Ph.D. thesis

APPLICATION OF THE POLYMERASE CHAIN REACTION IN THE HUNGARIAN VETERINARY DIAGNOSTICS

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1. INTRODUCTION AND THE AIM OF THE WORK

The polymerase chain reaction (PCR) has been widely applied for research purposes and as a diagnostic tool as well since its invention (Gelehrter and Collins, 1990). The popularity of PCR is primarily due to its apparent simplicity and high probability of success, thus it is not surprising that the number of applications of PCR seems to be infinite - and is still growing. They include direct cloning from genomic DNA or cDNA (Temesgen and Eschrich, 1996), *in vitro* mutagenesis and engineering of DNA (Higuchi, 1989b), genetic fingerprinting of forensic samples (von Beroldingen et al., 1989), prenatal determination of sex (Macháty et al., 1993) and diagnosis of genetic diseases (Gelehrter and Collins, 1990), analysis of allelic sequence variations (Timme and Thompson, 1994), analysis of RNA transcript structure (Ansari-Lari et al., 1996), genomic footprinting (Luo et al., 1994), direct nucleotide sequencing of genomic DNA and cDNA (Reeben and Prydz, 1994), and **assays for the presence of infectious agents** (Eeles et al., 1992; Berencsi and Minárovits, 1997).

The PCR has revolutionised the detection of bacterial and viral pathogens. The targeted amplification of nucleic acid sequences provides not only dramatic increases in the number of copies to be detected thus allowing their further manipulation but concomitantly provides a nearly equivalent reduction in the amount of the nucleic acid to be probed. Either DNA or RNA (following the production of complementary DNA using reverse transcriptase) can be used as a template for amplification. Since PCR is a target amplification rather than a signal amplification system, the benefit of the variety of procedures developed over the years to amplify signal can be exploited coincidentally. These aspects of PCR allow ready detection of single pathogenic organisms or virus particles, an accomplishment provided earlier by procedures employing the *in vitro* propagation of such pathogens. However, since the

exponential amplification of PCR is catalysed by a biochemically simple cyclical process requiring less than several minutes per cycle, this procedure promises to supplant the culturing of a pathogen which frequently requires a total time of days to weeks. However, from either point of view, for the accurate diagnosis and further investigation of a pathogen (i.e., epidemiological studies) still needs and presumably will need the isolation of the causative agent, whenever it is possible. It is a known fact that pathogens not capable of *in vitro* propagation, for example because of the inability to culture a specific host cell for a virus, are refractory to detection using culturing approaches. Polyclonal and monoclonal antibodies used in a diagnostic setting typically recognise haptens found in multiple copies on a pathogen to circumvent the need to replicate the desired pathogen. Unfortunately, the cross-reactivity of these antibodies with host haptens and related but medically distinct pathogens as well as their often low avidity has compromised the convenient and broader use of these diagnostic reagents. In addition, some viruses establish latent infections in which active viral replication is substantially reduced thereby obviating procedures requiring the detection of proteins (Welch et al., 1992; Ballagi-Pordány, et al., 1992).

Although most diagnostic assays are based on sample collection by non-invasive means, invasive sampling is sometimes unavoidable. Optimal invasive sampling requires the collection of minuscule quantities of material. Even needle punch biopsies and aspirates of various types provide sufficient material for PCR analysis. Last but not least, the PCR can occasionally be used even if the state of the starting material is not sufficient for other diagnostic methods, e.g., because of its putrefaction.

Based on the above-mentioned features of PCR it is inevitable to introduce this technique into the routine veterinary diagnostic work. There have been numerous protocols applying PCR as a diagnostic tool (reviewed by Belák and Ballagi-Pordány, 1993). The aim of

my work was to establish a diagnostic PCR laboratory in the Veterinary Institute, Debrecen. For this purpose, preliminary studies and model experiments on setting up different systems of PCR in the Veterinary Medical Research Institute of the Hungarian Academy of Sciences, Budapest, under the leadership of Dr. Balázs Harrach and in the Department of Virology-Immunology, the University of Liege, Belgium, under the leadership of Professor Etienne Thiry. There are methods that were adopted from the literature with some modifications, others were combined from different sources and some others were used by us for different applications than originally described.

Since this work represents one of the very first experiments in the applied veterinary diagnostic area in Hungary it will hopefully give some guidelines which are advisable to follow for the future users. Therefore, in the beginning, an overview will be given on general methods of PCR then on the different applications of the technique and finally the detailed protocols will be described that have been applied as used in our institute.

REWIEV 2. PRINCIPLES OF THE PCR

The PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Saiki, 1990).

Reduced to its most basic terms, the PCR basically merely involves combining a DNA sample with oligonucleotide primers, deoxynucleotide triphosphates, and a thermostable DNA polymerase in a suitable buffer. A repetitive series of cycles involving **template denaturation**, **primer annealing**, and the **extension of the annealed primers** by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Because the primer extension products synthesised in one cycle can serve as templates in the next, the number of target DNA copies approximately doubles at every cycle. Thus, 30 cycles of PCR yields about 2^{28}-fold (270 million-fold) amplification. This method was originally applied to the amplification of human β-globin DNA and to the prenatal diagnosis of sickle-cell anaemia (Mullis et al., 1986; Mullis and Faloona, 1987; Saiki et al., 1985; Saiki et al., 1988).

In fact, the PCR is a relatively complicated and, as yet, incompletely understood biochemical process where constantly changing kinetic interactions among the several components determine the quality of the products obtained. Although the results will be good in most cases, there are a number of parameters that can be explored if better results are required or if the reaction fails altogether.

2.1. ELEMENTS OF THE PCR

2.1.1. Sample preparation

Sample preparation for PCR can be as simple and rapid as adding cells directly to the PCR. Things are not quite this easy, however, for all samples and applications of PCR.

In the simplest case mentioned above, sufficient DNA from a small number of tissue-culture cells can be made accessible to PCR merely by the lysis of the cells during the heat-denaturation step. Because of the ability of PCR to produce detectable amounts of product even from a few genome equivalents of DNA, it is not absolutely necessary to make the DNA available for PCR by a particularly efficient process. However, it is reasonable to do this e.g., if the PCR amplification is not particularly specific or efficient, or the target sequences are present infrequently among a large number of cells, the yield of product will be inadequate without the appropriate amount of the target DNA.

For the direct, cell-lysis protocol, the easiest way to increase the number of templates available to PCR should be to increase the number of cells added to the reaction. However, as the number of cells added goes above 600, the yield of product, unlike the yield from an equivalent amount of purified genomic DNA, does not increase with the addition of more cells, and begins to decline at 4800 cells. Due to the observed "cell-debris", it has been inferred that inhibition of the PCR process is occurring (Higuchi, 1989a).

For some applications, this limit on available template is unacceptable. This is true for the screening of samples for infectious agents such as retroviruses, especially HIV, which occurs in only a few white blood cells out of many thousands. One needs to efficiently screen all the DNA from tens of thousands of cells in order to pick up the HIV DNA from the few infected cells (Zimmermann et al., 1996), or there is an other application, where this is important, the screening of transgenic organisms for the transgene, if the transgene is present in only a minority of the cells of a certain tissue (Schlegel et al., 1996).

The problem is to find conditions that simultaneously release DNA and/or RNA from large numbers of cells in a form suitable for PCR while preserving the activity of *Taq* DNA polymerase. Methods of DNA purification from animal cells often use detergent to **solubilize cell components** and a proteolytic enzyme to **digest proteins**, probably mainly histones that would otherwise remain strongly bound to the DNA. This procedure is usually followed by extraction with organic solvents to **remove residual proteins** and membrane components, followed by steps such as **precipitation of nucleic acids** by ethanol to remove traces of the organic solvents. Since *Taq* DNA polymerase activity is not significantly affected by some non-ionic detergents and Proteinase K could be inactivated by heat, cells can be added directly to a PCR mixture containing non-ionic detergents and Proteinase K, but not yet the *Taq* DNA polymerase. Proteinase K needs some time to perform the digestion, and after the residual proteinase activity is destroyed by incubation at 95 °C for 10 min, *Taq* DNA polymerase is added and amplification cycles can be started.

Following the above-mentioned steps, good results can be obtained using tissue culture cells or density gradient purified (and washed) peripheral mononuclear cells as starting material for the PCR. However, if the same procedure is attempted with whole blood rather than purified mononuclear cells, inhibition of PCR occurs with the addition as little as 1 µl blood to a 0.1 ml reaction (Higuchi, 1989a). There is a noticeable precipitate in the reaction tubes and purified DNA "spiked" into this material will not yield amplification product. The testing of various blood components indicates that porphyrin compounds derived from heme may be the most inhibitory substances found in blood. Hematin has been found to inhibit PCR at a level as low as 0.8 micromolar (Higuchi, 1989a).

To separate porphyrin compounds quickly from nuclear DNA, the relevant protocols depend on the osmotic lysis of cells and the pelleting of nuclei and cell debris. Hemoglobin released from red blood cells is washed away in several pelleting and washing steps.

A simpler protocol exists that uses boiling to simultaneously lyse cells, release DNA, and precipitate hemoglobin. DNA found in the supernatant is added directly to the PCR. However, both the amount of DNA released to the supernatant and the amount of supernatant that can be added to a PCR without inhibition is limited.

For the preparation of DNA for PCR from tissues rather than blood, the same principles apply. Given the amplification potential of PCR, a small amount of DNA can be adequate. Crude lysates can be used adding amounts small enough to avoid inhibition (Kiss et al., 1996b). However, if screening for targets not present in every cell is required or if the PCR system used is not efficient additional effort may be necessary to make more template DNA available. There are protocols using non-ionic detergents and Proteinase K that can be used to screen for viral infections in epithelial cells collected as clinical swabs. It should be mentioned as well, that there are different fast-working kits, that use different kinds of resins or silica to prepare PCR-ready DNA thus eliminating deproteinization, organic extraction, and alcohol precipitation. The same approach can be applied to remove contaminating factors (i.e., primers, excess dNTPs, and salts) from an accomplished PCR mixture when further process is to be carried out (Smith et al., 1995; Higuchi, 1989a).

2.1.2. Primer selection

Unfortunately, the approach to the selection of efficient and specific primers remains somewhat empirical. There is no set of rules that will definitely ensure the synthesis of an effective primer pair. Yet it is the primers more than anything else that determine the success or failure of an amplification reaction. In order to design functioning primers the following guidelines should be kept in mind.

1. When possible, primers should be selected with a random base distribution and with a GC content similar to that of the fragment being amplified. It is important to avoid primers with stretches of polypurines, polypyrimidines, or other unusual sequences.

2. It is advisable to avoid sequences with significant secondary structure, particularly at the 3'-end of the primer.

3. The primers should be checked against each other for complementarity. In particular, avoiding primers with 3' overlaps will reduce the incidence of "primer dimer" (see below).

4. The stability of a hybridized primer/probe is expressed as the melting temperature or T_m, which is the temperature at which the primer dissociates from the target DNA. The T_m can be approximated from the equation of Meinkoth and Wahl (1984):

$T_m = 81.5^oC + 16.6\ (logM) + 0.41\ (\%GC) - 0.61\ (\%form) - 500/L$

where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, %form is the percentage of the formamide in the (hybridization) solution, L is the length of the hybrid in base pairs. (It should be noticed that there is an extended form of this equation which reckons with the mismatches between the primer and template sequences). By definition, using primers with higher T_m higher specificity can be achieved.

Sometimes there will be constraints on where the primers can be located. For example, perhaps only a limited amount of sequence information is available. Under those circumstances, it is worthwhile to go ahead and try the primers.

Most primers are between 20 and 30 bases in length and the optimal amount to use in an amplification will vary. Longer primers may be synthesized but are seldom necessary. Sequences not complementary to the template can be added to the 5'-end of the primers. These exogenous sequences become incorporated into the double-stranded PCR product and provide a means of introducing restriction sites or regulatory elements (e.g., promoters) at the ends of the amplified target sequence. If required, shorter primers or degenerate primers can be used as long as the

thermal profile of the reaction is adapted to reflect the lower stability of the primed template (Kiss et al., 1996a). For highly degenerate primers, it is preferable that the most unambiguous sequence be situated at the 3'-end of the primer, even to the extent of synthesizing a multiple series in which the various permutations of the 3' sequence are held constant. In general, concentrations ranging from 0.05 to 0.5 μM of each oligonucleotide should be acceptable (Saiki, 1989).

"**Primer dimer**" is an amplification artifact often observed in the PCR product, especially when many cycles of amplifications are performed on a sample containing very few initial copies of template. It is a doublestranded fragment whose length is very close to the sum of the two primers and appears to occur when one primer is extended by the polymerase over the other primer. The resulting concatenation is an extremely efficient PCR template that can, if it occurs at an early cycle, easily overwhelm a reaction and become the predominant product.

The exact mechanism by which primer dimer is formed is not completely clear. The observation that primers with complementary 3'-ends are predisposed to dimer formation suggests that transient interactions that bring the terrnini in close proximity are the initiating event. Several polymerases, including *Taq*, have been shown to have a weak non-template directed polymerization activity which can attach additional bases to a blunt-ended duplex or to nicks and gaps in the double stranded DNA as well. If such an activity were also to occur on a single-stranded oligonucleotide, there is a good chance that the extension would form a short 3' overlap with the other primer sufficient to promote dimerization. In any event, if dimers present an obstacle, they can be reduced somewhat by using minimal concentrations of primers and enzyme.

Although most primers will work with varying degrees of success, occasional primers will be synthesized that completely fail to amplify their intended target. The reason for this remain somewhat obscure, but in many of these instances, simply moving the primers by a few bases in either direction will solve the problem (Saiki, 1989, 1990).

Fortunately, the process of primer designing is not as complicated as it could be assumed from the aforementioned aspects. There have been several computer programs developed during the recent years to do this job quickly and reliably and some of them are accessible even via the Internet (Lowe et al., 1990; Montpetit et al. 1992).

2.1.3. The PCR buffer

The most widely used PCR buffer consists of 500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.0 and is supplied at 10x concentrated form. Changes to the PCR reaction buffer will usually effect the outcome of the amplification. In particular, the **concentration of $MgCl_2$** can have a profound effect on the specificity and yield of an amplification. Concentrations of about 1.5 mM are usually optimal (with 200 µM each dNTP), but in some circumstances, different amounts of Mg^{++} may prove to be necessary. Generally, excess Mg^{++} will result in the accumulation of non-specific amplification products and insufficient Mg^{++} will reduce the yield. It has been shown that the reduction or elimination of KCl can be beneficial (Saiki, 1989). Some protocols include 10% **dimethyl sulfoxide** (DMSO) ostensibly to reduce the secondary structure of the target DNA; however, it has been shown that DMSO can be slightly inhibitory to *Taq* polymerase and decrease the overall yield of amplification product mainly when GC-rich segments (>55%) are to be amplified. In this case, inclusion of **formamide** at a maximum concentration of 10% in the reaction mixture can eliminate non-specific products and increase the efficiency of the amplification (Sarkar et al., 1990).

The deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP) are usually present at 50 to 200 µM of each. Higher concentrations may tend to promote misincorporations by the polymerase (i.e., "thermodynamic infidelity") and should be avoided.

As deoxynucleotide triphosphates appear to quantitatively bind Mg^{++}, the amount of dNTPs present in a reaction will determine the amount of free magnesium available. In the standard

reaction, all four triphosphates are added to a final concentration of 0.8 mM; this leaves 0.7 mM of the original 1.5 mM $MgCl_2$ not complexed with dNTP. Consequently, if the dNTP concentration is changed significantly, a compensatory change in $MgCl_2$ concentration may be necessary (Innis and Gelfand, 1990).

2.1.4. Enzymes used in the PCR

Initially, the PCR used the **Klenow fragment of *E. coli* DNA polymerase I** to extend the annealed primers (Saiki et al, 1988). This enzyme was inactivated by the high temperature required to separate the two DNA strands at the outset of each PCR cycle. Consequently, fresh enzyme had to be added during every cycle. The initial PCR method based on DNA synthesis by the Klenow enzyme at 37 ^{0}C was not highly specific. Although a specific target fragment could be amplified up to a million-fold, most of what was synthesized in the PCR was not, in fact, this fragment.

The introduction of the **thermostable DNA polymerase, *Taq* polymerase** isolated from *Thermus aquaticus* strain YT1, which is a thermophilic, eubacterial microorganism capable of growth at 70°-75°C, and was isolated from a hot spring in Yellowstone National Park (Brock and Freeze, 1969) transformed the PCR into a simple and robust reaction which could now be automated by thermal cycling device. The reaction components (template, primers, *Taq* polymerase, dNTPs, and buffer) could all be assembled and the amplification reaction carried out by simply cycling the temperature within the reaction tube (Gelfand, 1989).

Taq DNA polymerase activity is sensitive to the **concentration of magnesium ion** as well as to the nature and concentration of monovalent ions. Generally, 2.0 mM magnesium chloride maximally stimulates *Taq* polymerase activity at 0.7-0.8 mM total dNTP concentration. Higher concentrations of Mg^{++} are inhibitory, with 40-50% inhibition at 10 mM $MgCl_2$ (Saiki, 1989).

Low, balanced **concentrations of dNTPs** have been observed to give satisfactory yields of PCR product, to result frequently in improved specificity, to facilitate labelling of PCR products with radioactive or biotinylated precursors and to contribute to increased fidelity of *Taq* polymerase. In a 100-µl PCR with 40 µM each dNTP, there are sufficient nucleotide triphosphates to yield 2.6 µg of DNA when only half of the available dNTPs are incorporated into DNA (Gelfand, 1989). It is likely that very low dNTP concentrations may adversely affect the processivity of *Taq* DNA polymerase.

Taq DNA polymerase does not contain an **inherent 3'-5'-exonuclease activity**. Single nucleotide incorporation/misincorporation, biochemical fidelity measurements have indicated that the ability of "non-proofreading" DNA polymerases to misincorporate a deoxynucleotide triphosphate is determined critically by the concentration of that triphosphate. Although not yet measured kinetically, *Taq* DNA polymerase appears to extend a mismatched primer/template significantly less efficiently than a correct primer/template. *Taq* DNA polymerase, however, has a DNA synthesis-dependent, strand replacement, **5'-3'-exonuclease activity** (Gelfand, 1989).

