Sect. B, 92: 209-211.

Baldino, F. Jr, Chesselet, M.-F. and Lewis, M.E. 1989. High-resolution *in situ* hybridization histochemistry. Methods Enzymol., 168: 761-767.

Ballagi-Pordány, A. and Belák, S. 1996. The use of mimics as internal standards to avoid false negatives in diagnostic PCR. Mol. Cell. Probes, 10: 159-164.

Barker, R.H. Jr. 1994. Use of the PCR in the field. Parasitology Today, 10: 117-119.

Bates, J.H. 1979. Diagnosis of tuberculosis. Chest, 76: 757-763.

Bej, A.K., Steffan, R.J., DiCesare, J.L., Haff, L. and Atlas, R.M. 1990. Detection of coliform bacteria in water by polymerase chain reaction and genes probes. Appl. Environ. Microbiol., 56: 307-314.

Belák, S., Ballagi-Pordány, A., Flensburg, J. and Virtanen, A. 1989. Detection of pseudorabies virus DNA sequences by the polymerase chain reaction. Arch. Virol., 108: 279-286.

Belák, S. and Ballagi-Pordány, A. 1993. Experiences on the application of the polymerase chain reaction in a diagnostic laboratory. Mol. Cell. Probes, 7: 241-248.

Belák, S., Ballagi-Pordány, A. and Ros, C. 1994. Application of the polymerase chain reaction in veterinary diagnostic virology (Spa). Invest. Agr., Prod. y San. Anim., 9: 147-169.

Bell, D.A. and DeMarini, D. 1991. Excessive cycling converts PCR products to random-length higher molecular weight fragments. Nucleic Acids Res., 19: 5079.

Bockstahler, L.E. 1994. Overview of International PCR Standardization Efforts. PCR Methods Applic., 3: 263-267.

Boughton, E. 1979. Mycoplasma bovis mastitis. Vet. Bull., 49: 377-378.

Bölske, G., Mattsson, J.G., Ros Bascuñana, C., Bergström, K., Wesonga H. and Johansson, K.-E. 1996. Diagnosis of contagious caprine pleuropneumonia by detection and identification of *Mycoplasma capricolum* subsp. *capripneumoniae* by PCR and restriction enzyme analysis. J. Clin. Microbiol., 34: 785-791.

Caetano-Anollés, G., Bassam, B.J. and Gresshoff, P.M. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/Technology, 9: 553-557.

Caetano-Anollés, G. 1993. Amplifying DNA with Arbitrary Oligonucleotide Primers. PCR Methods Applic., 3: 85-94.

Capucci, L., Scicluna, M.T. and Lavazza, A. 1991. Diagnosis of viral haemorrhagic disease in rabbits and the European brown hare syndrome. Rev. Sci. Tech. Off. Int. Epiz., 10: 347-370.

Chang, G.J., Trent, D.W., Vorndam, A.V., Vergne, E., Kinney, R.M. and Mitchell, C.J. 1994. An integrated target sequence and signal amplification assay, reverse transcriptase-PCR-enzyme-linked immunosorbent assay, to detect and characterize flaviviruses. J. Clin. Microbiol., 32: 447-483.

Chenal, V. and Griffais, R. 1994. Chemiluminescent and colorimetric detection of a fluorescein-labelled probe and a digoxigenin-labelled probe after a single hybridization step. Mol. Cell. Probes, 8: 401-407.

Chien, A., Edgar, D.B. and Trela, J.M. 1976. Deoxyribonucleic Acid Polymerase from the Extreme Thermophile *Thermus aquaticus*. J. Bacteriol., 127: 1550-1557.

Chirwing, J.M., Przybyla, A.E., McDonald, R.J. and Rutter, W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, 18: 5294-5299.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 161: 156-159.

Chomczynski, P. 1993. A reagent for the single step simultaneous isolation of RNA, DNA and proteins from cells and tissue samples. BioTechniques, 15: 532-535.

Chou, Q., Russell, M., Birch, D.E., Raymond, J. and Bloch, W. 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy number amplifications. Nucleic Acids Res., 20: 1717-1723.

Cohen, N.D., Martin, L.J., Simpson, R.B., Wallis, D.E. and Neibergs, H.L. 1996. Comparison of polymerase chain reaction and microbiological culture for detection of salmonellae in equine feces and environmental samples. Am. J. Vet. Res., 57: 780-786.

Cottew, G.S., Breard, A., DaMassa, A.J., Ernø, H., Leach, R.H., Lefévre, P.C., Rodwell, A.W. and Smith, G.R. 1987. Taxonomy of the *Mycoplasma mycoides* cluster. Isr. J. Med. Sci., 23: 632-635.

DaMassa, A.J., Wakenell, P.S. and Brooks, D.L. 1992. Mycoplasmas of goats and sheep. J. Vet. Diagn. Invest., 4: 101-113.

Davies, E.B. and Beran, G.W. 1980. Spontaneous shedding of pseudorabies virus from clinically recovered postparturient sow. J. Am. Vet. Med. Assoc., 176: 1345-1347.

Defer, C., Agut, H., Garbarg-Chenon, A., Moncany, M., Morinet, F., Vignon, D., Mariotti, M. and LefrereJ.-J. 1992. Multicentre quality control of polymerase chain reaction for the detection of HIV DNA. AIDS, 6: 659-663.

DiCesare, J., Grossman, B., Katz, E., Picozza, E., Ragusa, R. and Woudenberg, T. 1993. A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantification. BioTechniques, 15: 152-157.

Ernø, H. 1987. Mycoplasmosis of ruminants: a general introduction. Rev. Sci. Tech. OIE, 6: 553-563.

Felsenstein, J. 1989. PHYLIP-phylogeny inference package (version 3.2). Cladistics 5: 164-166.

Felsenstein, J. 1993. PHYLIP: Phylogeny inference package (version 3.52). University of Washington, Seattle, Washington.

Fuchs, A. and Weissenböck, H. 1992. Comparative histopathological study of rabbit haemorrhagic disease (RHD) and European brown hare syndrome (EBHS). J. Comp. Pathol., 107: 103-113.

Gelfand, D.H. 1989. *Taq* DNA polymerase, in PCR technology (Erlich, H.A., ed.), pp. 17-22. Stockton Press, New York.

Gibson, U.E.M., Heid, C.A. and Williams, P.M. 1996. A novel method for real time quantitative competitive RT-PCR. Genome Res., 6: 995-1001.

Goldenberger, D., Perschil, I., Ritzler, M. and Altwegg, M. 1995. A simple 'universal' DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. PCR Methods Applic., 4: 368-370.

Gray, M.W., Sankoff, D. and Cedergren, R.J. 1984. On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. Nucleic Acids Res., 12: 5837-5852.

Greer, C.E., Peterson, S.L., Kiviat, N.B. and Manos, M.M. 1991. PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. Am. J. Clin. Pathol., 95: 117-124.

Guittre, C., Baginski, I., Le Gall, G., Prave, M., Trepo, C. and Cova, L. 1995. Detection of rabbit haemorrhagic disease virus isolates and sequence comparison of the N-terminus of the capsid protein gene by the polymerase chain reaction. Res. Vet. Sci., 58: 128-132.

Haase, A.T., Retzel, E.F. and Staskus, K.A. 1990. Amplification and detection of lentiviral DNA inside cells. Proc. Natl. Acad. Sci., USA. 87: 4971-4975.

Han, J.H., Stratowa, C. and Rutter, W.J. 1979. Isolation of full length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and DNA cloning. Biochemistry, 26: 1617-1625.

Heid, C.A., Stevens, J., Livak, K.J. and Williams, P.M. 1996. Real time quantitative PCR. Genome Res., 6: 986-994.

Hillier, L. Green, P. 1991. OSP: a computer program for chossing PCR and DNA sequencing primers. PCR Methods Applic., 1: 124-128.

Holland, P.M., Abramson, R.D., Watson, R. and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci., USA. 88: 7276-7280.

Hultman, T., Bergh, S., Moks, T. and Uhlén. M. 1991. Bidirectional solid-phase sequencing of in vitro-amplified plasmid DNA. BioTechniques, 10: 84-93.

Innis, M.A., Myambo, K.B., Gelfand, D.H. and Brow, M.A.D. 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Proc. Natl. Acd. Sci., USA. 85: 9436-9440.

Innis, M., Gelfand, D.H., Sninsky, J.J. and White, T.J., Eds. 1990. PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA.