Taq DNA polymerase has a **template-independent terminal transferase activity** by which it adds a single nucleotide (usually A) to the 3' end of each PCR product molecule (it is the basis for T/A cloning). It has been shown, that the extended nucleotide is influenced by the nucleotides present at near the 5' end of the primer molecules, e.g., the non-templated nucleotide addition adjacent to a 3' terminal C of the PCR product (thus, 5' G in the primer) is favoured and the addition of adjacent to a 3' terminal A (thus, 5' T in the primer) is not (Brownstein et al., 1996).

The **concentration of *Taq* polymerase** typically used in PCR is about **2.5 units per 100 µl** reaction. For amplification reactions involving DNA samples with high sequence complexity, such as genomic DNA, there is an optimum concentration of *Taq* polymerase, usually 1 to 4 units

per 100 µl. Increasing the amount of enzyme beyond this level can result in greater production of non-specific PCR products and reduced yield of the desired target fragment (Gelfand, 1989).

In addition to the isolation and purification of DNA polymerase from *T. aquaticus*, DNA polymerase activities from *Bacillus stearothermophilus*, several other *Thermus* species and several divergent archaebacterial species have been reported and partially characterized. These organisms can grow at temperatures ranging from 60 °C to 87 °C. The polymerases isolated from these bacteria possess different advantages, for example, ***Pwu* polymerase** (originating from the hyperthermophilic archaebacterium *Pyrococcus woesei*) is a **blunting DNA polymerase** (it does not extend the PCR product), and it exhibits a **strong proofreading function**, therefore it is a perfect tool for generating amplified fragments for cloning purposes, the same as ***UlTma*** (*Thermotoga maritima*) or ***Pfu*** (*Pyrococcus furiosus*) **DNA polymerases**. Other blunting enzymes are ***Pfu*** and ***Tli*** (*Thermococcus litoralis*) **DNA polymerases**. ***Tth* DNA polymerase** (*Thermus thermophilus*) demonstrates **reverse transcriptase activity** in the presence of manganese ions thus allowing the development of protocols for single-enzyme reverse transcription and PCR amplification. ***Tfl* DNA polymerase** (*Thermus flavus*) and *Tth* DNA polymerase has a template-independent terminal transferase activity similar to *Taq* DNA polymerase (Hinnisdaels et al, 1996; Kobs, 1997; Miller and Storts, 1995).

Since the ability of *Taq* DNA polymerase to amplify longer DNA fragments is restricted to several thousand base pairs owing to misincorporation of nucleotides, protocols for **long-range PCR** (or long and accurate "LA-PCR") employ a mixture of two thermostable DNA polymerases, one that is highly processive and one with 3' to 5' exonuclease activity, allowing proofreading of the product, or use a single (usually modified *Taq*, such as Takara LA *Taq* DNA polymerase) enzyme with high capacity of amplifying large fragments up to 35,000 base pairs (Hengen, 1994).

Thus, when choosing the suitable DNS polymerase for our experiments we can follow two approaches: it means the usage of an enzyme with high capacity (i.e. *Taq* DNA polymerase)

mainly for diagnostic purposes while for further processing (such as the cloning and sequencing the PCR product) one is advised to use enzymes with strong proof-reading activity (i.e. *Pwu*, *Pfu*, or *UlTma* DNA polymerase; Stock et al., 1995).

2.1.5. Cycling parameters

PCR is performed by incubating the samples at three temperatures corresponding to the three steps in a cycle of **denaturation**, **annealing**, and **extension**. This cycling can be accomplished either manually with pre-set water baths, or automatically with a thermal cycler machine.

In a typical reaction, the double-stranded DNA is denatured by briefly heating the sample to 90-95 °C, the primers are allowed to anneal to their complementary sequences by briefly cooling to 40-60 °C, followed by heating to 70-75 °C to extend the annealed primers with a thermostable DNA polymerase.

The time of incubation at 70-75 °C varies according to the length of target being amplified; allowing 1 min for each kilobase of sequence is almost certainly excessive, it is a good place to begin (Saiki, 1989). Shorter times should be tried once the other amplification conditions have been established. (The extension step can be eliminated altogether if the target sequence is approximately 150 bases or less. The polymerase retains significant activity at lower temperatures and complete extension will occur during the thermal transition from annealing to denaturation.)

The ramp time, or time taken to change from one temperature to another, depends on the type of equipment used. With some notable exceptions, this rate of temperature change is not important and the fastest ramps attainable are used to shorten the cycle time. However, in order to be certain that the samples reach the intended temperatures, the actual ramp times for a particular setup should be determined by measuring the sample temperature during a test amplification.

Insufficient heating during the denaturation step is a common cause of failure in a PCR reaction. It is very important that the reaction reaches a temperature at which complete strand separation occurs. A temperature of about 94 °C should be adequate in most cases. As soon as the sample reaches 94 °C, it can be cooled to the annealing temperature. Extensive denaturation is probably unnecessary and limited exposure to elevated temperatures helps maintain maximum polymerase activity throughout the reaction.

The temperature at which annealing is done depends on the **length and GC content of the primers**. A temperature of 55 °C is a good starting point for typical 20-base oligonucleotide primers with about 50% GC content; even higher temperatures may be necessary to increase primer specificity (Saiki, 1989). Because of the very large molar excess of primers present in the reaction mix, hybridization occurs almost instantaneously and long incubation at the annealing temperature is not required. (The 20 sec denaturation and annealing incubation times generally used with the thermal cyclers is the amount of time it takes for a 100-µl reaction in a 0.5-ml microcentrifuge tube to equilibrate with the block temperature.)

In some cases, primers of only 12 to 15 bases are available and an annealing temperature around 40-45 °C is needed. However, primers of that length are unlikely to remain annealed at the 72 °C extension temperature. The problem can be overcome by taking advantage of the partial enzymatic activity of the polymerase at lower temperatures to extend the primers by several bases and stabilize them. This is accomplished either by an **intermediate incubation** at 50-60 °C or by heating gradually from 40 °C to 72 °C. Degenerate primers will often have multiple mismatches with their target sequence and should be treated in a similar manner.

It is often possible to anneal and extend the primers at the same temperature. In addition to simplifying the procedure to a **two-temperature** cycle, simultaneously annealing and extending at a temperature greater than 55 °C may further improve the specificity of the reaction (Saiki, 1989).

2.2. AMPLIFICATION PLATEAU

The amplification reaction is not infinite. After a certain number of cycles the desired amplification fragment gradually stops accumulating exponentially and enters a linear or stationary phase. This second stage of the reaction is called the "plateau." The point at which a PCR reaction reaches its plateau depends primarily on the **number of copies of target** originally present in the sample and by the total **amount of DNA synthesized**. (As such, it is not completely informative to specify the performance of a reaction solely by degree of amplification without also indicating the initial template concentration.)

In addition to the mundane possibilities such as exhaustion of primer or dNTP or inactivation of polymerase or dNTP, (none of which is significant in a standard reaction), there remains three more exotic causes of plateau - substrate excess conditions, competition by non-specific products, and product reassociation.

Substrate excess is simply the result of having synthesized more DNA than the amount of *Taq* polymerase present in the reaction is capable of replicating in the allotted extension time. It can be overcome by **increasing the extension time and/or increasing the amount of enzyme** in the reaction. This is usually not practical, however, because each succeeding cycle would require the doubling of extension time and/or polymerase to continue exponential growth.

Competition by non-specific amplification products is closely related to substrate excess conditions. In this case, the unwanted DNA fragments compete with the desired fragment for the attention of the limiting DNA polymerase. Clearly, this problem can be alleviated by **increasing the specificity of the reaction** so that the non-target sequences are not allowed to accumulate to any significant degree.

Alternatively, further accumulation of product can be attenuated by **reassociation of the single-stranded PCR fragments** before the annealed primers can be extended. This limitation

usually occurs when the product concentration approaches 10 pmol per 100 µl and is difficult to avoid except by **dilution of the reaction**.

In most of these cases, the plateau is an unavoidable and inherent limitation of the PCR reaction. However, by the time it occurs sufficient amounts of product will have accumulated for almost any purpose. For those few situations where even more material is needed, it is probably much easier to **set up multiple reactions** than to try to evade the plateau (Saiki, 1989).

2.3. FACTORS AFFECTING SPECIFICITY

There are a number of factors that can affect the specificity of the amplification reaction. The stringency of the annealing step can be controlled to some extent by adjusting the annealing temperature. Minimizing the incubation time during the annealing and extension steps will limit the opportunities for mispriming and extension by molecules of otherwise idle DNA polymerase. Reducing primer and enzyme concentrations also serves to limit mispriming, particularly the type that leads to dimerization. Finally, changing $MgCl_2$ (and perhaps KCI) levels can further improve specificity, either by increasing the stringency of the reaction or by direct effects on the polymerase itself (activity, processivity, etc.) (Saiki, 1989).

2.4. PCR PRODUCT DETECTION

The most common method for analyzing PCR products is the **agarose gel electrophoresis**. During electrophoresis, **DNA fragments sort by size** in the gel. The porous gel matrix acts as a molecular sieve through which smaller molecules can move easily than larger ones; thus, the distance moved by a DNA fragment is inversely proportional to its molecular weight. In a given period of time, smaller fragments migrate relatively far from the origin compared to larger fragments.

An agarose matrix can efficiently separate larger DNA fragments ranging in size from 100 nucleotides to more than 50,000 nucleotides. DNA fragments in different size ranges can be separated by adjusting the agarose concentration. A low concentration (down to 0.3%) produces a loose gel that separates larger fragments, whereas a high concentration (up to 2%) produces a stiff gel that resolves small fragments (Westermeier, 1993).

Generally, a fluorescent dye, **ethidium bromide** is used to **stain DNA bands** in agarose gels. The fragment pattern is viewed directly under **ultraviolet (UV) light**. After that, the gel can be photographed or video tape record can be taken. Images gained by the latter way can be stored and further analyzed or edited in a computer possessing the required software(s). Processing the data by such softwares is very useful for standardizing genotype-analyses.This ethidium bromide based technique is quite sensitive; as little as 5 ng (0.005 μg) of DNA can be detected.

Besides DNA sequencing, **polyacrylamide gel electrophoresis** (PAGE) is used to separate PCR fragments, mainly where higher sensitivity is needed than that achieved in agarose gels, for example for single-strand conformational polymorphism (SSCP) assays (Selvakumar et al., 1997).

However, gel-based methods for the detection of PCR products may have a number of drawbacks. Agarose gel electrophoresis with ethidium bromide staining is simple and inexpensive but suffers from a lack of the desired sensitivity and specificity.

There is a new stain however, that proved to be an extremely useful reagent for qualitative as well as quantitative PCR applications where high sensitivity or reduction of PCR cycle numbers is required. This stain, the **SYBR Green I** exhibits exceptional affinity for DNA and a large fluorescence enhancement upon DNA binding - at least one order of magnitude greater than ethidium bromide. Also, the fluorescence quantum yield of the DNA/SYBR Green I complex is more than five times greater than that of DNA/ethidium bromide (Schneeberger et al, 1995).

Besides this dye, two alternative gel-based methods have been used to overcome the previously mentioned problems.

In one method, known as **Southern hybridization** after its inventor (Southern, 1975), PCR products in an agarose gel are transferred to a membrane by standard blotting techniques and are subsequently detected with a labelled probe of specific sequence. With proper hybridization conditions, this technique is sequence specific and will not detect non-specific amplification products. However, in some instances, a significant amount of the PCR product is not full length and the results may be ambiguous because the detected products will still appear as a smear. The sensitivity of blotting methods may vary, depending on the specific label and the detection method used. Radioactive labels or enzymatic labels coupled with chemiluminescent detection usually give the most sensitive results, typically, one to three orders of magnitude better than ethidium bromide staining. Blotting methods are also laborious and time consuming, and radioactive labels pose significant disposal problems.

An alternative to blotting-based methods is **nested-primer amplification**, followed by agarose gel electrophoresis and ethidium bromide staining. With this technique, specificity and sensitivity are enhanced by the amplification conditions while the detection method itself remains insensitive and non-specific. With the nested primer technique, a first round of amplification is performed in a normal way. A portion of the first-round amplification reaction is then used as the sample in a second round of amplification using primers that are internal, or nested, to the first

set. Although the sensitivity of agarose gel detection is low, the overall sensitivity is high, because the target has been subjected to two rounds of amplification. The specificity of amplification is also enhanced because the primers used in the second round of amplification will only amplify the first-round amplicons. The disadvantages of this method are the lengthy and tedious amplification procedures, the cost of amplification reagents, and the higher probability of amplicon contamination during the seeding of the second round of amplification (Belák and Ballagi-Pordány, 1993).

To overcome these drawbacks, advanced detection methods have been developed which exhibit superior sensitivity and specificity compared with traditional methods. Ten or fewer input target copies can be detectable after one round of amplification, and the detection is sequence specific. Primer-dimers and other non-specific amplification products are not detected, and to ensure against carryover contamination, these detection methods are capable of incorporating decontamination procedures.

Enzyme-linked immunosorbent assay (ELISA) based detection methods offer the most promising alternative to gel-based detection methods because ELISA assays are relatively standardized, and ELISA techniques are familiar to most laboratory. In addition, a wide range of materials, equipment, and supplies are commercially available to support ELISA-based technology, and many laboratories are already thoroughly equipped to run ELISA-based assays. Other advanced detection methods based on alternative technologies, such as **high performance liquid chromatography** (HPLC) or **capillary electrophoresis**, are currently in limited use or under development (Lazar, 1994; Oefner et al., 1994).

2.5. CONTROLS FOR THE PCR

Based on its extreme sensitivity, it is crucial for the PCR to use both suitable negative and positive controls. The **negative control** reaction ensures the investigator that contamination was

excluded during the PCR. Such a reaction mixture can simply be assembled by replacing the target DNA by distilled water or by samples from mock-infected animals.

The result of a **positive control** PCR shows if the system works as it is expected. In the simplest case reference positive sample can be used as target for the PCR in both ways, in a separate tube and by putting the different dilutions of the reference sample into reaction tubes containing different dilutions of the sample under investigation. The reference sample can be a purified DNA of the particular agent (or a closely related member from the same taxonomic group) that we are seeking for. Thus, the reference and the "wild-type" sample will share the same primer sequences. This way we may gain information about the effectiveness of the purification process applied and the presence of the potential inhibitors in the clinical material. For most of the routine diagnostic protocols these controls are satisfactory - not to mention their low cost.

In another approach, a different set of primers than the target is used to yield an internal positive control sequence that can be **endogenous** or **exogenous**. For the former, the internal control product is derived from endogenous cellular sequence. This kind of control is very useful in diagnostic applications since it gives more accurate information about the effectiveness of the sample preparation. Exogenous internal controls are designed and produced artificially and used for accurate quantitation of the sample. It is important when using such controls that the internal control PCR product should be different in size to be resolved easily from the PCR product of interest, but close enough in size so there is no concern about differences in transfer efficiency due to size differences. It should also be clearly distinguished in size from the artifactual "primer-dimer" product.

2.6. CONTAMINATION

It is critical in any procedure that can detect only a few molecules to be absolutely scrupulous in avoiding contamination from exogenous nucleic acids. There are several precautions to be taken to avoid contamination that are listed below in order of importance.

DNA or PCR reagents preparation or the assembling of PCR must not be carried out in the same place that is used to handle the large amounts of PCR products. PCR products are the worst possible contaminants because they give rise to more of themselves faster than normal DNA. If possible, the two **different procedures should be performed in different rooms**, avoiding even the presence of PCR products in the room where DNA is prepared or PCRs are assembled. In addition, DNA and PCR reagents preparation and the assembling of PCRs should be done in a **laminar flow hood equipped with a UV light** to inactivate contaminating DNA.

It is important, if possible, to use only **sterile disposable plasticware** for preparing and storing reagents. If glassware used, care should be taken to avoid contamination with genomic DNA.

One possible source of contamination occurs during pipetting by the aerosols produced. Therefore, **sterile disposable tips** should be used. A more expensive, but more convenient, alternative may be to use positive displacement pipetting devices with disposable tip and plunger, but even then, the same device must not be used for handling PCR products. There are pipet tips marketed to minimize aerosol contamination.

Reagents should be sterile and stored as small aliquots.

When working with PCR, **disposable gloves** should be used and frequently changed.

One must be careful of **aerosols** from opening tubes. All tubes should be closed except those receiving sample DNA. It is advised to prepare only equivalent DNA samples at the same time. The use of screw-cap tubes can be helpful. It is also useful to dedicate a microcentrifuge solely to PCR assembly.

It is critical to **include appropriate control(s)**, which can help assess reagent contamination, as well as controls for DNA preparation, as it was discussed above.

Aside from PCR products, one should be suspicious of **other sources of contamination**, including various pieces of laboratory apparatus that come into contact with large amounts of target sequence. This extends to tools used to extract tissue specimens. In addition, contamination from the investigator should be avoided (Belák and Ballagi-Pordány, 1993).

3. RNA DETECTING PCR (RT-PCR)

PCR has been used extensively to study gene expression by amplification from RNA sequences. This approach requires **conversion of the RNA** template **to cDNA by reverse transcription** (RT) and amplification of the cDNA by PCR (RNA PCR or RT-PCR). RT-PCR is now a major methodology for detecting the expression of genes. This method is particularly useful when analyzing low abundance mRNAs or when a limited amount of sample is available. Amplification of RNA and cloning of amplified DNA also provide a convenient alternative to conventional cDNA cloning. RT-PCR is also widely used as a qualitative and quantitative tool for studying the expression of genes and for the **detection of viral RNAs** (Berencsi and Minárovits, 1997).

The conditions of fundamental importance for RT-PCR are the **quality of the target RNA**, the **features of modifying enzymes** to be used, and the **priming strategy**.