Jones, G.E. 1992. Contagious caprine pleuropneumonia, in the OIE manual of standards for diagnostic tests and vaccines. (Truszczynsky, M., Pearson, J. and Edwards, S., Ed.), pp. 376-392. Office International des Epizooties, Paris.

Kaashoek, M.J., Moerman, A., Madic, J., Rijsewijl, F.A., Quak, J., Gielkens, A.L. and van Oirschot, J.T. 1994. A conventionally attenuated glycoprotein E-negative strain of bovine herpesvirus type 1 is an efficacious and safe vaccine. Vaccine, 12: 439-444.

Katz, J.B., Gustafson, G.A., Alstad, A.D., Adler, K.A. and Moser, K.M. 1993. Colorimetric diagnosis of prolonged bluetongue viremia in sheep, using and emzyme-linked oligonucleotide sorbent assay of amplified viral nucleic acids. Am. J. Vet. Res., 54: 2021-2026.

Kleppe, K., Ohstuka, E., Kleppe, R., Molineux, L. and Khorana, H.G. 1971. Studies on polynucleotides. XCVI. Repair replication of short synthetic DNA's as catalyzed by DNA polymerases. J. Mol. Biol., 56: 341-361.

Knoth, K., Roberds, S., Poteet, C. and Tamkun, M. 1988. Highly degenerate, inosine-containing primers specifically amplify rare cDNA using the polymerase chain reaction. Nucleic Acids Res., 16: 10932.

Kohne, D.E. 1989. The use of DNA probes to detect and identify microorganisms, in Rapid Methods in Clinical Microbiology (Kleger, B., Jungkind, D., Hinks, E. and Miller, L.A., eds.), pp. 11-35. Plenum Press, New York.

Kwok, S., Kellog, D.E., McKinney, N., Sugimoto, N., Caruthers, M.H., Neilson, T. and Turner, D.H. 1990. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus 1 model studies. Nucleic Acids Res., 83: 9373-9377.

Lambert, M. 1987. Contagious agalactia of sheep and goats. Rev. Sci. Tech., OIE. 6: 677-711.

Lavazza, A. and Vecchi, G. 1989. Osservazioni su alcuni episodi di mortalita nella lepre evidenziazione al microscopio elettronico di una particella virale. Nota preliminare. Estr. Sel. Vet., 30: 461-468.

Leach, R.H., Ernø, H. and MacOwan, K.J. 1993. Proposal for designation of F38-type mycoplasmas as *Mycoplasma capricolum* subsp. *capripneumoniae* subsp. nov. and consequent obligatory relegation of strains currently classified as *M. capricolum* (Tully, Barile, Edward, Theodore, and Ernø) to an additional new subspecies, *M. capricolum* subsp. *capricolum* subsp. nov. Int. J. Syst. Bacteriol., 43: 603-605.

Lehtovaara, P., Uusi, O.M., Buchert, P., Laaksonen, M., Bengstrom, M. and Rank, M. 1993. Quantitative PCR for hepatitis B virus with colorimetric detection. PCR Metohds Applic., 3: 169-175.

Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W. and Deetz, K. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Applic., 4: 357-362.

Longo, M.C., Berninger, M.S. and Hartley, J.L. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reaction. Gene, 93: 125-128.

Lowe, T., Shareifkin, J., Yang, S.Q. and Dieffenbach, C.W. 1990. A computer program for selection of oligonucleotide primers for polymerase chain reaction. Nucleic Acids Res., 18: 1757-1761.

Lucas, K., Busch, M., Mossinger, S. and Thompson, J.A. 1991. An improved microcomputer program for finding gene- or gene family-specific oligonucleotides suitable as primers for polymerase chain reaction or as probes. CABIOS, 7: 525-529.

Maniloff, J., McElhaney, R.N., Finch, L.R., Baseman, J.B., Eds. 1992. Mycoplasmas: molecular biology and pathogenesis. ASM Press, Washington, D.C.

Mantero, G., Zonaro, A., Albertini, A., Bertolo, P. and Primi, D. 1991. DNA enzyme immunoassay: general method for detecting products of polymerase chain reaction. Clin. Chem., 37: 422-429.

Mattsson, J.G., Gersdorf, H., Göbel, U.B. and Johansson, K.-E. 1991. Detection of *Mycoplasma bovis* and *Mycoplasma agalactiae* by oligonucleotide probes complementary to 16S rRNA. Mol. Cell. Probes, 5: 27-35.