3.1. QUALITY OF THE RNA

Successful cDNA synthesis begins with the isolation of **high-quality RNA**. One of the most important factors preceding the synthesis of cDNA is isolation of intact RNA. Because quality of the RNA dictates the maximum amount of sequence information that can be converted into cDNA, it is important to optimize the isolation of RNA from a given biological source and to prevent adventitious introduction of ribonucleases into the RNA preparation or other reagents. RNA can be isolated for use in RT-PCR by many methods. The **guanidine isothiocyanate/acid-phenol method** described by Chomczynski and Sacchi (1987), which yields nondegraded RNA from cultured cells or whole tissue samples, has been used extensively. The amount of starting material for the preparation of RNA may be varied from 1 x 10^7 cells to as little as 1 x 10^3 cells, which is advantageous in applications where only small amounts of cellular material are available.

Typically, oligo(dT) selection for poly(A)$^+$ RNA is not necessary (moreover, in the case of RNA viruses that do not possess poly(A)$^+$ tail it is impossible), although incorporating this step may improve the yield of specific cDNAs and thus simplify subsequent amplification steps. Oligo(dT) selection is more useful in cloning applications of RT-PCR. Poly(A)$^+$ RNA selection may also reduce the likelihood of genomic DNA contamination, which is undesirable when studying the expression of genes.

Frequently, RNA preparations contain small amounts of genomic DNA that subsequently may be amplified along with the target cDNA. Some applications may require removal of all genomic DNA from the RNA preparation, and this can be accomplished by DNase I digestion of the RNA preparation (Liedtke et al., 1994).

3.2. CHOICE OF REVERSE TRANSCRIPTASE

A variety of reverse transcriptases are available for synthesis of cDNA prior to RT-PCR. These enzymes include Moloney Murine Leukemia Virus reverse transcriptase (**MMLV RT**), Avian Myeloblastosis Virus reverse transcriptase (**AMV RT**), and the ***Tth* polymerase** from *Thermus thermophilus*. There are also two **ribonuclease-H$^-$ mutants of MMLV RT**.

All of these enzymes have been used successfully in RT-PCR. Because the first step in RT-PCR involves reverse transcription of RNA into DNA, maximum conversion of RNA into DNA is of critical importance to the success of RT-PCR. Depending on the purpose and design of the RT-PCR, the requirement for synthesis of full-length cDNA varies. For cloning applications, it is generally better if the cDNA is full length. This is also true if the region of target to be amplified is near the 5' end of mRNA. The ribonuclease-H$^-$ derivatives of MMLV RT can convert a greater proportion of the RNA into cDNA and can synthesize longer cDNAs than other enzymes, because of a lack of ribonuclease-H activity. These enzymes also operate at a higher temperature (50 °C)

than their wild-type counterparts and AMV RT. This property allows synthesis of longer cDNAs from mRNA templates with secondary structure that are difficult to copy at lower temperatures.

The DNA polymerase from the thermophilic eubacterium *Thermus thermophilus* (*Tth* polymerase) exhibits a reverse transcriptase activity in the presence of Mn^{2+}. The thermophilic nature of this enzyme allows RT of RNA at high temperatures, which alleviates secondary structures present in the RNA template. More recently, bicine (2-hydroxyethyl-glycine) buffer has been used to develop a one-step RT-PCR method that utilizes the *Tth* polymerase as reverse transcriptase as well as DNA polymerase for amplification (Miller and Storts, 1995).

There is an **important feature of reverse transcriptases**, especially of MMLV RT, that having accomplished the reverse transcription the residual **reverse transcriptase adds nucleotides to the 3' end of primers** before PCR amplification begins, thereby extending short complementary regions in primers facilitating their interaction. In this case **accumulation of primer-dimers** and **inhibition of amplification of a specific DNA product** occurs. Hence, when cDNA is used to prime PCR, reverse transcriptase should be inactivated either by phenol extraction or heating, or in the case of r*Tth* polymerase by chelating manganase ions with EGTA (ethylene-glycol-tetraacetic-acid) (Chumakov, 1994).

3.3. CHOICE OF PRIMER FOR CDNA SYNTHESIS

Choice of primer for cDNA synthesis is largely dictated by specific application of the RT PCR. A first-strand cDNA synthesis reaction may be primed using three different methods. The relative specificity of each primer for RNA influences the amount and variety of cDNA synthesized.

1. The most non-specific of the primers, **random hexamers**, are typically used when a particular mRNA is difficult to copy in its entiretyi, because of the presence of sequences that cause the RT to abort synthesis. With this method, all RNAs in a population are templates for first

strand cDNA synthesis, and the PCR primers confer the needed specificity during the PCR amplification reaction. Generally **96% of all cDNA synthesized** using random hexamers is **from rRNA**. To maximize the size of cDNA synthesized using random hexamers, the ratio of primers to RNA may need to be determined empirically for each RNA preparation.

2. A method specific for mRNA is to use **oligo(dT)** as the primer. When the primer is hybridized to 3' poly(A) tails, which are found in the vast majority of eukaryotic mRNAs, only the mRNA is transcribed. Because poly(A)$^+$ RNA constitutes **~1-2% of a total RNA population**, the amount and complexity of cDNA is considerably less than when random hexamers are used. Because of its high specificity, oligo(dT) priming generally does not require optimization.

3. The most specific of the priming methods is to use an **oligonucleotide** containing sequence information **complementary to the target RNA**. If the PCR amplification reaction uses two specific primers, first-strand synthesis can be primed with the amplification primer that hybridizes to the 3' terminus of the mRNA. The advantage of using a specific primer is that the specific cDNA is produced, resulting in a more specific PCR amplification. In addition, regardless of the location of the amplification target in the mRNA, the cDNA represents the amplification region. It should be noted, however, that the use of the PCR antisense primer for the reverse transcription often results in the accumulation of non-specific amplified products in the ensuing PCR. This drawback can be overcome by the use of short (12-15 nucleotides long) sequence-specific RT **primer(s) located next to the antisense PCR primer** (< 100 bp). Since the T_m of these short oligonucleotides are much lower than that of the PCR primers they do not interfere with the subsequent amplification but remarkably enhance its specificity (Pfeffer et al., 1995; Rashtchian, 1994; Nuovo, 1995; Kawasaki, 1990).

Last but not least, it should be mentioned, that there have been a recently developed method mainly for the detection of RNA viruses and this is the NASBA (nucleic acid sequence-based amplification) technique. This method does not require the cycling of the temperature during the

reaction and it "simulates" the life-cycle of the retroviruses. It means that it amplifies the respective segment of the genome of a certain virus by the successive usage of three enzymes (the reverse transcriptase, the ribonuclease-H, and the RNA polymerase). Since these steps can be accomplished in the lack of a thermal cycler and comparable product yield can be gained (both in amount and in specificity) to the RT-PCR it is believed that the NASBA technique will soon be a real competitor of PCR based RNA (or even DNA) detecting protocols (Carrino and Lee, 1995).

4. CONSIDERATIONS OF DIAGNOSTIC PCR

4.1. ANALYTICAL SENSITIVITY AND SPECIFICITY

The analytical sensitivity of PCR as a biochemical process itself is dependent on the specificity at which the primers initiate polymerization from the targeted site relative to other non-specific regions of the nucleic acid. Altering the concentration of the various reaction components such as the enzyme, primers and metal cation (magnesium chloride) has a marked effect on the yield of the desired PCR product. Additionally, the temperatures and times of the annealing and extension steps of each cycle also affect sensitivity and specificity of the amplifications. Although primers of 20 to 30 residues are commonly used, synthetic oligonucleotides containing as few as twelve bases have been utilized. Since pathogen detection is often carried out in the presence of vast quantities of host nucleic acid, the ability of the primers to be pathogen-specific is critical to sensitive and specific amplifications. Until a larger proportion of the sequence of the hosts' genome has been determined, the design of optimal primers for specific pathogens will require significant empirical experimentation. It should be noted, however, that in the systems best optimized to date (e.g., HIV-detecting PCR) the detection of one to ten molecules of DNA in the context of highly complex nucleic acids from the equivalent of one million cells can be accomplished using thirty cycles of amplification and standard detection strategies. Of course, as the number of copies of a nucleic acid that one wishes to detect in a sample approaches one, the reproducibility of detection is compromised because of the decreased likelihood that all aliquots of the sample will contain the desired target and because of stochastic effects of the amplification reaction.

The utility of any diagnostic assay is based on its clinical sensitivity and specificity (Gelehrter and Collins, 1990). It is important to evaluate PCR from epidemiological point of view, when the sensitivity and specificity of the test are exteriorized to larger scales, i.e., to herds or flocks in the veterinary diagnostic application. Sensitivity is defined as the ability of

the PCR to correctly identify each animal infected with the particular pathogen, while specificity is defined as the ability of the PCR to correctly identify the uninfected animals. To define sensitivity and specificity, results of the PCR must be compared with those of other diagnostic methods used for the diagnosis of the particular pathogen. These may include serology, culture procedures, histological and electron microscopic examinations, etc. (Lauerman et al., 1993).

4.2. HETEROGENEITY OF THE PATHOGENES

Viral genomes particularly those of RNA viruses (i.e., bovine viral diarrhea virus) and viruses that replicate through an RNA intermediate (i.e., retroviruses) contain multiple base alterations, additions, duplications, and deletions (Grieser-Wilke et al., 1993; Sugden, 1993). The variability of these viruses has been attributed to the low fidelity and lack of proofreading functions or template switching of the polymerases responsible for their replication (Meyers et al., 1991). In addition, since RNA polymerase II plays a critical role in the retroviral life cycles, both the fidelity of this enzyme and the reverse transcriptase must be taken into consideration. Repeated rounds of infection further magnify variability. The role that these viral variants play in the natural history of infection is only beginning to be understood and appears to vary with each class of virus. This variability may lead to problems when using PCR since some individuals may harbor viral variants incapable of amplification due to sequence alterations in the region recognized by the primers. As a result, continued evaluation of the clinical sensitivity of a primer pair and probe system and the development of primers that may tolerate mismatches more efficiently should be an integral part of the evaluation of a diagnostic assay for several types of viruses.

4.3. THE DESIGN OF A DIAGNOSTIC PCR

There are numerous aspects that should be evaluated when setting up a diagnostic PCR protocol. First and foremost its feasibility is to be considered. Of course, in the case of certain pathogens the expenses should not limit the use of this fast, specific and extremely sensitive method (i.e., HIV in human medicine, some notable diseases of livestock species). The use of the PCR is reasonable too if the particular pathogen is difficult to isolate or it takes a long time and the other diagnostic methods (i.e., gross- and histopathology, serology, etc.) are less informative. However, PCR can be a unique tool even in the case of pathogens that are easy to detect because the amplified products can serve as a base for further epidemiological (and of course taxonomic) investigations if it is needed.

When it is decided upon which pathogen is targeted the study of its literature is indispensable. It can be stated that virtually all of the known relevant pathogens have been targeted by PCR by now, therefore, one can find safe "pathways" to follow, thus avoiding unnecessary pitfalls.

Depending on the pathogen(s) to be detected and identified, PCRs can be designed of different specificity. When a defined pathogen is solely to be targeted, one may use not merely species specific but even serotype or genotype specific system by amplifying fragments from variable regions of the genome. The another approach which allows identification at a higher taxonomic level exploits the presence of highly conserved regions in the genome of these organisms. The result can be refined afterwards by a more specific PCR or by several other methods, including restriction fragment length polymorphism assay, Southern blotting, or the most precise method the nucleotide sequencing. Either approach is chosen it needs for the knowledge of the respective part(s) of the genome. Besides the published sequences it is more convenient to reach for the database of the GenBank and collect sequences of interest. Moreover, this latter kind of information can directly be handled by a primer designer software.

After selecting an appropriate region to be targeted from the diagnostic point of view the thermodynamic considerations should be balanced for primer selection - as it was detailed in a previous chapter. Having settled upon the targeted sequence it should definitely be checked again by the GenBank in order to eliminate the possibility of amplifying similar sequences from uninterested sources that could present false positive results in the future. It may happen even in the case when only one primer is affected and low-stringency conditions are used (Hiltunen et al., 1994).

The selected primers should then be purchased from any of the numerous suppliers and checked in practice. It is advisable to start with purified target DNA to see the yield-capacity of the primers; besides, the Mg^{++} concentration should be optimized. Having performed these preliminary experiments clinical samples are to be tried. Depending on the source of the samples purification procedures are to be tested concerning the reliability, reproducibility, simplicity, and the cost of them. Working with RNA viruses reverse transcriptase is necessary to produce cDNA. There are several possibilities to do this, from the RT reaction mixtures assembled on one's own through the specifically designed RT kits to the pre-assembled reaction mixes that allow RT and the subsequent PCR in the same tube using a single enzyme.

Whichever method is chosen for the abovementioned processes it is very important to set up a protocol that will be standardized. This also applies to the reaction components (i.e., dNTPs, enzyme, buffer). Thus, the occurrent differences between the results obtained by different labs could at least be minimized.

It should be emphasized again that follow-up studies must be carried out from time to time concerning the sequence variation of the particular and other pathogens, thus reserving the value of the given procedure for diagnostic applications.

5. EXPERIMENTS

5.1. Detection of homologous DNA sequences in animal adenoviruses by PCR

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INTRODUCTION

The DNA restriction enzyme (RE) analysis of different serotypes of bovine (Kurokawa et al., 1978; Belák et al., 1983; Hu et al., 1984b; Benkő et al., 1988), porcine (Garwes and Xuan, 1989; Benkő, 1990; Kleiboeker et al., 1993; Reddy et al., 1993, 1995; Tuboly et al., 1995), and ovine (Benkő, 1990; Boyle et al., 1994) adenoviruses did not indicate the existence of very close genetic relationships among these animal adenoviruses and any types of HAVs. The only exception was BAV-9, with RE patterns resembling those of group C HAVs (Benkő, 1990). Southern hybridization experiments revealed DNA homology between HAVs, BAVs, and PAVs, except subgroup 2 (a serologically and biologically different cluster) BAVs (Bartha, 1969), which showed no homology to HAVs or subgroup 1 BAVs (Benkő 1990; Benkő and Harrach, 1990), instead, members of the subgroup show a close phylogenetic relationship with the egg drop syndrome virus and ovine adenovirus 287 isolate (Harrach et al., 1997; Harrach and Benkő, in press). Sequence data of bovine adenoviruses available to date suggest, that the genome organization, the relative size and location of some important genes, especially those of the genes of the late mRNAs are comparable in the human and animal adenoviruses (Hu et al., 1984a; Mittal et al., 1992; Elgadi et al., 1993; Esford and Haj-Ahmad, 1994; Salmon and Haj-Ahmad, 1994).

A primer pair and a PCR method were earlier elaborated for the general detection of HAVs (Allard et al., 1990). The 301 base pair (bp) long sequence flanked by the two primers (HexAA1885 and HexAA1913) encodes for the basal part of the β-barrel-forming P1 domain of the hexon protein (Roberts et al., 1986). The DNA sequence of the primers were taken from the most conserved parts of this region, where all the known HAV hexon sequences were identical. By the use of these primers, all the 18 examined HAV serotypes representing all the six subgenera A through F could be detected by PCR (Allard et al., 1990). Based on the available sequence data of bovine adenoviruses, we have altered these general human adenovirus primers by introducing some degeneracy.

In the present work, the modified primers were tested successfully on certain adenoviruses of animal origin.

MATERIALS AND METHODS

Source of viruses. The designation of virus strains examined are listed in Table 1. The references of prototype BAVs, PAVs, and OAVs were recently reviewed, by Benkő (1990), Derbyshire (1989), and Belák (1990), respectively. Beside the prototype strain of BAV-10, four additional isolates from Northern Ireland (Adair et al., 1996) were included into our studies.

Preparations of viral DNA. Phenol-extracted and alcohol-precipitated viral DNA (at a concentration of 6 to 10 μg/ml estimated on agarose gels), as well as crude preparations of adenovirus infected cell lysates were used. The DNA extraction methods were described earlier (Benkő et al., 1988). Crude preparations were made from inoculated test tube cultures. When the cytopathic effect was maximal, the tubes were twice frozen and thawed, then 0.5 ml of each cell suspension was boiled in an Eppendorf tube for 10 minutes. After 5 minutes of

centrifugation at maximum rpm in a microcentrifuge, 1 µl of the supernatants were used in the PCR assay. For control purposes, uninfected MDBK cell culture were treated the same way. The viral DNA of PAV-5 was kindly provided by T. Tuboly.

Table 1. Designations and recent references of the adenovirus strains examined.

Serotype	Subgroup	Isolate	Reference	
BAV-1	1	B-10	Benkő et al.	1988
BAV-2	1	B-19	Benkő et al.	1988
BAV-3	1	WBR-1	Benkő et al.	1988
BAV-4	2	THT/62	Benkő et al.	1988
BAV-5	2	B4/65	Benkő et al.	1988
BAV-6	2	671130	Benkő et al.	1988
BAV-7	2	Fukuroi	Benkő et al.	1988
BAV-8	2	285	Benkő et al.	1988
BAV-9	1	Sofia-4/67	Benkő et al.	1988
BAV-10	1	Ruakura	Horner et al.	1989
BAV-10	1	Belfast 1	Adair et al.	1996
BAV-10	1	Belfast 2	Adair et al.	1996
BAV-10	1	Belfast 3	Adair et al.	1996
BAV-10	1	Belfast 4	Adair et al.	1996
OAV-1		S1	Belák	1990
OAV-2		PX515	Belák	1990
OAV-3		PX611	Belák	1990
OAV-4		7769	Belák	1990
OAV-5		SAV	Belák	1990
OAV-6		WV419	Belák	1990
PAV-1		25R	Derbyshire	1989
PAV-3		6618	Derbyshire	1989
PAV-5		HNF70	Hirahara et al.	1990

Primers. The nucleotide sequence of the 25-mer left primer (HexAA1885) and the 23-mer right primer (HexAA1913) designed for HAV detection is shown in Table 2. Taking into

consideration the known hexon gene sequence of BAV-3 (Hu et al., 1984a) and earlier observed AT-rich coding strategy of BAV-4 (Benkő and Harrach, 1994), we have altered these primers by introducing wobbles at positions 7 and 13 (left primer: HexAdB), and at positions 5, 6, 7, and 22 (right primer: HexAdJ), as shown in details in Table 2. Thus the extent of degeneracy was four- and sixteen-fold in the left and right primers, respectively. In the degenerate primers, however the sequence of coded amino acids was preserved, except the 5' end of the right primer, where even BAV-3 and the HAVs contain different amino acids (Toogood et al., 1988). The primers were synthesized on a Gene Assembler Special oligonucleotide synthesizer (Pharmacia LKB), their concentration was measured by a GeneQuant Calculator (Pharmacia LKB).

Table 2. DNA sequence of the PCR primers

Primer	Sequence	Amplimer length (bp)
HexAA1885	5' GCCGCAGTGGTCTTACATGCACATC 3'	301
HexAdB	5' GCCGCAGTGGTCTTACATGCACATC 3' A C	301
HexAA1913	5' CAGCACGCCGCGGATGTCAAAGT 3'	301
HexAdJ	5' CAGCACGCCGCGGATGTCAAAGT 3' GTA A	301

HexAA1885 and HexAA1913 are the original primers designed for the detection of human adenoviruses, while HexAdB and HexAdJ are the modified (left and right) primers used in this work. Underlined letters show the nucleotides at which wobbles were allowed. The alternative bases are presented below the original sequence.

Polymerase chain reaction. The PCR was carried out in 50 µl of reaction mixture containing 5l of 10X buffer, (500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.0), 50 pmol of each primer, 1 U of *Taq* DNA polymerase, 300 µM of each of the four

deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Pharmacia LKB), and 1 to 5 µl of the target DNA. The volume of the reaction mixture was adjusted to 50 µl by adding distilled water. For positive control, HAV-2 total genomic DNA was used, while a lysate of uninfected MDBK cells served as negative control.

The DNA amplification was repeatedly carried out in two machines: either in a DNA Thermal Cycler, (Perkin Elmer Cetus) at the Veterinary Institute of Debrecen, or in a PDR 91 DNA reproducer (BLS Ltd., Hungary) at the Veterinary Medical Research Institute, Hungarian Academy of Sciences, in Budapest. The first cycle consisted of denaturation of target DNA at 94 °C for 10 minutes, annealing at 55 °C for 30 seconds, and polymerization at 72 °C for 30 seconds. Cycles 2 to 34 consisted of the same three steps, but only 30 seconds denaturation time was applied. For the last, 35th cycle, an extended elongation step (72 °C) of 5 minutes was used. In several experiments lowered annealing temperatures (45 and 50 °C) were also tested.

Detection of PCR products. The amount and size of the amplification products were analyzed by the electrophoresis of 8 µl of the reaction mixture on a 1.5% agarose gel (SeaKem) in TBE buffer containing ethidium bromide (0.5 µg/ml) at a constant voltage of 110 V for 45 minutes. For molecular weight marker *Pst* I cleaved λ phage DNA (Fermentas, Lithuania) was loaded on the same gel. The gel was visualized by ultraviolet light at 302 nm wavelength and photographed on Polaroid 665 and/or 667 films.

RESULTS

Agarose gels containing the products of different PCRs performed with annealing temperature of 55 °C are shown in Fig. 1. and 2. As it can be seen, specific amplification of an approximately 300 bp DNA fragment could be achieved using the modified primers from

each of the examined animal adenovirus serotypes. A variation in the intensity of the bands can however be observed indicating that the amount (final concentration) of the amplification products is not equal in each reaction. Less product was consistently obtained when testing the members of the subgroup 2 BAVs (Fig. 1). Also, the performance of crude preparations was generally inferior than that of purified DNA (Fig. 2, lanes 2 and 3).

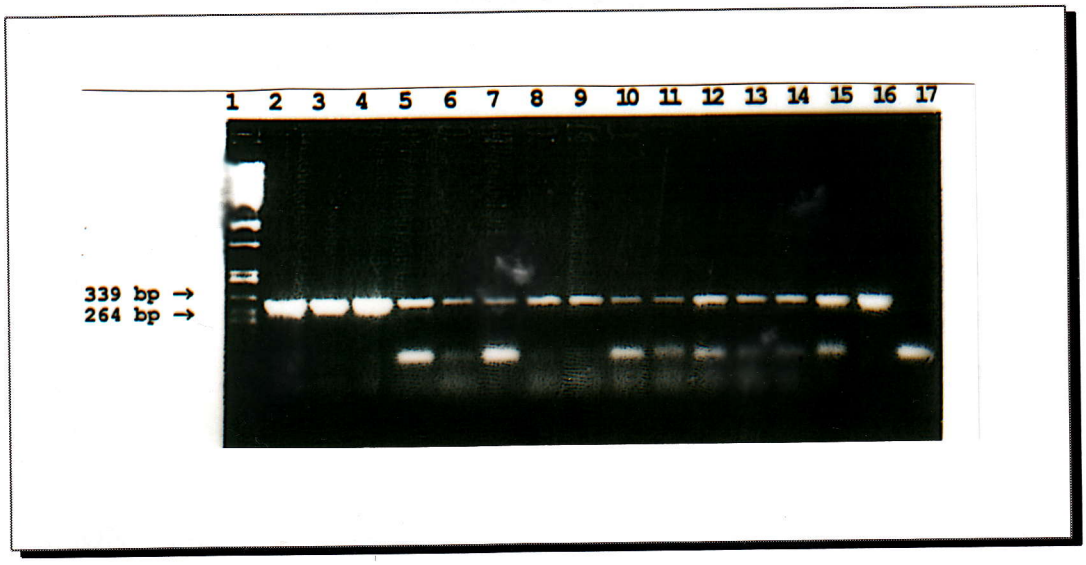

Figure 1. Gel electrophoresis of the PCR amplification products from different bovine adenovirus serotypes with primers HexAdB and HexAdJ. Lane 1: Molecular weight marker: λ phage DNA digested with *Pst*I, fragment sizes as marked; Lane 2: BAV-1 DNA; Lane 3: BAV-2 DNA; Lane 4: BAV-3 DNA; Lane 5: BAV-9 infected cell lysate; Lane 6: BAV-4 infected cell lysate; Lane 7: BAV-5 infected cell lysate; Lane 8: BAV-6 DNA; Lane 9: BAV-7 DNA; Lane 10: BAV-8 DNA; Lane 11: prototype BAV-10 (strain Ruakura) infected cell lysate; 12-15: DNA of the different isolates of BAV-10, namely: Belfast-3, Belfast-2, Belfast-4, and Belfast-1, respectively; Lane 16: Positive control: HAV-5 DNA; Lane 17: Negative control: uninfected MDBK cell lysate.

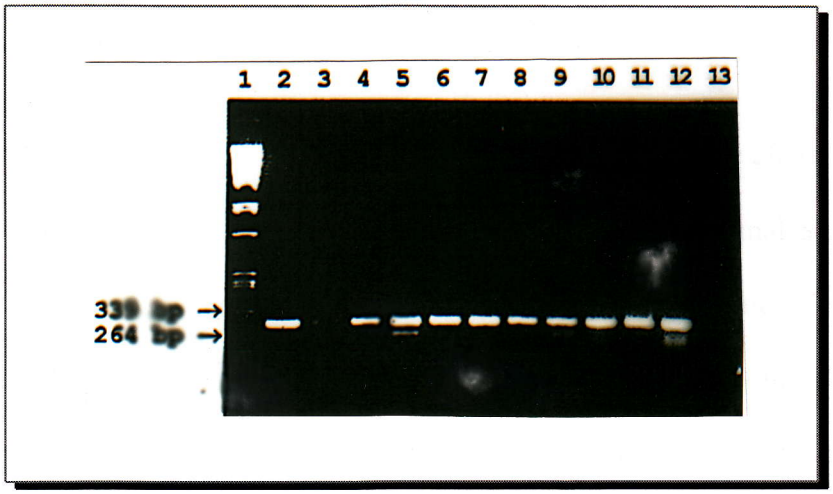

Figure 2. Gel electrophoresis of PCR amplification products from ovine and porcine adenoviruses. Lane 1: Molecular weight marker: λ phage DNA digested with *Pst*I; Lane 2: OAV-6 DNA; Lane 3: OAV-6 infected cell lysate; Lane 4: OAV-5 infected cell lysate; Lane 5: OAV-1 DNA; Lane 6: OAV-2 DNA; Lane 7: OAV-3 infected cell lysate; Lane 8: OAV-4 infected cell lysate; Lane 9: PAV-5 infected cell lysate; Lane 10: PAV-1 DNA; Lane 11: PAV-3 DNA, Lane 12: Positive control: HAV-5 DNA; Lane 13: Negative control: uninfected MDBK cell lysate.

DISCUSSION

Recently, up-to-date molecular biological techniques such as DNA hybridization, restriction fragment length polymorphism (RFLP) analysis, or *in vitro* amplification of DNA by PCR are being applied in identifying microorganisms of interest. In the present study, we have used BAVs, OAVs and PAVs as models to test the applicability and feasibility of PCR in the detection of different animal adenovirus serotypes. In preliminary experiments (Allard, 1990; Benkő 1990), a primer pair designed for the general detection of HAVs was found feasible for the detection of bovine, ovine, and porcine adenoviruses too. The identity of the amplification products was then confirmed even by Southern-blot hybridizations (Benkő, 1990). However, a significant reduction was observed in the yield of the amplification

products resulting from the subgroup 2 BAV serotypes. This reduced yield could partially be overcome by reducing the annealing temperature to 45 °C (Benkő, 1990).

In order to enhance the performance of the human primers, some alterations have been introduced into the primers on the basis of the presently known animal adenovirus DNA sequences (Hu et al., 1984a; Benkő and Harrach, 1994). With the modified primer pair, amplification products similar in size and quantity could be obtained from HAV-2 and from the examined serotypes of porcine, ovine, and subgroup 1 bovine adenoviruses. The yield of PCR products resulting from subgroup 2 BAVs was still slightly less (Fig. 1), but superior to the amount obtained earlier with the original human primers (Benkő, 1990).

Our experiments produced further evidence of the existence of well-preserved coding regions in the hexon gene of different animal adenoviruses. This finding might be exploited in orientating restriction site maps of adenovirus genomes, since selected clones (or separated DNA fragments) can directly be tested by PCR for the presence of the hexon-coding gene. Moreover, this PCR procedure might also be introduced into phylogenetic studies. Computer-aided phylogenetic analysis can be performed on nucleic acid and protein sequence data of limited size if they originate from corresponding genomic regions (Bailey and Mautner, 1994). For this purpose, however, further modification of the method, i.e. selection of other primers flanking longer stretches of DNA might be needed.

An obvious field of practical application of the described PCR method would be, in veterinary diagnostics, the detection of adenoviruses which are difficult to isolate and grow. These viruses comprehend above all the members of subgroup 2 BAVs, several OAVs, PAVs, and especially the most recently described BAV-10, which seems to bear outstanding pathological and phylogenetic importance (Benkő et al., 1995; Matiz, 1997; Matiz et al.,

submitted). Moreover, the method described above was just reported to have successfully used for the amplification of the adenovirus specific sequences from a cat (Lakatos et al., 1997). The evaluation of the applicability and performance of the method on clinical samples needs to be performed, and a possibly simple procedure for the pre-treatment of clinical samples appropriate for routine laboratory use has to be elaborated.

In veterinary diagnostics, type classification of the detected adenoviruses might be of interest. According to the results of several pilot experiments on BAVs, restriction endonuclease digestion and subsequent gel electrophoresis of the cleaved amplification products might be feasible in type identification. Nevertheless, for such purposes, another PCR method resulting in larger (500-1000 bp long) products might also be indispensable.

5.2. Infectious canine hepatitis: detection of canine adenovirus type 1 by polymerase chain reaction

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INTRODUCTION

Canine adenovirus type 1 the causative agent of infectious canine hepatitis, can provoke severe disease in dogs and other carnivores. Death from infectious canine hepatitis is usually sporadic, although small outbreaks may occur among young dogs kept in kennels. Fatalities seldom occur in dogs older than 2 years of age. Vaccination has greatly reduced the frequency of the disease, and nowadays it is rare in many countries where it used to be endemic. Although the clinical symptoms, gross and histopathological findings of fatal cases are usually rather characteristic (Kelly 1993), the confirmation of the presence of CAV-1 is necessary for exact diagnosis. Isolation of CAV-1 on MDCK or vascular epithelial cells is often difficult and may require several blind passages before the cytopathic effect becomes obvious.

In the present work, a PCR method is described, which might serve as a useful alternative to virus isolation in the rapid confirmation of the diagnosis of infectious canine hepatitis.

MATERIALS AND METHODS

Case history and clinical observations. Case 1. A four-month-old Caucasian shepherd male dog was sent for *post mortem* examination to the Veterinary Institute, Debrecen, at the Spring of 1995. The puppy had been vaccinated against canine parvovirus and canine

distemper virus only. The first clinical symptoms of vomiting, high fever, and signs of abdominal pain appeared 2 days earlier. The puppy was found dead on the third morning.

Gross and histopathological examinations. Gross and histological examinations, including light microscopic examination of haematoxylin and eosin stained sections of the liver were carried out at the Veterinary Institute, Debrecen. Organ samples taken from the liver were stored at -20 °C before being subjected to PCR.

Case 2. A similar case had been examined at the Central Veterinary Institute, Budapest in 1992. Organ sample taken from the liver of the affected dog had been passed to the Department of Epizootology and Microbiology, University of Veterinary Science, Budapest, where an adenovirus was isolated on MDCK cell line. The isolate was identified as CAV-1 by virus neutralization test with hyperimmune serum kindly provided by Professor L. E. Carmichael, Cornell University, Ithaca, USA. The isolated virus strain and liver specimen were kept frozen at -20 °C for over three years. These samples were used as specific positive controls.

Sample preparation. In order to expose the viral DNA we have used two different procedures. The first one (Procedure A) was a standard DNA purification method including Proteinase K digestion, phenol/chloroform extraction, and alcohol precipitation steps (Benkő, 1990). The other method (Procedure B) consisted of the following simple steps: approximately one gram of liver specimen was homogenized and diluted to 1:10 in PBS. After three cycles of freezing at -20 °C and thawing at room temperature, the sample was boiled for 10 minutes. One ml of the solution was transferred into an Eppendorf tube and was spun in a microcentrifuge at maximum speed (app. 13000 rpm) for 5 minutes. An aliquot of 2 µl of the supernatant was used in the PCR mixture as target DNA solution.

Controls. Purified DNA of human adenovirus type 5 (HAV-5) served as positive control, while liver sample taken from an uninfected dog carcass and treated as described above was used as negative control.

Primers. The sequence of the primers (HexAdB and HexAdJ) was described before (Kiss et al., 1996). The primers were synthesized on a Gene Assembler Special oligonucleotide synthesizer (Pharmacia LKB).

PCR. The amplification was carried out in a reaction mixture of 50 µl volume containing 5 µl 10X reaction buffer (500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.0), 50 pmol of each primer, 1 U of thermostable *Taq* DNA polymerase, 300 µM of each of the four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Pharmacia LKB), and 2 µl of sample solution. A DNA Thermal Cycler (Perkin-Elmer Cetus Instruments) was used. The "Step-Cycle" program was set to denature the DNA at 94 °C for 30 seconds, to anneal the primers at 55 °C for 30 seconds, and to extend the sequence at 72 °C for 30 seconds, for a total of 35 cycles.

Detection of PCR products. The amplification products were detected by agarose gel electrophoresis. An aliquot of 8 µl from each reaction was run on 1.5% agarose gels containing ethidium bromide (5 µg/ml) at a constant voltage of 110 V for 45 minutes. For molecule weight marker λ phage DNA digested with *Pst*I enzyme was used. The gels were placed on a UV transilluminator and photographed on Polaroid 665 films.

RESULTS

Gross and histopathological examinations. The necropsy of the Caucasian shepherd dog revealed an excess of bloody fluid in the abdomen, petechial haemorrhages and slight jaundice

on the serous membranes. The liver was enlarged, turgid, and friable. The wall of the gallbladder was oedematous. The histology of the liver showed haemorrhages, and hepatic necrosis. There were some large, amphophilic intranuclear inclusions in the hepatic parenchymal cells indicating the presence of CAV-1. Similar gross and histopathological changes were seen at the Case 2.

PCR. Fig. 1 shows an agarose gel containing the PCR products from positive control (HAV-5 DNA and Case 2) samples. The amount of the amplified product from CAV-1 appears to be less than that from HAV-5. Apart from this, it can clearly be seen that the size of the amplified DNA fragment corresponds to the size of the amplified segment of HAV-5.

Fig. 2 shows the result of the PCR amplification of the tissue homogenate samples. In this experiment we applied the simple method (Procedure B) for to expose the viral DNA, and found it satisfactory for our purpose. As it can be seen on Fig. 1 (Lanes 6 and 7), there was only minor difference in the intensity of the two bands obtained from the same sample pre-treated by the two different ways. Obviously, the DNA concentration in the extracted sample can be much higher, but since it is contaminated with chromosomal DNA of cellular origin, if the sample is used undiluted, the performance of the reaction can be considerably diminished.

DISCUSSION

PCR is a recently described *in vitro* method for primer-directed enzymatic amplification of specific target DNA sequences. (Saiki, 1989). It has gained wide popularity in different fields of biology from basic research to routine diagnostics. In the developed countries, PCR became an important part of medical diagnostic methods, and will certainly be introduced into certain fields of veterinary routine diagnostics too (Belák and Ballagi-Pordány, 1993). With

appropriate cautiousness, PCR can represent invaluable help in rapid identification of specific pathogens which are difficult to grow.