McConlogue, L., Brow, M.A. and Innis, M.A. 1988. Structure-independent DNA amplification by PCR using 7-deaza-2'-deoxyguanosine. Nucleic Acids Res., 16: 9869.

McCully, M.A. and Brock, K.V. 1992. Development of a DNA hybridization probe for detection of *Mycoplasma bovis*. J. Vet. Diagn. Invest., 4: 464-467.

McGarrity, G.J., Sarama, J. and Vanaman, V. 1985. Cell culture techniques. ASM News, 51: 170-183.

Mettenleiter, T.C., Klupp, B.G., Weiland, F. and Visser, N. 1994. Characterization of a quadruple glycoprotein-deleted pseudorabies virus mutant for use as a biologically safe live virus vaccine. J. Gen. Virol., 75: 1723-1733.

Meyers, T.W. and Gelfand, D.H. 1991. Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. Biochemistry, 30: 7661-7666.

Meyers, T.W., Sigua, C.L. and Gelfand, D.H. 1994. High temperature reverse transcription and PCR with *Thermus thermophilus* DNA polymerase. Miami Short Reports, 4: 87.

Mitro, S. and Krauss, H. 1993. Rabbit hemorrhagic disease: a review with special reference to its epizootiology. Eur. J. Epidemiol., 9: 70-78.

Morris, J.A. and Ivanyi, J. 1985. Immunoassays of field isolates of *Mycobacterium bovis* and other mycobacteria by use of monoclonal antibodies. J. Med. Microbiol., 19: 367-373.

Mullis, K.B., Faloona, F.A., Scharf, S.J., Saiki, R.K., Horn, G.T. and Erlich, H.A. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol., 51: 263-273.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol., 155: 335-350.

Mullis, K.B. 1990. The unusual origin of the polymerase chain reaction. Sci. Am., 262: 56-65.

Naif, H.M., Daniel, R.C., Cougle, W.G. and Lavin, M.F. 1992. Early detection of bovine leukemia virus by using an enzyme-linked assay for polymerase chain reaction-amplified proviral DNA in experimentally infected cattle. J. Clin. Microbiol., 30: 675-679.

Nascimento, E.R., Yamamoto, R. and Khan, M.I. 1993. *Mycoplasma gallisepticum* F-vaccine strain-specific polymerase chain reaction. Avian diseases, 37: 203-211.

Nash, K.A., Klein, J.S. and Inderlied, C.B. 1995. Internal controls as performance monitors and quantitative standards in the detection by polymerase chain reaction of herpes simplex virus and cytomegalovirus in clinical specimens. Mol. Cell. Probes, 9: 347-356.

Nowotny, N., Fuchs, A., Schilcher, F. and Loupal, G. 1990. Zum Auftreten der Rabbit Haemorrhagic Disease (RHD) in Österreich: I. Pathomorphologische und virologische Untersuchungen. Wien. Tierärztl. Mschr., 77: 19-23.

Nowotny, N., Steineck, Th., Tataruch, F., Schilcher, F. and Weissenböck, H. 1991. European Brown Hare Syndrome (EBHS)-Experimentelle Untersuchungen. Wien. Tierärztl. Mschr. 78: 370-378.

Numberg, J.H., Wright, D.K., Cole, G.E., Petrovskis, E.A., Post, L.E., Compton, T. and Gilbert, J.H. 1989. Identificatiom of the thymidine kinase gene of feline herpesvirus: use of degenerate oligonucleotides in the polymerase chain reaction to isolate herpesvirus gene homologs. J. Virol., 63: 3240-3249.

Nuovo, G.J., Gallery, F., MacConnell, P., Becker, J. and Bloch, W. 1991. An improved technique for the detection of DNA by *in situ* hybridization after PCR-amplification. Am. J. Pathol., 139: 1239-1244.

Nuovo, G.J. 1994. PCR *in situ* Hybridization: Protocols and Applications, 2nd ed. Raven Press, New York.

Olsen, G. and Woese, C. 1993. Ribosomal RNA: a key to phylogeny. FASEB J., 7: 113-123.

Osterrieder, N., Hubert, P.H., Brandmuller, C. and Kaaden, O.R. 1994. A touchdown PCR for the differentiation of equine herpesvirus type 1 (EHV-1) field strains from the modified live vaccine strain Rach. J. Virol. Meth., 50: 129-136.