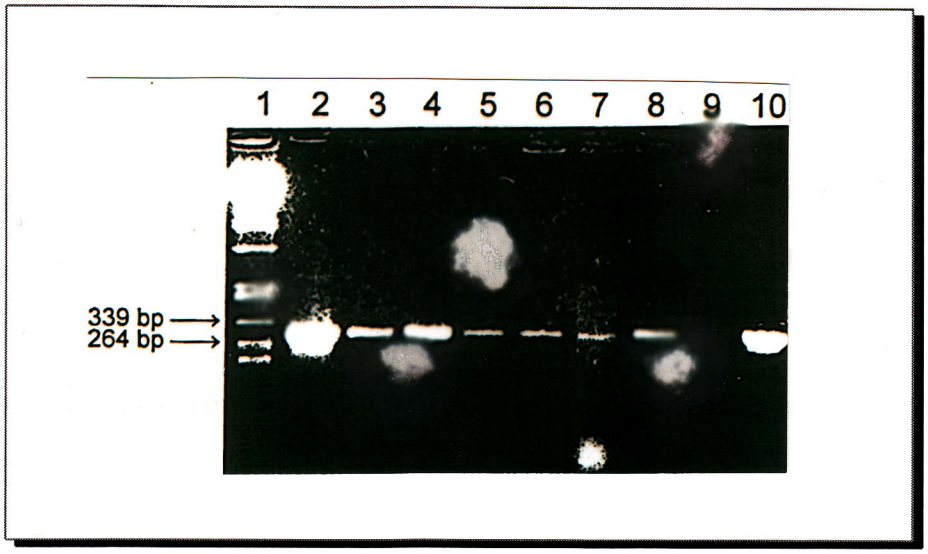


Figure 1. Agarose gel containing PCR amplification products from samples pre-treated by different procedures.

Lane 1: Molecule weight standard: λ phage DNA digested with *Pst*I enzyme; Lanes 2 and 10: Positive control: HAV-5 DNA; Lane 3-5: Tissue culture fluid of the third passage of the isolated CAV-1 strain, treated either by procedure B (see in the text, Lane 3), or by procedure A (see in the text, Lane 4 and 5). Lane 5 contains the PCR product from sample 100 times prior to the reaction; Lane 6-8: liver tissue homogenate from Case 2, treated either by procedure B (Lane 6), or by procedure A (Lane 7-8). Lane 8: same as Lane 7, but the sample was 100 times diluted prior to the PCR; Lane 9: Negative control: tissue homogenate (treated by procedure B) from the liver of an uninfected dog.

In the present study, the performance of PCR was tested in the detection of the presence of canine adenovirus type 1 in clinical samples. The applied primers were originally designed and tested for the detection of human adenoviruses (Allard et al., 1990), then with some modification for bovine, ovine, and porcine adenoviruses (Kiss et al., 1996a). We have now

demonstrated, that CAV-1 might also be efficiently detected by these primers either in tissue culture (especially in early passages of attempted virus isolation) or in clinical samples.

The advantage of PCR over virus isolation lays in its rapidity and simplicity (Kiss et al., 1997). Successful amplification of certain segments of CAV-1 and CAV-2 DNA from inoculated cell culture supernatant without any chemical pre-treatment was recently reported (Harasawa et al., 1994). We have also found, that even a very simple treatment of the organ samples and tissue homogenates is sufficient for obtaining fast and reliable results. It might even be advisable to avoid the more labour-effective DNA extraction procedure, since co-precipitated chromosomal DNA (and other impurities of cellular origin) or even just a too high concentration of target DNA seem to hamper the amplification reaction (Fig. 2, lanes 7 and 8). We have not intended however to address the problems of quantitative conditions in the framework of this study.

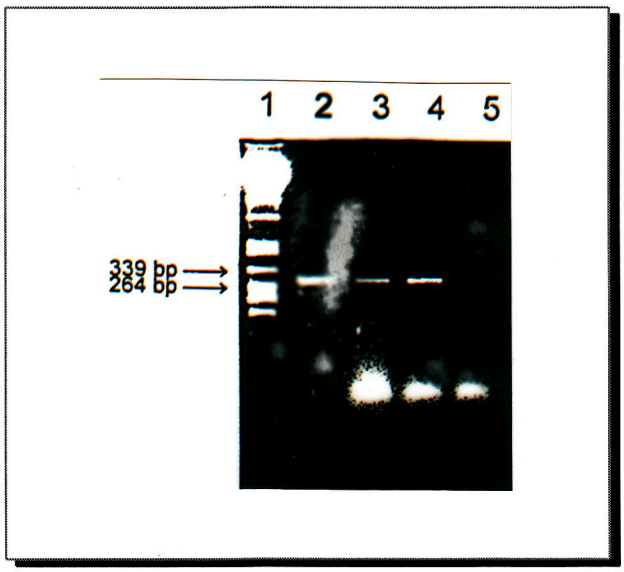


Figure 2. A 1.5% agarose gel containing PCR amplification products from tissue homogenate samples (procedure B) as listed below.

Lane 1: Molecular weight marker: λ phage DNA digested with *Pst*I enzyme; Lane 2: Positive control: HAV-5 DNA; Lane 3: Specific positive control: Unextracted liver tissue homogenate from Case 2. Lane 4: Unextracted liver tissue homogenate from Case 1; Lane 5: Negative control: Unextracted liver tissue homogenate from an uninfected dog.

Because of the widely used preventive vaccination, infectious canine hepatitis became a rare disease in Hungary by nowadays. Consequently, there are fewer fatalities to be seen. Since the preparation of this manuscript, a suspected case of adenovirus caused canine hepatitis turned up. The PCR performed on the very day of the *post mortem* examination however gave negative result. The histopathological examination next day did also fail to demonstrate the characteristic inclusion bodies, implying that the hepatitis of the dog was not of adenoviral origin. We believe, that the PCR method described here might be useful even in the exclusion of the presence of adenoviral DNA.

5.3. Detection and identification of avian mycoplasmas by polymerase chain reaction and restriction fragment length polymorphism assay

In press in: I. Kiss, Katalin Matiz, Éva Kaszanyitzky , Yleana Chávez, and K.-E., Johansson (1997): Veterinary Microbiology

INTRODUCTION

Infections caused by mycoplasmas may result in economic losses in the poultry industry. Mycoplasmal infections are usually diagnosed by serological procedures that are sometimes hampered by interspecies cross-reactions and non-specific reactions. Moreover, there are some mycoplasma species e.g. *M. iowae* showing antigenic heterogeneity and poor immune response that makes the development of reliable serological methods for detection more difficult (Jefferey et al., 1995). *In vitro* isolation of the organisms is usually used to confirm serological results. However, confirmation of the identity of an isolate by growth inhibition requires considerable additional time, and is hampered by bacterial and fungal contamination. However, the principal disadvantage of growth inhibition methods in mycoplasma specification is the requirement for monospecific, high-titered antisera. There are a few sources of reference reagents accessible to many workers at the present time, but commercial sources for working volumes are virtually nonexistent (Avakian et al., 1988, Fan et al., 1995).

Numerous useful procedures have been applied during the last two decades, i.e. acetic acid-urea polyacrylamide gel electrophoresis (Rhoades et al., 1974), SDS-PAGE (Khan et al., 1987), Western blotting (Thomas and Sharp, 1988), different ELISA systems (Thomas and Sharp, 1988, Stipkovits et al., 1993, Kaszanyitzky et al., 1994), immunofluorescence (Talkington and Kleven, 1983), analysis of membrane proteins by monoclonal antibodies

(Czifra et al., 1993) and IgG receptor detection (Lauerman et al., 1993b). However, from the aspect of detecting different protein profiles, these techniques did not bring a real breakthrough, since considerable time and labour are still needed. Recently, the rapidly developing nucleic acid based molecular biological techniques have been employed with the same purpose and these methods have proved to be excellent tools for fast and effective identification of mycoplasma strains indicating previously unnoticed heterogeneity among them. These techniques included the analysis of the whole genomic DNA by restriction enzyme analysis (Kleven et al., 1988, Ley and Avakian, 1992), but later the 16S rRNA and its gene have become the subject of many investigations. For instance hybridization with DNA probes (Yogev et al., 1988, Fernández et al., 1993, Johansson, 1993), PCR with either species specific (Jefferey et al., 1995) and genus specific or broad-range primers (Van Kuppeveld et al., 1992), the latter in combination with RFLP (Ros Bascuñana et al., 1994, Fan et al., 1995, Garcia et al., 1995). More recently the arbitrarily primed PCR (AP-PCR) technique has been used and was found to be highly sensitive for detection of genetic variants of organisms including mycoplasmas (Geary et al., 1994, Boerlin et al., 1995).

In this study, we investigated PCR method: a species specific PCR for detection of *M. gallisepticum*, and a broad-range PCR in combination with RFLP for detection and identification of *M. iowae*, *M. meleagridis*, and *M. synoviae*. These species are very important in clinical and economical situations in poultry husbandry.

MATERIALS AND METHODS

Mycoplasma cultures and specimen preparation. The type strains of *Mycoplasma gallisepticum*, *M. iowae*, *M. meleagridis*, and *M. synoviae* obtained from the National Veterinary Institute, Uppsala, Sweden, and field isolates of the same mycoplasma species

originating from the Veterinary Institute, Debrecen, Hungary were used in this study. Mycoplasma culture was performed as described previously (Frey et al., 1968; Jordan, 1983; Kaszanyitzky et al., 1994). Growth inhibition assays were done as described by Clyde, Jr. (1983) with *M. gallisepticum*, *M. iowae, M. meleagridis*, and *M. synoviae* antisera obtained from the Central Public Health Laboratory, London, England. The broth cultures of isolated and identified mycoplasmas were stored at -20°C. One microliter of the broth culture of mycoplasmas was added to the PCR mixture.

In order to investigate the possibility of obtaining specific result without the need for culturing techniques we sampled six flocks that had previously been infected with *M. gallisepticum* (one flock), *M. meleagridis* (three flocks), and *M. synoviae* (two flocks) as indicated by serological examinations. The results were confirmed by isolation and identification of the organisms. We checked our method on eleven new outbreaks, performing both the conventional isolation and identification procedures and the PCR and RFLP-based technique. For the latter approach, the samples were prepared as described by Ley et al. (1992) and Jefferey et al. (1995). Briefly, samples were collected by swabbing the tracheae, air sacs, and pericardial regions, and homogenizing lung and brain tissues of turkeys or chickens during necropsy. Frey agar and Frey broth were inoculated with each swab. One milliliter of Frey broth cultures was centrifuged at 12,000 × g for 5 min. The supernatants were removed and pellets were suspended in 50 µl of sterile water, and 1 µl of the suspensions was used for PCR. The cultures were incubated at 37°C, and aliquots were removed daily from the broth cultures for PCR analysis, and agar plates were examined daily for mycoplasma colonies.

Primer selection. The general primer pair was designed by van Kuppeveld et al. (1992) and was kindly provided by Heleen Gerritsma, Department of Virology, Leiden University, Leiden, Holland. The sequence of primer GPO-3 was 5'-GGG AGC AAA CAG GAT TAG

ATA CCC T-3'. The sequence of primer MGSO was 5'-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3'.

For the detection/exclusion of *Mycoplasma gallisepticum* we used a species specific primer pair. The sequence of the forward primer was: 5'-AAC ACC AGA GGC GAA GGC GAG G-3'; the sequence of the reverse primer was: 5'-ACG GAT TTG CAA CTG TTT GTA TTG G-3'.

Before the experiments we examined the complementarity of the primer sequences to the sequence data available concerning the 16S rRNA gene of the four avian mycoplasmas by using the sequence analysis software package entitled Primer Designer-Version 2.0 (Scientific and Educational Software, Ser. No.: 50178, P. O. Box 440 State Line, Pa. 17263, USA).

PCR procedure. The PCR was carried out in 50 µl volumes. Each reaction mixture contained 5 µl of 10 x PCR buffer (500 mM KCl, 200 mM Tris-HCl, [pH 8.4]), 1.5 µl of 10 mM of each nucleotide (dATP, dCTP, dGTP, and dTTP; Pharmacia Biotech, Uppsala, Sweden), 2.5 µl of each of the general mycoplasma primer (60 ng/µl), and 1 µl of each of the *M. gallisepticum* specific primer (35 pmol/µl) 0.2 µl (2 U) of *Taq* DNA polymerase, and 1 µl of the broth culture of mycoplasmas. The reaction mixtures were adjusted to the total volume by adding distilled water. All DNA amplifications were performed in a Perkin-Elmer Cetus DNA Thermal Cycler (Norwalk, Conn. U.S.A.) that was programmed to heat the DNA at 94°C for 5 minutes, followed by 35 cycles at three different temperatures and times (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) that corresponded to target DNA denaturation, primer annealing, and primer extension. The final step was 72°C for 10 min.

Detection of PCR products. The amplified DNA products were analysed by electrophoresis on 2.0% agarose gels (SeaKem ME, FMC Bioproducts, Rockland, Maine, USA) in TAE buffer (40 mM Tris acetate, 1 mM EDTA) containing 0.5 µg/ml ethidium

bromide. Gel electrophoresis was performed at a constant voltage of 110 V for 45 min. The gels were photographed under UV transillumination (at 302 nm) on "Polaroid 667" film. 100 bp DNA ladder (Gibco BRL, Gaithersburg, MD, USA) was used as a molecular weight standard.

RFLP analysis. The mycoplasmal 16S rRNA sequences were obtained from GenBank and the EMBL nucleotide sequence data library (Weisburg et al., 1989, Grau et al., 1991, Scamrov and Beabealashvilli, 1991, Pettersson et al., 1994). Restriction site analyses were performed by using the above sequence analysis software package. Based on the alignment studies two restriction endonucleases (*Bam*H I, and *Rsa* I) appeared to be suitable for the distinction of the amplified (approximately 270 bp long) fragments from the examined mycoplasmas. Digestions were performed according to the manufacturer's recommendations (New England Biolabs, Beverly, Mass. U.S.A.) The DNA fragments were visualized in comparison with a DNA size marker (100 bp DNA ladder) by electrophoresis of the total volume of the reaction mixture in 2.0% agarose gels in TAE buffer containing 0.5 μg/ml ethidium bromide. The result was documented as above.

RESULTS AND DISCUSSION

Fig. 1 shows the result of the PCR that was performed with the general mycoplasma primers and the *M. gallisepticum* specific primers in the same reaction mixture on the reference strains of the four avian mycoplasmas. In the case of each of *M. iowae*, *M. meleagridis*, and *M. synoviae* we obtained a DNA fragment of about 270 bp after PCR while from the two reference strains of *M. gallisepticum* we obtained an amplicon of about 530 bp in size. We assumed from the sequence data available that, due to the inadequacy of one or both primers, we were able to obtain amplification product from neither *M. gallisepticum*

reference strains with the general primer pair nor from the other three mycoplasmas with the *M. gallisepticum* specific primer pair.

The results of the digestions of the amplicons generated by the broad-range primers from *M. iowae, M. meleagridis*, and *M. synoviae*, with the two chosen restriction endonucleases are shown in Fig. 2.

Fig. 3 represents the result of the PCR of the broth culture sample of some avian mycoplasma strains isolated previously or during this study at the Veterinary Institute, Debrecen, and Fig. 4 shows the RFLP pattern of the amplified products. Three out of the examined seventeen mycoplasma isolates proved to be *M. gallisepticum*, one *M. iowae*, four *M. synoviae*, and nine *M. meleagridis* by both the PCR and RFLP-based and the conventional methods. The PCR gave positive results after 48 hours of incubation of the broth cultures in all cases while the conventional methods needed for the usual length of time (a few weeks) to be completed.

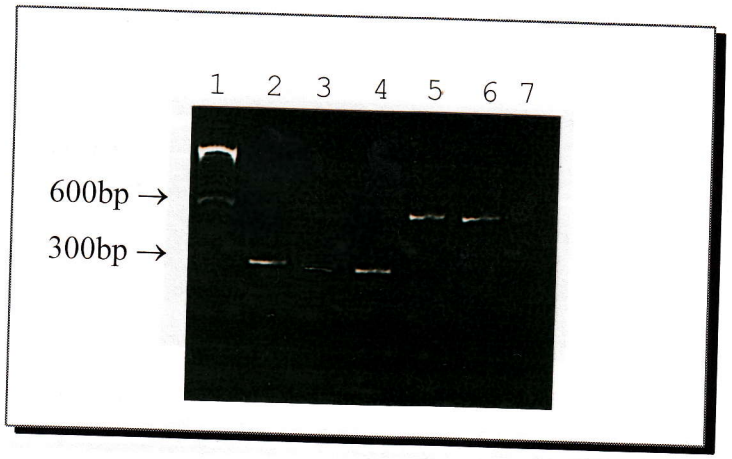


Figure 1.

Electrophoretogram of the PCR products of the reference strains of the four avian pathogenic mycoplasmas generated by the broad-range primer pair and by the species specific primer pair. Lane 1: Molecular weight marker: 100 bp DNA, Lane 2-6: amplicons generated with the mixture of the four primers (Lane 2: *M. iowae*, Lane 3: *M. meleagridis*, Lane 4: *M. synoviae*, Lane 5: *M. gallisepticum* S6, Lane 6: *M. gallisepticum* X95), Lane 7: negative control (uninoculated broth culture).

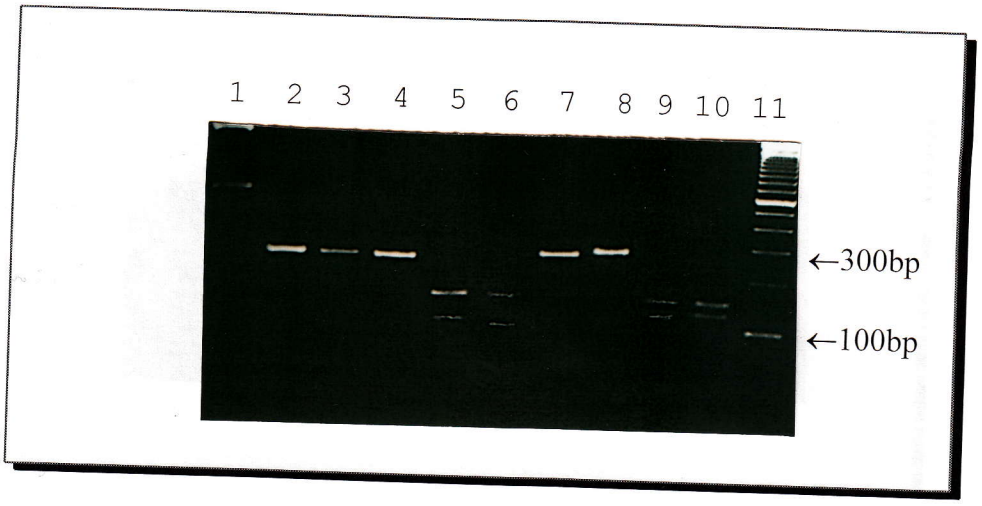


Figure 2.