Panet, A. and Khorana, H.G. 1974. Studies on Polynucleotides. J. Biol. Chem., 249: 5213-5221.

Peeters, B., Bouma, A., de Bruin, T., Moormann, R., Gielkens, A. and Kimman, T. 1994. Non-transmissible pseudorabies virus gp50 mutants: a new generation of safe live vaccines. Vaccine, 12: 375-380.

Persing, D.H., Smith, T.F., Tenover, F.C. and White, T.J. 1993. Diagnostic molecular biology. Principles and applications. Mayo Foundation, Rochester, MN, USA, 3-641.

Pettersson, B., Leitner, T., Ronaghi, M., Bölske, G., Uhlén, M. and Johansson, K.-E. 1996. Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. J. Bacteriol., 178: 4131-4142.

Pfeffer, M., Wiedmann, M. and Batt, C.A. 1995. Applications of DNA amplification techniques in veterinary diagnostics. Vet. Res. Comm., 19: 375-407.

Picavet, D.P. 1991. La pleuropneumonie contagieuse de la chévre (PPCC). Rev. Méd. Vét., 142: 377-387.

Plikaytis, B.B., Plikaytis, B.D., Yakrus, M.A., Butler, W.R., Woodley, C.L., Silcox, V.A. and Shinnick, T.M. 1992. Differentiation of Slowly Growing *Mycobacterium* Species, Including *Mycobacterium tuberculosis*, by Gene Amplification and Restriction Fragment Length Polymorphism Analysis. J. Clin. Microbiol., 30: 1815-1822.

Podzorski, R.P. and Persing, D.H. 1995. Molecular detection and identification of microorganisms, in Manual of Clinical Microbiology. (Murray, P.R., ed.), pp. 130-157. ASM Press, Washington, D.C.

Razin, S. 1987. Appealing attributes of mycoplasmas in cell biology research. Isr. J. Med. Sci., 23: 318-325.

Reubel, G.H., Ramos, R.A., Hickman, M.A., Rimstad, E., Hoffman, D.E. and Pedersen, N.C. 1993. Detection of active and latent feline herpesvirus-1 infections using the polymerase chain reaction. Arch. Virol., 132: 409-420.

Reubel, G.H., Crabb, B.S. and Studdert, M.J. 1995. Diagnosis of equine gammaherpesvirus 2 and 5 infections by polymerase chain reaction. Arch. Virol., 140: 1049-1060.

Rodwell, A.W. 1982. The protein fingerprints of mycoplasmas. Rev. Infect. Dis., 4: 8-17.

Rychlik, W. and Rhoads, R.E. 1989. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. Nucleic Acids Res., 17: 8543-8551.

Rychlik, W., Spencer, W.J. and Rhoads, R.E. 1990. Optimization of annealing temperature for DNA amplification *in vitro*. Nucleic Acids Res., 18: 6409-6412.

Saiki, R.F., Scharf, S., Faloona, F.A., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. 1985. Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230: 1350-1354.

Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B. and Erlich, H.A. 1986. Analysis of enzymatically amplified ß-globin and HLA- DQ DNA with allele-specific oligonucleotide probes. Nature (London), 324: 163-166.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239: 487-491.

Saitou, N. and Nei, M. 1986. The number of nucleotides required to determine the branching order of three species, with special reference to the human-chimpanzee-gorilla divergence. J. Mol. Evol., 24: 189-204.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning. A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sardelli, A.D. 1993. Plateau effect. Understanding PCR limitations. Amplifications, 9: 1-3.

Sarkar, G. and Sommer, S.S. 1989. Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. Science, 21: 331-334.

Shindo, M., Di, B.A., Silver, J., Limjoco, T., Hoofnagle, J.H. and Feinstone, S.M. 1994. Detection and quantitation of hepatitis C virus RNA in serum using the polymerase chain reaction and a colorymetric enzymatic detection system. J. Virol. Meth., 48: 65-72.

Simms, D. 1993. Rapid micro-scale isolation of RNA for PCR or translation. Focus, 15: 6-11.

Slater, J.D., Borchers, K., Thackray, A.M. and Field, H.J. 1994. The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in horse. J. Gen. Virol., 75: 2007-2016.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., 98: 503-517.

Stegeman, J.A., Tielen, M.J., Kimman, T.G., Van Oirschot, J.T., Hunneman, W.A. and Berndsen, F.W. 1994. Intensive regional vaccination with a gI-deleted vaccine markedly reduces pseudorabies virus infections. Vaccine, 12: 527-531.

Steineck, Th. and Nowotny, N. 1993. European Brown Hare Syndrome (EBHS) in Österreich: Epizootiologische Untersuchungen. Tierärztl. Umschau., 48: 225-229.

Suggs, S.V., Hirose, T., Myake, E.H., Kawashima, M.J., Johnson, K.I. and Wallace, R.B. 1981. ICN-UCLA symposium for developmental biology using purified gene. (Brown, D.D., ed.), vol. 23, pp. 683-693. Academic Press, New York.

Swofford, D.L. 1991. PAUP: Phylogenetic analysis using parsimony. Version 3.1.1. Illinois Natural History Survey, Champaign, Illinois.

Ter Laak, E.A., Wentink, G.H. and Zimmer, G.M. 1992. Increased prevalence of *Mycoplasma bovis* in the Netherlands. Vet. Quart., 14: 100-104.

Tola, S., Rizzu, P. and Leori, G. 1994. A species-specific DNA probe for the detection of *Mycoplasma agalactiae*. Vet. Microbiol., 41: 355-361.

Totsch, M., Brommelkamp, E., Stucker, A., Fille, M., Gross, R., Wiesner, P., Schmid, K.W., Bocker, W. and Dockhorn, D.B. 1995. Identification of mycobacteria to the species level by automated restriction enzyme fragment length polymorphism analysis. Virchows Arch., 427: 85-89.

Tully, J.G., Bové, J.M., Laigret, F. and Whitcomb, R.F. 1993. Revised taxonomy of the class *Mollicutes*: proposed elevation of a monophyletic cluster of arthropod-associated mollicutes to ordinal rank (*Entomoplasmatales* ord. nov.), with provision for familial rank to separate species with nonhelical morphology (*Entomoplasmataceae* fam. nov.) from helical species (*Spiroplasmataceae*), and emended descriptions of the order *Mycoplasmatales*, family *Mycoplasmataceae*. Int. J. Syst. Bacteriol., 43: 378-385.

Uwatoko. K., Sunairi, M., Nakajima, M. and Yamaura, K. 1995. Rapid method utilizing the polymerase chain reaction for detection of canine parvovirus in feces of diarrheic dogs. Vet. Microbiol., 43: 315-323.

van Engelenburg, F.A.C., Maes, R.K., van Oirschot, J.T. and Rijsewijk, F.A.M., 1993. Development of a rapid and sensitive polymerase chain reaction assay for detection of bovine herpesvirus type 1 in bovine semen. J. Clin. Microbiol., 31: 3129-3135.

van Eys, G.J.J.M., Gravekamp, C., Gerritsen, M.J., Quint, W., Cornelissen, M.T.E., Ter Scheggat, J. and Terpstra, W.J. 1989. Detection of leptospires in urine by polymerase chain reaction. J. Clin. Microbiol., 27: 2258-2262.

van Kuppeveld, F.J.M., van der Logt, J.T.M., Angulo, A.F., van Zoest, M.J., Quint, W.G.V., Niesters, H.G.M., Galama, J.M.D. and Melchers, W.J.G. 1992. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. Appl. Environ. Microbiol., 58: 2606-2615.

Veres, G., Gibbs, R.A., Scherer, S.E. and Caskey, C.T. 1987. The molecular basis of the sparse fur mouse mutation. Science, 237: 415-417.

Welsh, J. and McClelland, M. 1991. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., 18: 7213-7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Wilton, S. and Cousins, D. 1992. Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube. PCR Methods Applic., 1: 269-273.

Wirblich, C., Meyers, G., Ohlinger, V.F., Capucci, L., Eskens, U., Haas, B. and Thiel, H.-J. 1994. European brown hare syndrome virus: relationship to rabbit hemorrhagic disease virus and other caliciviruses. J. Virol., 68: 5164-5173.

Wirz, B., Tratschin, J.D., Muller, H.K. and Mitchell, D.B. 1993. Detection of hog cholera virus and differentiation from other pestiviruses by polymerase chain reaction. J. Clin. Microbiol., 31: 1148-1154.