Electrophoretograms of the amplicons and the restriction fragment length polymorphism patterns for the reference strains of *M. iowae*, *M. meleagridis*, and *M. synoviae* obtained with two restriction enzymes. Lane 1 and 11: Molecular weight marker: 100 bp DNA ladder, Lane 2: uncut amplicon from *M. iowae*, Lane 3: from *M. meleagridis*, and Lane 4: from *M. synoviae*; Lane 5: *M. iowae*, Lane 6: *M. meleagridis*, and Lane 7: *M. synoviae* amplicon cut by *Rsa*I; Lane 8: *M. iowae*, Lane 9: *M. meleagridis*, and Lane 10: *M. synoviae* amplicon cut with *Bam*HI.

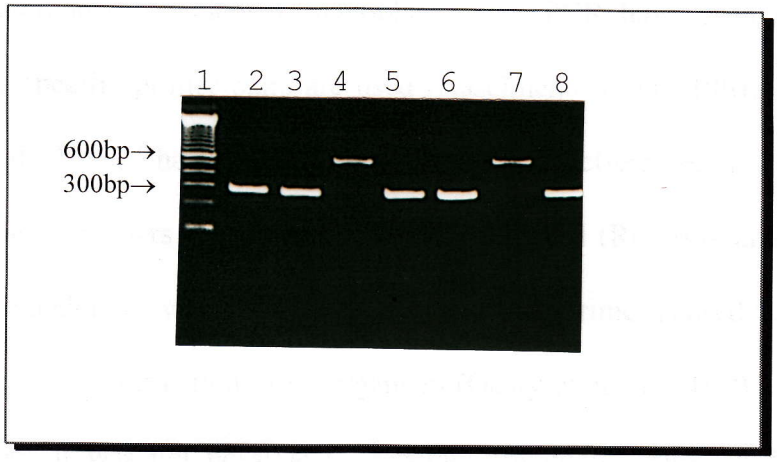


Figure 3.

Electrophoretogram of the PCR products of some field isolates of avian pathogenic mycoplasmas. Lane 1: Molecular weight marker: 100 bp DNA, amplicon from: Lane 2: *M. iowae*, Lane 3: from *M. meleagridis*, Lane 4: from *M. gallisepticum*, Lane 5: from *M. synoviae*, Lane 6: from *M. synoviae*, Lane 7: from *M. gallisepticum*, Lane 8: from *M. meleagridis*.

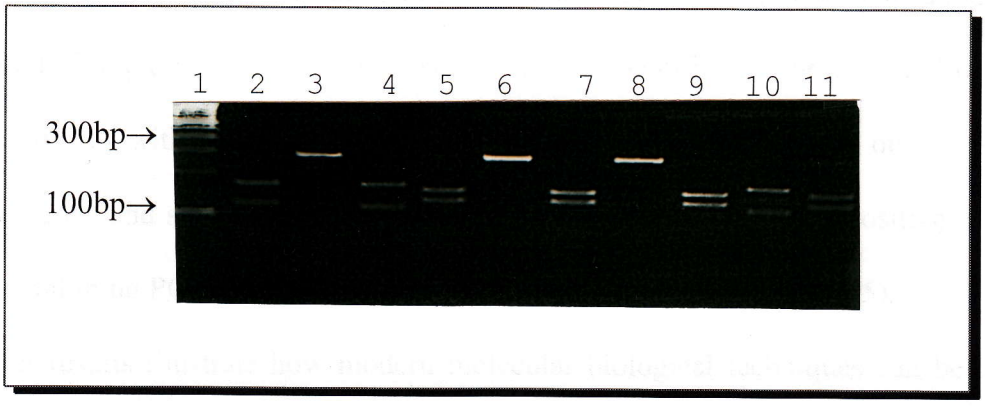


Figure 4.

Restriction enzyme analysis of the amplicons shown in Fig. 3, (excluding the PCR products generated from the isolates of *M. gallisepticum*) and cleaved with two restriction enzymes, *Rsa*I and *Bam*HI, respectively. Lane 1: 100 bp DNA ladder, Lane 2 and 3: restriction pattern of the *M. iowae* amplicon, Lane 4 and 5: *M. meleagridis*, Lane 6 and 7: *M. synoviae*, Lane 8 and 9: *M. synoviae*, and Lane 10 and 11: restriction pattern of the *M. meleagridis* amplicon.

CONCLUSION

Different approaches to identify mycoplasmas by PCR have been reported. In one approach, species specific primer pairs are used (Nascimento et al., 1991, Lauerman et al., 1993a, Slavik et al., 1993, Zhao and Yamamoto, 1993, and Jefferey et al., 1995). In another approach broad-range primers combined with RFLP are used (Ros Bascuňana et al., 1994). Finally, the RAPD technique where a single, usually 10-mer primer is used has been shown to give accurate data for identification of an organism (Geary et al., 1994). We applied the first two methods, since it was not possible to generate amplicons from the *M. gallisepticum* strains with the broad-range primer pair. The species specific primer pair is effective with this organism. On the other hand, our experiments showed that the primer pair described by Van Kuppeveld et al. (1992) is suitable for amplifying a similar product in size from other avian mycoplasmas. Distinction can be made among the amplicons by RFLP with two restriction endonucleases. It has been clear from these data that the method described herein or any similar protocol based on PCR and RFLP (e.g. Fan et al., 1995) can serve as a simple, rapid, and easy technique in routine mycoplasma detection-identification procedure. Specific result can be achieved with samples taken by swabbing different organs, within one working day in positive cases, and significantly shorter time is sufficient for definitive positive and negative results combining PCR with culture than culture alone (Jefferey et al., 1995).

Our results illustrate how modern molecular biological techniques can be applied for routine diagnostic purposes. Studies on bovine and porcine mycoplasmas using the described procedures are underway at our institute in Debrecen. We believe, that such techniques should be involved in and facilitate the diagnosis of diseases caused by agents that are time consuming to isolate and difficult to identify.

5.4. Diagnosing the Derzsy's disease by polymerase chain reaction

INTRODUCTION

Goose parvovirus (GPV) infection (Derzsy's disease) is one of the major diseases of goslings and Muscovy ducklings (Kisary, 1993). The disease occurs worldwide and is economically important to goose and Muscovy duck producers. The Derzsy's disease is prevalent in our investigational area too, providing grounds for litigation between vendors and breeders year by year. The diagnosis of the disease has been based on the characteristic clinical course, age incidence (the disease is absent in goslings after the fourth week of life), and gross and histological lesions for a long time (Mészáros, 1985; Kecskeméti et al., 1996). However, there have been observations recently that indicated the occurrence of the disease at older age with the lack of unambiguous clinical and histopathological findings. Therefore, it is often required now to confirm the diagnosis by detecting the causative agent in such cases. The detection of the viral antigens in infected tissues has some difficulties: to isolate the virus, several blind passages in embryonating goose or Muscovy duck eggs are required (Gough et al., 1981; Takehara et al., 1994). Further in samples contaminated with other avian viruses, such as reoviruses or adenoviruses, the contaminant viruses are often isolated instead of GPV (Derzsy, 1967; Csontos, 1967). Electron microscopy can be used to demonstrate GPV in the nuclei of infected cells in certain organs such as the heart and the bursa (Bergmann, 1987). Also, Roszkowski et al. (1982) developed an immunoperoxidase technique for detecting GPV antigens in goslings, but they could only detect the viral antigens in the liver tissues. However, since the nucleotide sequence for Muscovy duck parvovirus FM, and a GPV strain B is available (Zádori et al., 1995) we have been applying the PCR - similarly to

others (Limn et al., 1996) - for the detection of the GPV DNA in clinical samples since the early summer of 1996.

MATERIALS AND METHODS

Sample preparation. The exposition of the viral DNA was based on the simple protocol that we found suitable for the detection of canine adenovirus one (Kiss et al., 1996b) and was carried out as follows. Approximately one gram of heart and liver specimen of each cadaver was homogenized and diluted to 1:10 in bidistilled water. After three cycles of freezing at -20 ^{o}C and thawing at room temperature, the sample was boiled for 10 minutes. One ml of the solution was transferred into an Eppendorf tube and was spun in a microcentrifuge at maximum speed (app. 13000 rpm) for 5 minutes. An aliquot of 1 µl of the serially diluted (on a ten times scale) supernatants were used in the PCR mixture as target DNA solutions.

Controls. 100 $TCID_{50}$ of the GPV strain B (Kisary et al., 1978) served as the stock for positive controls. Four positive control tubes contained 1 µl of the tenfold dilutions of the B virus stock. 1 µl of a mixture of 10 µl of the 100 times diluted stock virus solution and a 300 µl amount of the commixture of the samples was added to a next positive control tube in order to reveal the presence of inhibiting materials to the PCR. Heart and liver tissue samples taken from uninfected goose carcasses were used as negative controls. All control tubes were treated as described at the sample preparation.

Primers. The sequences of the primers are applied for patent by the RekombiVet Ltd., Budapest, therefore these are not presented here;

PCR. The amplifications were carried out in a reaction mixture of 50 µl volume containing 5 µl 10X reaction buffer (500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.0), 50 pmol of each primer, 1 U of thermostable *Taq* DNA polymerase, 300 µM of each of

the four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Pharmacia LKB), and 1 µl of sample solution. A DNA Thermal Cycler (Perkin-Elmer Cetus Instruments) was used. The "Step-Cycle" program was set to denature the DNA at 94 °C for 30 seconds, to anneal the primers at 55 °C for 30 seconds, and to extend the sequence at 72 °C for 30 seconds, for a total of 35 cycles.

Detection of PCR products. The amplification products were detected by agarose gel electrophoresis. An aliquot of 8 µl from each reaction was run on 1.5% agarose gels containing ethidium bromide (5 µg/ml) at a constant voltage of 110 V for 45 minutes. For molecule weight marker a 100 base pair DNA ladder was used. The gels were placed on a UV transilluminator and photographed on Polaroid 665 films.

Virus isolation. The procedure was carried out after Kisary et al., 1978. Briefly, the samples that were described beforehand were supplemented with antibiotics and 200 µl of them were inoculated into the allantoic cavity of embryonated goose eggs on the 10th day of incubation. The inoculated eggs were incubated for a further 14 days and candled twice daily. The dead embryos were examined for macroscopic myocardial and hepatic lesions. Where the characteristic lesions were not developed, phenol/chloroform extraction and alcohol precipitation of the different dilutions of the allantoic fluids were performed and subjected to PCR.

RESULTS AND CONCLUSIONS

A representative gel from a GPV infected flock is shown in Fig. 1. It clearly exemplifies a tipical finding to the effect that in the positive flocks usually 2 to 10 out of the examined animals give positive result in the PCR. Fig. 2. shows a case when the dilution of the sample was necessary to eliminate a PCR inhibiting component from the starting material.

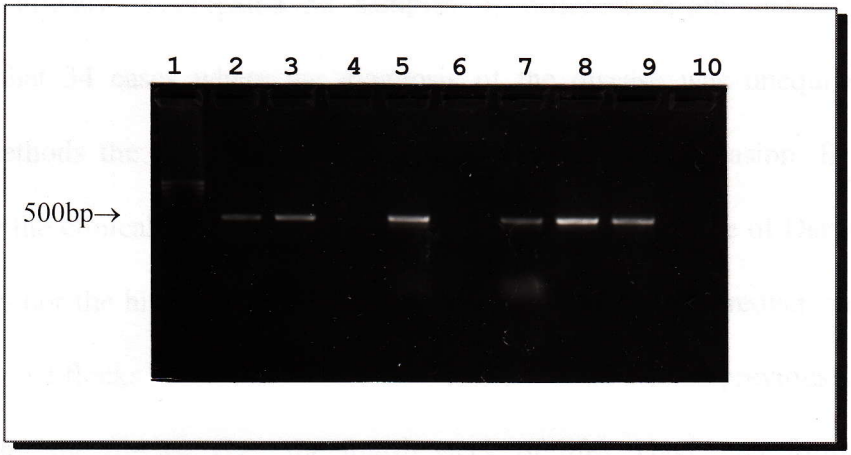


Figure 1. PCR results of the heart- and liver homogenates of eight geese originating from the same flock for the presence of GPV.
1: Molecular weight marker: 100 bp DNA ladder; 2-9: PCR amplicons from the individual organ samples. 10: negative control (DNA replaced by distilled water as target).

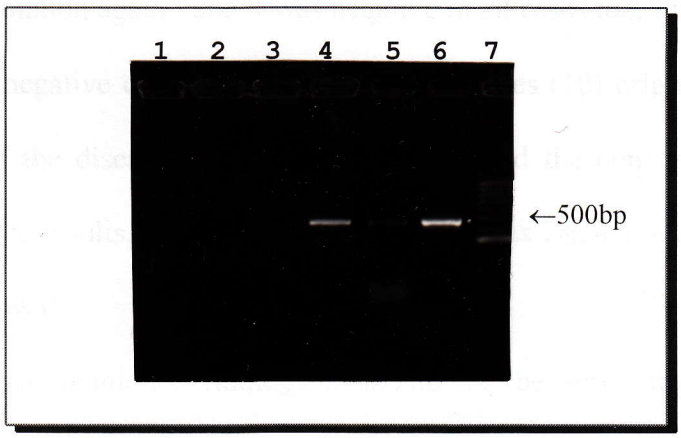


Figure 2. PCR-inhibitor in the sample. The results of the samples diluted and assembled diversely.

1: sample-mixture consisting of 10 individual samples from the same flock; 2: positive control (1µl from the following mixture: 300µl from the before-mentioned sample + 10µl of the stock of the "B" virus); 3: negative control (DNA replaced by distilled water as target); 4: 10^4-fold dilution of the "B" virus stock; 5: positive kontrol: 1µ l from the 10^3-fold dilution of the sample described at Lane 1 + 10µl of the "B" virus stock, and 6: PCR result of the 10^3-fold dilution of the sample-mixture described at Lane 1; 7: Molecular weight marker: 100 bp DNA ladder.

We have already investigated 58 samples of different origin and we found the followings: in that 34 cases where the diagnosis of the disease was unequivocal by the conventional methods the PCR agreed with these results in each occasion. In four cases, however, where the clinical findings raised the suspicion of the presence of Derzsy's disease, neither the gross nor the histopathology could definitely confirm it. Moreover, in these cases most of the affected flocks had been some weeks older than that of the previously considered age limitation for the disease (i.e., the fourth week of life). Having performed PCR on samples from these flocks they proved to be positive. To confirm the results, we attempted and succeeded in isolating the GPV from the examined tissues in embrionated goose eggs. There had been ten another intriguing cases, namely where the conventional methods indicated the presence of Derzsy's disease and the PCR remained negative in all cases. We called upon the virus isolation again - and it was negative in all cases too. Finally, we checked the GPV-PCR on real negative controls as well. These samples (10) originated from flocks where the suspicion of the disease had not even arisen - and the conventional diagnostic techniques gave negative results, of course. The PCR then was carried out blind and it was negative in all cases as well.

Based on the abovementioned findings it should not be surprising that during the introduction of the PCR into the routine tools for the diagnosis of Derzsy's disease the "unusual" results were confirmed further by virus isolation in embryonated goose eggs. Since the results of both methods agreed in all cases we have been continuing only with the much faster and simpler PCR since the spring of 1997.

Our data support the recent findings of our Department of Avian Diseases that depending on several factors, such as the inappropriate use and quality of vaccine(s) and sera, the GPV infection can lead to the manifestation of the disease at an older age without the

characteristic clinical and pathological changes but with the marked retardation of the animals. Therefore, it is obvious that both the breeders and the vendors need the most accurate diagnosis as far as possible in commercial cases. The PCR proved to be a very useful tool for this purpose. However, it should be emphasized that the traditional methods (i.e., evaluation of the clinical history, the epidemiological data, the pathological and histopathological findings, serology, and recently, the antigen capturing ELISA) cannot be replaced by the PCR, for its unique sensitivity could present an unexplainable situation if the results of all the other investigations were not taken into account adequately.

5.5. Pestivirus differential PCR

INTRODUCTION

Pestiviruses are important pathogens of cattle, swine and sheep (Baker, 1987). Classified as a genus within the Flaviviridae, these small, enveloped, predominantly noncytopathogenic positive-stranded RNA viruses cause a wide range of clinical conditions in their natural hosts. Four viral species or genotypes are currently recognized (Becher et al., 1995; Paton et al., 1995; Harasawa, 1996). Classical swine fever virus (CSFV) occurs naturally only in pigs; bovine viral diarrhea virus (BVDV) genotype I is the predominant pestivirus of cattle, but also occurs in sheep and pigs; BVDV genotype II (also known as "atypical pestivirus", Paton, 1995) has been described most often in cattle, but also in sheep and pigs; and border disease virus (BDV) is a mainly ovine pathogen, occasionally infecting pigs.

In most bovine populations, a high rate of pestivirus infection is maintained by virus shed from persistently infected immunotolerant animals (Baker, 1987). Generally, most acute pestiviral infections of cattle are subclinical, or result in full recovery, but clinically severe disease has also been described (Corapi et al., 1990; Pellerin et al., 1994). A consequence of the high worldwide incidence of bovine pestivirus infections is frequent contamination of fetal calf serum with both virus and virus-specific antibodies (King and Harkness, 1975; Baker, 1987). Besides complicating routine diagnostic work in laboratories (Belák and Ballagi-Pordány, 1993), BVDV types I and II have also contaminated vaccines, both human (Harasawa and Tomiyama, 1994) and veterinary. In the latter case, this has led to harmful pestivirus infections in swine, goats, sheep and cattle (Wensvoort and Terpstra, 1988; LØken et al., 1991).

The clinical consequences of outbreaks of classical swine fever (CSF, synonym hog cholera) are usually far more serious than infections with other pestiviruses. CSFV strains of high, moderate and low virulence have been described (Van Oirschot, 1988), but since even low-virulent strains are foetopathogenic, and cause significant losses in pig breeding units, outbreaks of CSFV are often controlled by statutory destruction of infected animals. Since other pestiviruses can also infect pigs under natural circumstances, sometimes with symptoms indistinguishable from low-virulent CSFV strains (Terpstra and Wensvoort, 1988: Paton et al., 1992), reliable laboratory tests able to distinguish between CSFV and other pestiviruses are of great importance.

Reference methods for pestivirus diagnostic work include use of cell cultures and specific antisera for virus isolation and serology. The introduction of monoclonal antibodies (mAbs) against pestiviruses has improved the quality of the diagnostic tests considerably, primarily by their ability to distinguish between CSFV and other pestiviruses (Edwards et al., 1991), but also by allowing the development of cell-culture independent enzyme immunoassays to be used for routine screening of large numbers of samples (Wensvoort et al., 1988; Entrican et al., 1995). Nevertheless, cell culture-based techniques remain the standard reference methods for all pestivirus diagnostic work, and with them, the need for continuous monitoring of cell cultures and media for viral contamination.

Several investigators have used the polymerase chain reaction (PCR) to detect pestivirus nucleic acid (reviewed by Belak and Ballagi-Pordany, 1993; Ridpath et al., 1993; Schmitt et al., 1994; Da Silva et al., 1995; Hamel et al., 1995; Horner et al., 1995; Radwan et al., 1995), or for analysis of PCR amplified cDNA for identification of pestiviruses (Boye et al., 1991; De Moerlooze et al., 1993; Hofmann et al., 1994; Vilček et al., 1994). Using species-specific PCR primers, cDNAs of the protein-encoding parts of the pestivirus genome have been

amplified for selective detection of CSFV (Katz et al., 1993; Wirz et al., 1993; Lowings et al., 1994) or to discriminate between different BVDV strains and BDV (Sullivan and Akkina, 1995). Recently, Canal et al. (1996) designed primers from the 5' untranslated region (5'-UTR) for selective amplification of CSFV and BVDV.

The function of the 5'-UTR is not completely understood, but there are indications that it is of importance for efficient translation initiation. A proposed secondary structure model of the 5'-UTR indicated loop structures of striking similarity between different pestiviruses (Deng and Brock, 1993), one of which is also structurally similar to an internal ribosome entry site identified within the 5'-UTR of hepatitis C virus (Le et al., 1995; Poole et al., 1995). Since the similarity of these loop structures is maintained by base-pairing of self complementary runs of up to seven nucleotides, this implies a more strict conservation of some parts of the 5'-UTR sequence compared to the flexibility permitted by codon degeneracy within protein-encoding parts of the genome. Exploiting this conservation, there have been PCR protocols that use this region as a target for Pestivirus specific amplification while other primer pairs target species, genotype, or even recombinant specific regions of the viral genome thus allowing more specific identification.

MATERIALS AND METHODS

Grouping of the samples examined on the grounds of classical swine fever. The following sortment was applied during the examinations:

1. lymphocytes separated from the blood of living animals that were suspect of suffering from the disease;

2. animals showing the characteristic clinical signs and pathological picture of the acute form of the disease;

3. animals showing ambiguous clinical sings and characteristic pathological pictures of the late form of the disease;

4. atypical cases;

5. animals that were assigned to the institue with the suspect of the disease, which, however, was excluded finally on the base of pathological/histological and virological investigations.

To determine the sensitivity of the system we applied the serial of the tenfold dilutions of the tissue homogenates.

In the case of the CSFV we compared our results to those that were obtained in the Central Veterinary Institute by immunofluorescence.

For the BVDV-system we used the NADL (National Animal Disease Laboratory) reference strain as a positive control.

Sample preparation and RNA extraction. RNA extraction is performed as follows: samples are taken from tonsils, kidneys, spleen, lymph nodes, brain, and buffy coat for CSFV detection, and from lesion material, spleen, mesenteric lymph nodes, Peyer's patches, buffy coat for BVDV detection. The extraction of total RNA is based on the method that was described by Chomczynski and Sacchi (1987) as follows. Tissue samples are homogenized in sterile distilled water (10%), the white blood cells are simply dispersed in 150 µl bidistilled water. 150 µl of tissue homogenates are mixed with 400µl of 6M guanidine isothiocyanate (Sigma Chemical, St. Louis, MO, USA), vortexed and left at room temperature for 10 min. After that, 220µl of 5M sodium chloride is added, vortexed for a short time and put on ice for 10 minutes and extracted with chloroform. After centrifugation for 10 min. at 15,800 g, 600 µl of water phase is transferred into a new tube. The RNA is precipitated with 360 µl of 95% ethanol at -75°C for 20 minutes. The spun down RNA precipitates are washed carefully two

times with 70% ethanol, left to dry and dissolved in 30 µl of diethyl-pyrocarbonate-treated (DEPC, Sigma Chemical, St. Louis, MO, USA) distilled water. All centrifugation steps are performed at 4°C.

MDBK cells infected with the NADL BVDV reference strain were destroyed as described before for the adenoviruses and they were further processed as it was detailed above.

Primers. Because of its great economic importance, the protocol we use for the detection and identification of Pestiviruses in our laboratory is based on two approaches. It means that we use a pestivirus specific primer pair which is the same as Wirz et al., used (1993) and is capable of amplifying a 74 bp product; to differentiate CSFV from BVDV (as the BDV virtually non existent in our investigational area) we use type specific nested priming sites from the E2 (the major envelope glycoprotein) region and priming sites from the region coding for the p80, in the CSFV and in the BVDV genome, respectively (Katz et al., 1993; Da Silva et al., 1995; Hertig et al., 1991). The selected part of the E2 region of the CSFV genome proved to be highly specific and conservative for a large number of CSFV isolates and vaccine strains as well. For the BVDV, it is known that the two most conservative parts of the genome are the 5' UTR and the p80 coding region. We chose the latter because a much longer fragment can be amplified from this sequence which is easy to recognize on a gel.

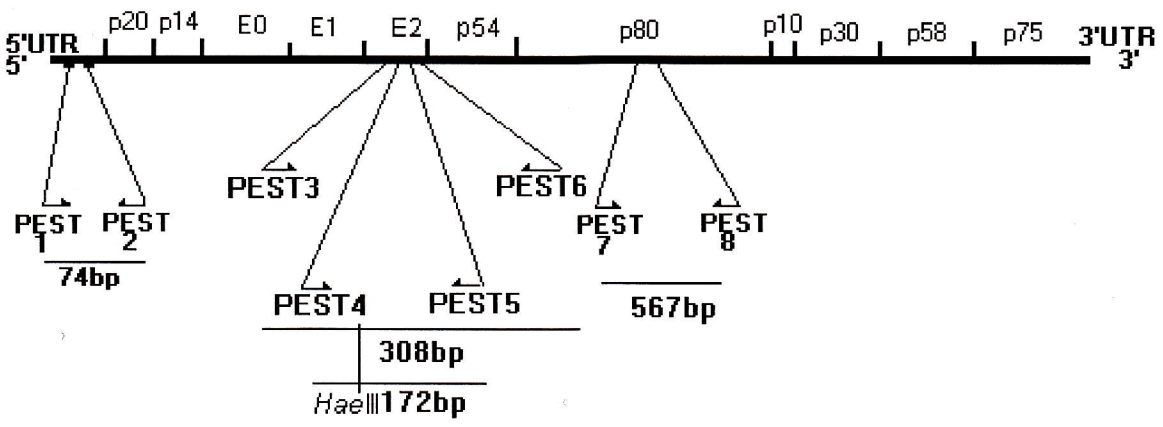
The suitability of the primer candidates was checked on the published pestivirus sequences using the before-mentioned software (Primer Designer-Version 2.0).

The sequence of the primers are shown in Table 1. and their location on the schematic pestivirus genome in Fig. 1.

Table 1. Primers used for the detection and identification of BVDV and CSFV.

primers' code	sequence	position (nucleotides)	specificity
PEST 1	5'-CCTGATAGGGTGCTGCAGAG-3'	(300-319)	genus
PEST 2	5'-TCAACTCCATGTGCCATGTAC-3'	(373-353)	genus
PEST 3	5'-ATATATGCTCAAGGGCGAGT-3'	(3378-3398)	CSFV
PEST 4	5'-CTGTGGCTAATAGTGACCTAC-3'	(3499-3519)	CSFV
PEST 5	5'-CATTTCTTTATGGGCTCATC-3'	(3650-3671)	CSFV
PEST 6	5'-ACAGCAGTAGTATCCATTTCTTTA-3'	(3664-3686)	CSFV
PEST 7	5'-AAGAAGCTAAAAGCTAARGGCTAYAA-3'	(6668-6693)	BVDV
PEST 8	5'-CGGGACCTGGACTTCATAGC-3'	(7255-7235)	BVDV

Figure 1. Location of the "PEST"-primers on the schematic *Pestivirus* genome.



cDNA synthesis. The cDNA synthesis is carried out by using a commercial kit (cDNA synthesis kit, Pharmacia) in 15 µl volume. For priming the reaction we use both random hexamer primers and the backward PCR primer in the same reaction mixture in 25 pmol amount per reaction, respectively. 1 µl of the accomplished reaction is used as target for the PCR.

PCR amplification and product detection. The amplification is carried out in a reaction mixture of 50 µl volume containing 5 µl 10X reaction buffer (500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.0), 25 pmol of each primer, 1 U of thermostable *Taq* DNA polymerase, 300 µM of each of the four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Pharmacia LKB), and 2 µl of sample solution. A DNA Thermal Cycler (Perkin-Elmer Cetus Instruments) is used. The "Step-Cycle" program is the same we use for the above mentioned applications (adenovirus and mycoplasma identification, etc.) The amplification products are detected by agarose gel electrophoresis.

RESULTS AND CONCLUSIONS

The results are shown on Fig. 2. and 3. The specificity of the primers we used was proved beforehand by their authors. Accordingly, we did not find any "cross-reaction" between them ourselves (data not shown). The estimated sensitivity of the CSFV-system proved to be similar to those that can be found in the literature, i.e., appr. 1-10 $TCID_{50}$ viral particles (Katz et al., 1993; Wirz et al., 1993). Moreover, PCR had the advantage that we could detect the presence of CSFV in the buffy coat of suspected animals being alive, therefore the necessary measures could be carried out in good time. On the other hand, we found that samples that are not accessible for other diagnostic methods like some day-old thus autolyzed corpses of wild boars could even be used as targets for the PCR.

Out of the total number of 57 pig samples examined by us 2 belonged to group 1, 27 blonged to group 2, 12 belonged to group 3, and 7 belonged to group 4. All these samples were positive in the RT-PCR while the 9 samples belonging to group five were consistently negative.

The results we have obtained using CSFV-PCR were always in accordance with those of the other diagnostic methods (i.e., immunoflourescence, antigen-capturing ELISA). Taking into account all these aspects we state that PCR is a really powerful tool for diagnosing or excluding CSF in a quick and precise way. However, the isolation of the virus is necessary, since the present epidemiological status of Europe needs for the most detailed investigation of each outbreak. This kind of investigation means nowadays the determination of the nucleotide sequence of some parts (or the whole) of the viral genome wherethrough undoubting evidence can be obtained concerning the source/identity of the actual virus isolate. Follow up studies can warn the investigator when new genomic types begin to emerge for developing more adequate vaccines.

We found the BVDV specific PCR system reliable as well. We performed retrospective analyses on stored organ samples and lymphocytes originating from four BVD suspected animals and our findings confirmed the diagnoses which were based on pathological examinations. We use the reference strain NADL as a positive control for these tests. The results are confirmed through culturing the sample on primary bovine testicle cells and subsequent analysis of the tissue culture supernatant by a p80/p125 specific antigen-capturing ELISA (Rhone Merieux, Lyon, France).

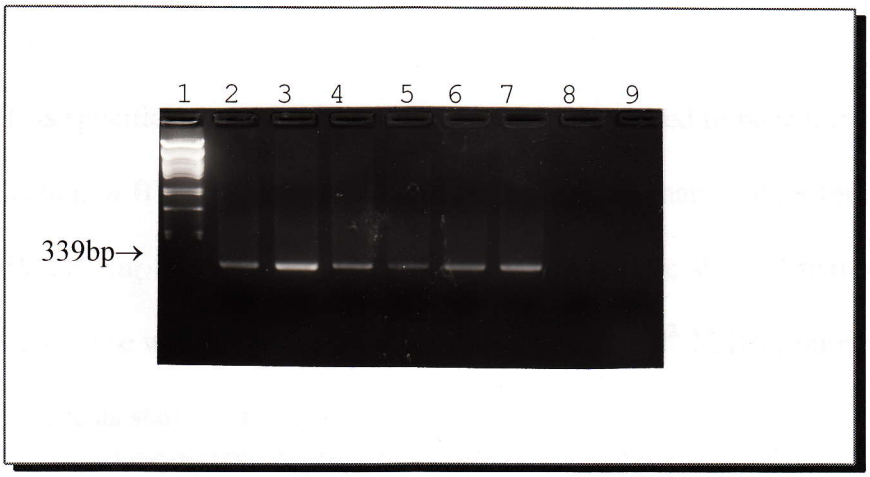


Figure 2. RT-PCR results form the samples originating from the different pathological groups using the CSFV specific "outer" primers.

1: Molecular weight marker: λ DNA cut with *Pst* I; 10^2-fold dilutions of the samples originating from 2 and 3: the 1st, 4 and 5: the 2nd, 6: the 3rd, 7: the 4th, and 8: from the 5th pathological group. 9: negative control (target cDNA replaced by distilled water).

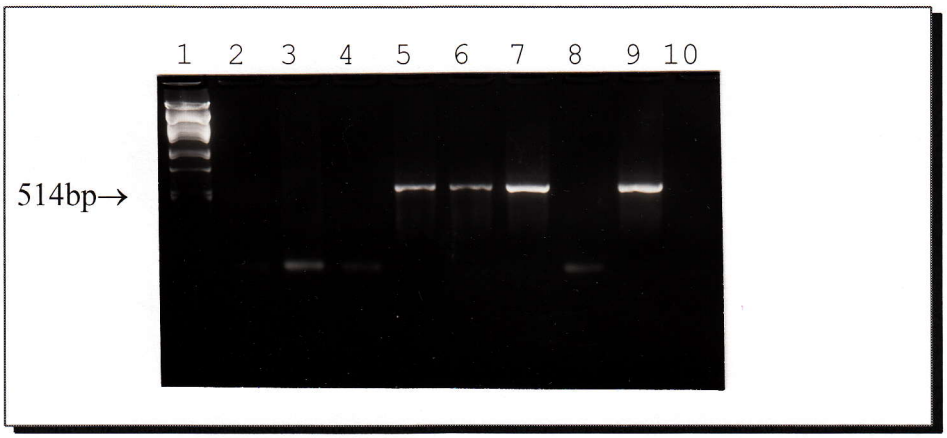


Figure 3. RT-PCR results of organ samples from cattle on the grounds of BVD using the pestivirus specific and the BVDV specific primers.

1: Molecular weight marker: λ DNA cut with *Pst* I; 2-4: amplicons from the organ samples of 3 animals of different origin generated by the genus specific primers, and 5-7: by the BVDV specific primers. 8-9: RT-PCR results by the same primers on the tissue culture supernatant of MDBK cells infected with the NADL reference strain. 10: negative control (target cDNA replaced by distilled water).

CONCLUSIONS

On account of its specificity, senstivity, and speed the PCR proved to be suitable for the fast and reliable detection of BVDV and CSFV. We did not have the chance of performing the titration of the CSFV but our follow up studies based on the literature showed that we were able to detect - in accordance with the published data - as few as 10-10^2 $TCID_{50}$ amount of the virus using 100 mg tissue as starting material.

Since the CSFV was readily detectable even from chronic cases and from tissue samples that were stored at room temperature for two weeks or from lymphocytes we consider the method to be a highly effective tool for the accurate and at the same time fast diagnosis of classical swine fever.

6. SUMMARY

The polymerase chain reaction (PCR) has been widely used in basic research and in diagnostics as well since its invention. Based on its unique sensitivity, specificity and speed the routine diagnostic application of the technique is steadily growing. However, the PCR has not been used for the purpose of veterinary diagnostics in Hungary till now. Thus, the aim of my work was to introduce this technique into those fields of the diagnostic work where it is reasonable and the advantages of it could properly be taken.

We established our PCR laboratory in 1994 and settled to work with model experiences in animal adenoviruses of different origin. We found that after the modification of a primer pair that was originally designed for the universal detection of human adenovirus (HAV) serotypes of all subgenera it can adequately be used for the detection of different bovine, ovine, and porcine adenovirus (BAV, OAV and PAV, respectively) serotypes. Apparently, in the examined viruses, parts of the DNA sequence coding for the basal part of the hexon protein are conserved enough for being applicable in polymerase chain reaction (PCR) as primers. Positive amplification could be obtained even from the so-called subgroup 2 BAVs, which viruses do not cross react with HAVs or subgroup 1 BAVs in Southern hybridization. In order to test the primers and the method for the detection of the causative agent of the Rubarth's disease of dogs (canine adenovirus type 1, CAV-1) in the infected liver tissues we elaborated a simple, effective sample processing method and used it successfully not only for the exposition of CAV-1 DNA, but for goose parvovirus (GPV) DNA or mycoplasma genomic DNA as well in the ensuing protocols.

The isolation and identification of mycoplasmas has its well-known difficulties, such as the long incubation period of these microorganisms, the cross reactivity of certain antigens,

and occasionally the low sensitivity of the serological assays. Therefore, it seemed obvious to introduce the PCR in this process, where the improved speed of the identification presents the most valuable account of the technique - from practical point of view. Primers complementary to the 16S rRNA genes was used to detect avian mycoplasmas. This primer pair was designed for the detection of human and rodent mycoplasmal species and we examined their ability to detect the most important avian mycoplasmas. After testing the respective reference strains we found, that *Mycoplasma iowae*, *M. meleagridis*, and *M. synoviae* could be detected by PCR with this primer pair, and distinction could be made among them by restriction fragment length polymorphism (RFLP) assay with two restriction enzymes (*Bam*HI, and *Rsa*I). For the detection of *M. gallisepticum* by PCR we needed species specific primers. The results of the PCR and RFLP-based identification procedures of seventeen different field isolates agreed with those obtained by conventional methods. Thus, our experiments showed that the primer pair originally designed for the detection of human and rodent mycoplasmas is suitable for amplifying a similar product in size from other, avian mycoplasmas. It has been clear from our data that the method based on PCR and RFLP can serve as a simple, rapid, and easy technique in routine mycoplasma detection-identification procedure. Specific result can be achieved with samples taken by swabbing different organs, within one working day in positive cases, and significantly shorter time is sufficient for definitive positive and negative results combining PCR with culture than culture alone.