Wolff, C., Hörnschemeyer, D., Wolff, D. and Kleesiek, K. 1995. Single-tube nested PCR with room temperature stable reagents. PCR Methods Applic., 4: 376-379.

Wright, D.K. and Manos, M.M. 1990. Sample preparation from paraffin-embedded tissues, in PCR protocols: A Guide to Methods and applications (Innis, M.A., Gelfand, D.H., Svinsky, J.J. and White, T.J., eds.), pp. 153-158. Academic, New York.

Yap, E.P.H., Lo, Y.-M.O., Fleming, K.A. and McGee, J. O'D. 1994. False-positives and contamination in PCR, in PCR Technology: current innovations. (Griffin, H.G. and Griffin, A.M., eds.), pp. 249-257. CRC Press, Florida.

Yeager, H. Jr., Lacy, J., Smith, L.R. and LeMaistre, C.A. 1967. Quantitative studies of mycobacterial populations in sputum and saliva. Am. Rev. Resp. Dis., 95: 998-1004.

Zaaijer, H.L., Cuypers, H.T.M., Reesink, H.W., Winkel, I.N., Gerken, G. and Lelie, P.N. 1993. Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet, 341: 722-724.

Acknowledgements

I would like to express my sincere gratitude to all the people working at the Departments of Bacteriology and Virology of The National Veterinary Institute, where the work of the present thesis has been performed.

My very special thanks to professor Karl-Erik Johansson, 'Kaggen', for guiding, helping and encouraging me all the time and for his wonderful friendship. You and Irja have been like my family in 'cold' Sweden.

Special thanks to professor Sándor Belák, my first supervisor, for accepting me as a student in the Department of Virology, for his excellent advise, trust, friendship and fabulous discussions.

The PCR group, Annie, Anna-Karin, Maria Elena, Claudia, Harry, Karin and Anders, for help, fun, productive collaboration, and being excellent colleagues at work.

The Mycoplasma group, Stefan, Marianne, Elisabeth, Katrin and Eva 'Puck'. For your help and wonderful working atmosphere that all of you created. And Joakim Holmdahl, 'Juka', for good times inside and outside the lab.

Professor Bengt Hurvell and professor Bror Morein, for trust and freedom in my work.

My co-authors, Jens Mattsson and Andras Ballagi, for excellent help in my first steps in molecular biology from the veterinary clinic.

Göran Bölske, for valuable discussion and help with the Mycoplasma strains.

Lena Bjömerot, for help 'fighting' with the sows and J-Å Robertsson for trust, freedom of work and kindness.

Norbert Nowotny, for his contagious enthusiasm in research and good humour.

Katinka Belák, for valuable '*in situ*' discussions and tasteful dinners.

Tibor Soos, for very nice talks about life and science, in spanish.

All the co-authors.

Helene Larsson and Barbro Sjödin-Stensmo, for their excellent secretarial work.

Laszlo Egyed, for his helping hand during virus isolation.

Hans-Joakim Rziha and Mathias Uhlén and their groups, for excellent collaboration.

Gustav Jansson and all the members of the 'Fundación Berndt Wistedt-Villa de Madrid', for giving me the oportunity to come to Sweden.

And a very special word of gratitude to:

My best friend in Sweden, Javier Martinez. I was very lucky to have had you as a friend all these years.

Claudia Rabino, for the way you are, always in good mood.

Manolo 'el cubanska', for being such a nice person. Extended to all the cubans I met in Uppsala, for their unique way of understanding life, with 'salsa'.

Carlos Concha, for your friendship and for let me win from time to time in tennis and ping-pong.

All the mixture of latin friends that I met in Sweden, and non-latin, Shariar and Leif.

My old friends in Spain, Vidal, Luis, Pedro, Cecil, Jesus 'chinchon' and Ignacio.

My parents, Manolo y Mari, for being everything in my life and for having understood my long absence.

Claude y Maud, for your patience with my 'français'.

Anne, my Sun in Sweden, for love, understanding, constant smile and contagious energetic personality.

Acta Universitatis Agriculturae Sueciae
presents doctoral theses from the faculty of agriculture, landscape architecture and horticulture, the faculty of forestry and the faculty of veterinary medicine at the Swedish University of Agricultural Sciences. Each faculty has its own subseries, Agraria, Silvestria and Veterinaria, respectively, with separate numbering.

ISSN 1401-6257
ISBN 91-576-5247-3