We have been applying the PCR for the detection of the GPV DNA in clinical samples since the early summer of 1996. We have already investigated as many as 57 samples and we found sometimes that the PCR gave the decisive result for the diagnosis of the disease. During the introduction of the PCR into the routine tools for the diagnosis of Derzsy's disease the positive results were confirmed further by virus isolation in embryonated goose eggs.

However, the isolation of GPV in embryonated eggs is quite difficult under the recent circumstances, i.e., most of the eggs contain maternal antibodies against GPV, the SPF eggs are virtually non-existent in the Hungarian market or they are very expensive, the availability of the goose eggs is seasonable, and last but not least, these are time consuming investigations. Therefore, as the results of both methods agreed in all cases we have been continuing only with the much faster and simpler PCR to prove the presence of GPV in the samples examined since the spring of 1997.

The close antigenic relationship of the Pestiviruses and their ability to cross-infect ruminants and suidae can lead to diagnostic problems. Taking into account both the aforementioned fact and the great economical importance of classical swine fever virus infections it is reasonable to use the highly specific PCR (combined with reverse transcription) to differentiate these viruses. In our approach two sets of primers are used for the detection and identification of CSFV and BVDV. One is a general pestivirus-primer that flanks to a highly conserved region of the virus genome (the 5' UTR) and detects all the known pestiviruses in a very sensitive way. For the species specific detection a nested primer set in the genome region coding for the E2 (main envelope glycoprotein) is used for CSFV while a primer pair within the p80 (nonstructural protein) coding region is used for BVDV identification. BDV has not been targeted in our experiments since it is virtually non-existent in our investigating area.

We tested our PCR protocol using different samples and we could detect the presence of CSFV in the buffy coat of suspected animals being alive, therefore the necessary measures could be carried out in good time. On the other hand, we found that samples that are not accessible for other diagnostic methods like some day-old thus autolyzed corpses of wild boars could even be used as targets for the PCR. Taking into account all these aspects we state

that PCR is a really powerful tool for diagnosing or excluding CSF in a quick and precise way.

We found the BVDV specific PCR system reliable as well, and we performed retrospective analyses on stored samples originating from four BVD suspected animals and our findings confirmed the diagnoses which were based on pathological examinations.

Beyond the detection of particular pathogens PCR serves as a very useful tool for epidemiological investigations where different scales of the identification can be gained through the analysis of the PCR product, i.e. by restriction fragment length polymorphism assay, by single-strand conformational polymorphism assay, or by the most informative way, the nucleotide sequencing.

Our experiences showed that the PCR can be a valuable tool for the veterinary diagnostics based on its most important features like sensitivity, specificity and speed. However, these are the very characteristics of PCR that present the potential danger of contamination. Therefore, the maximum care should be taken during the PCR experiments to prevent either fals positive or negative results. It should be emphasized too, that the PCR does not replace the conventional isolation techniques. Nevertheless, we have successfully extended our facilities with this technique for the detection of microorganisms that are difficult to isolate, or for situations when rapid diagnosis is necessary.

The most important results of the work

1. We have organised the first PCR laboratory in the field of veterinary diagnostics in Hungary, at the Veterinary Institute of Debrecen.

2. We showed, that the slightly modified primer pair that was originally designed to detect human adenoviruses can equally be applied to detect different animal adenoviruses, including the isolates of the recently described bovine adenovirus type 10.

3. We assisted the diagnosis of the infectious canine hepatitis in several cases by using the PCR that was applied for the detection of animal adenoviruses either way confirming or excluding the presence of the causative agent. We elaborated a simple physical procedure for exposing the viral DNA that lacks the usage of the harmful chemicals.

4. We introduced the PCR into the diagnosis of the economically important avian mycoplasmoses. The method considerably reduced the time that is necessary for the identification of the four most important avian mycoplasmas.

5. The PCR technique has routinely been applied for solving the uncertanities and contradictions in the diagnosis of Derzsy's disease. We revealed that the virus isolation can be replaced by PCR in this case.

6. PCR has been used successfully for the demonstration and identification of classical swine fever virus from leukocytes and other samples unfit for other investigations even within one day.

7. The presence of the bovine viral diarrhoea virus has been detected successfully in clinical cases confirming the suspicion of the disease.

7. REFERENCES

Adair, B. M., McKillop, E. R., Smyth, J. A., Curran, W. L., and McNulty, M. S. (1996): Bovine adenovirus type 10: properties of viruses isolated from cases of bovine haemorrhagic enterocolitis. Vet. Rec. 138, 2250-2252.

Allard, A. (1992): Enteric adenovirus type 41. Genome organisation and specific detection procedures. Ph.D. Thesis, Department of Virology, University of Umeå, Umeå, Sweden

Allard, A., Girones, R., Juto, P., and Wadell, G. (1990): Polymerase chain reaction for the detection of adenoviruses in stool samples. J. Clinical Microbiol. 28, 2659-2667.

Ansari-Lari, M. A., Jones, S. N., Timms, K. M., and Gibbs, R. A. (1996): Improved ligation-anchored PCR strategy for identification of 5' ends of transcripts. BioTechniques 21, 34-38.

Avakian, P. A., Kleven, S. H., and Glisson, J. R. (1988): Evaluation of the specificity and sensitivity of two commercial enzyme-linked immunosorbent assay kits, the serum plate agglutination test, and the hemagglutination-inhibition test for antibodies formed in response to *Mycoplasma gallisepticum*. Avian Dis. 32, 262-272.

Bailey, A. and Mautner, V. (1994): Phylogenetic relationships among adenovirus serotypes. Virology 205, 438-452.

Baker, J. (1987): Bovine viral diarrhea virus: a review. J. Am. Vet. Med. Assoc. 190, 1449-1458.

Ballagi-Pordány, A., Klintevall, K., Merza, M., Klingeborn, B., and Belák, S. (1992): Direct detection of bovine leukemia virus infection: practical applicability of a double polymerase chain reaction. J. Vet. Med. B. 39, 69-77.

Bartha, A. (1969): Proposal for subgrouping of bovine adenoviruses. Acta Vet. Acad. Sci. Hung. 19, 319-321.

Becher, P., König, M., Paton, D. J., and Thiel, H.-J. (1995): Further characterization of border disease virus isolates: evidence for the presence of more than three species within the genus pestivirus. Virology 209, 200-206.

Belák, S. (1990): Ovine adenoviruses. In: Dinter, Z., Morein, B. (eds.) Virus Infections of Ruminants. Virus Infections of Vertebrates 3. Elsevier Science Publishers BV, Amsterdam, Chap. 17, 171-185.

Belák, S. and Ballagi-Pordány, A. (1993): Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology. Vet. Res. Comm. 17, 55-72.

Belák, S., Berencsi, Gy., Rusvai, M., Lukács K., and Nász, I. (1983): DNA structure and hemagglutination properties of bovine adenovirus type 2 strains which bypass species specificity. Arch. Virol. 77, 181-194.

Benkő, M. (1990): Comparison and characterisation of adenoviruses of domestic *Artiodactyles* by means of nucleic acid analysis (in Hungarian). Ph.D. Thesis, Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary.

Benkő, M. and Harrach, B. (1990): Restriction enzyme mapping of bovine adenovirus type 1. Acta Vet. Hung. 38, 281-284.

Benkő, M. and Harrach, B. (1994): Identification of the proteinase gene of bovine adenovirus type 4. Acta Microbiol. Immunol. Hung. 41, 323.

Benkő, M., Bartha, A., and Wadell, G. (1988): DNA restriction enzyme analysis of bovine adenoviruses. Intervirology 29, 346-350.

Benkő, M., Harrach, B., and D'Halluin, J. C. (1990): Molecular cloning and physical mapping of the DNA of bovine adenovirus serotype 4; study of the DNA homology among bovine, human and porcine adenoviruses. J. Gen. Virol. 71, 465-469.

Benkő, M., Smyth, J. A., Adair, B. M., and Harrach, B. (1995): Genome typing of adenovirus isolates from fatal enterocolitis cases of cattle for the elaboration of a rapid diagnostic method. In: Schwyzer, M., Ackermann, M., Bertoni, G., Kocherhans, R., McCullough, K., Engels, M., Wittek, R. and Zanoni, R. (eds.): Immunobiology of viral infections. Proc. 3rd Congress Europ. Soc. Vet. Virol. 484-488.

Berencsi, Gy. and Minárovits, J. (1997): Virális genomok kimutatása. In: Kopper, L., Marcsek., Z., and Kovalszky, I. (eds.): Molekuláris Medicina. Medicina, Budapest pp. 81-85.

Bergmann, V. (1987): Pathology and electron microscopical detection of virus in the tissues of goslings with Derzsy's disease (parvovirus infection). Arch. Experiment. Vet. Med. 41. 212-221.

Boerlin, P., Bannerman, E., Ischer, F., Rocourt, J., and Bille, J. (1995): Typing *Listeria monocytogenes*, a comparison of random amplification of polymorphic DNA with 5 other methods. Res. Microbiol. 146, 35-49.

Boye, M., Kamstrup, S. and Dalsgaard, K. (1991): Specific sequence amplification of bovine virus diarrhea virus (BVDV) and hog cholera virus and sequencing of BVDV nucleic acid. Vet. Microbiol. 29, 1-13.

Boyle, D. B., Pye, A. D., Kocherhans, R., Adair, B. M., Vrati, S., and Both, G. W. (1994): Characterisation of Australian ovine adenovirus isolates. Vet. Microbiol. 41, 281-291.

Brock, T. D. and Freeze, H. (1969): Thermus aquaticus gen. n. and sp. n., a nonsporulating extreme thermophile. J. Bacteriol. 98, 289-297.

Brownstein, M. J., Carpten, J. D., and Smith, J. R. (1996): Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications that facilitate genotyping. BioTechniques 20, 1004-1010.

Canal, C. W., Hotzel, I., de Almeida, L. L., Roehe, P. M., and Masuda, A. (1996): Differentiation of classical swine fever virus from ruminant pestiviruses by reverse transcription and polymerase chain reaction (RT-PCR). Vet. Microbiol. 48, 373-379.

Carrino, J. J. and Lee, H. H. (1995): Nucleic acid amplification methods. J. Microbiol. Methods 23, 3-20.

Chomczynski, P. and Sacchi, N. (1987): Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

Chumakov, K. M. (1994): Reverse transcriptase can inhibit PCR and stimulate primer-dimer formation. PCR Methods and Applications 4, 62-64.

Clyde, Jr. W. A., (1983). Growth inhibition tests. In: S. Razin and J. G. Tully (eds): Methods in Mycoplasmology Vol. 1., Academic Press pp. 405-410.

Corapi, W. V., Donis, R. O., and Dubovi, E. J. (1990): Characterization of a panel of monoclonal antibodies and their use in the study of the antigenic diversity of bovine viral diarrhea virus. Am. J. Vet. Res. 51, 1388-1394.

Csontos, L. (1967): Isolation of adenoviruses from geese. Acta Vet. Hung. 17, 217-219.

Czifra, Gy., Tuboly, T., Sundquist, B. G., and Stipkovits, L. (1993): Monoclonal antibodies to *Mycoplasma gallisepticum* membrane proteins. Avian Dis. 37, 689-696.

Da Silva, N., Zardoya, R., Santurde, G., Solana, A., and Castro, J. M. (1995): Rapid and sensitive detection of the bovine viral diarrhea virus genome in semen. J. Virol. Methods 55, 209-218.

De Moerlooze, L., Lecomte, C., Brown-Schimmer, S., Schmetz, D., Guiot, C., Vandenbergh, D., Allaer, D., Rossius, M., Chappuis, G., Dina, D., Renard, A., and Martial, J.A. (1993): Nucleotide sequenee of the bovine viral diarrhoea virus Osloss strain: comparison with related viruses and identification of specific DNA probes in the 5' untranslated region. J. Gen. Virol. 74, 1433-1438.

Deng, R. and Brock, K. V. (l993): 5' and 3' untranslated regions of pestivirus genome: primary and secondary structure analysis. Nucleic Acids Res. 21, 1949-1957.

Derbyshire, J. B. (1989): Porcine adenovirus. In: Pensaert, M. B. (ed.) Virus Infections of Porcines. Virus Infections of Vertebrates 2. Elsevier Science Publishers BV, Amsterdam, Chap. 5, 73-80.

Derzsy, D. (1967): A viral disease of goslings. I. Epidemiological, clinical, pathological and aetiological studies. Acta Vet. Hung. 17, 443-448.

Edwards, S., Moennig, V., and Wensvoort. G. (1991): The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. Vet. Microbiol. 29, 101-108.

Eeles, R. A., Warren, W., and Stamps, A. (1992): The PCR revolution. Eur. J. Cancer 28, 289-293.

Elgadi, M., Rghei, N., and Haj-Ahmad, Y. (1993): Sequence and sequence analysis of E1 and pIX regions of the BAV-3 genome. Intervirolgy 36, 113-120.

Entrican, G., Dand, A., and Nettleton, P. F. (1995): A double monoclonal antibody ELISA for detecting pestivirus antigen in the blood of viraemic cattle and sheep. Vet. Microbiol. 43, 65-74.

Esford, L. E. and Haj-Ahmad, Y. (1994): Sequence analysis of the putative E3 region of bovine adenovirus type 2. Intervirology 37, 277-286.

Fan, H. H., Kleven, S. H., Jackwood, M. W., Johansson, K.-E., Pettersson, B., and Levisohn, S. (1995): Species identification of avian mycoplasmas by polymerase chain reaction and restriction fragment length polymorphism assay. Avian Dis. 39, 398-407.

Fernández, C., Mattsson, J.G., Bölske, G., Levisohn, S., and Johansson, K.-E. (1993): Species-specific oligonucleotide probes complementary to 16S rRNA of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. Res. Vet. Sci. 55, 130-136.

Frey, M. L., Hanson, R. P., and Anderson, D. P. (1968): A medium for the isolation of avian mycoplasmas. Am. J. Vet. Res. 29, 2163-2171.

Garcia, M., Jackwood, M. W., Levisohn, S., and Kleven, S. H. (1995): Detection of *Mycoplasma gallisepticum*, *M. synoviae*, and *M. iowae* by multi-species polymerase chain reaction and restriction fragment length polymorphism. Avian Dis. 39, 606-616.

Garwes, D. J. and Xuan, H. (1989): Genome typing of three serotypes of porcine adenoviruses. Intervirology 30, 234-236.

Geary, S. J., Forsyth, M. H., Saoud, S. A., Wang, G., Berg, D. E., and Berg, C. M. (1994): *Mycoplasma gallisepticum* strain differentiation by arbitrary primer PCR (RAPD) fingerprinting. Mol. Cell. Probes 8, 311-316.

Gelehrter, T. A. and Collins, F. S. (eds., 1990): Principles of medical genetics. Williams & Wilkins, Baltimore, pp. 255-286.

Gelfand, D. H. (1989): *Taq* DNA polymerase. In: Erlich, H., A. (ed.) PCR Technology: Principles and applications for DNA amplification. Stockton Press, New York, pp. 17-22.

Gough, R. E., Spackman, D., and Collins, M. S. (1981): Isolation and characterisation of a parvovirus from goslings. Vet. Rec. 108, 399-400.

Grau, O., Laigret, F., Carle, P., Tully, J. G., Rose, D. L., and Bové, J. M. (1991): Identification of a plant-derived mollicute as a strain of an avian pathogen, *Mycoplasma iowae*, and its implications for mollicute taxonomy. Int. J. Syst. Bacteriol. 41, 473-478.

Grieser-Wilke, I., Haas, L., Dittmar, K., Liess, B., and Moennig, V. (1993): RNA insertions and gene duplications in the nonstructural protein p125 region of pestivirus strains and isolates *in vitro* and *in vivo*. Virology 193, 977-980.

Harrach, B., Meehan, B. M., Benkő, M., Adair, B. M., and Todd, D. (1997): Close genetic relationship between egg drop syndrome virus, bovine adenovirus serotype 7, and ovine adnovirus strain 287. Virology 229, 302-306.

Harrach, B. and Benkő, M.: Phylogenetic analysis of adenovirus sequences; proof of necessity of establishing a third genus in the Adenoviridae family; In: Wold, W. S. M. (ed.) Adenovirus Methods and Protocols. Methods in Molecular Biology. Totowa, NJ, Humana Press (in press)

Hamel, A. L., Wasylyshen, M. D., and Nayar, G. S. (1995): Rapid detection of bovine viral diarrhea virus by using RNA extracted directly from assorted specimens and a one-tube reverse transcription PCR assay. J. Clin. Microbiol. 33, 287-291.

Harasawa, R. (1996): Phylogenetic analysis of pestivirus based on the 5'-untranslated region. Acta virologica 40, 49-54.

Harasawa, R. and Tomiyama, T. (1994): Evidence of pestivirus RNA in human virus vaccines. J. Clin. Microbiol. 32, 1604-1605.

Harasawa, R., Tohya, Y., Yoshida, M., Kataoka, Y., and Katae, H. (1994): Two genomic variations in the E1 region of canine adenovirus type 2 strains. Vet. Microbiol. 40, 373-378.

Hengen, P. N. (1994): Long and accurate PCR. Trends in Biochemical Science 19, 341.

Hertig, C., Pauli, U., Zanoni, R., and Peterhans, E. (1991): Detection of bovine viral diarrhea (BVD) virus using the polymerase chain reaction. Vet. Microbiol. 26, 65-76.

Higuchi, R. (1989a): Simple and rapid preparation of samples for PCR. In: Erlich, H., A. (ed.) PCR Technology: Principles and applications for DNA amplification. Stockton Press, New York, pp. 31-38.

Higuchi, R. (1989b): Using PCR to engineer DNA. In: Erlich, H., A. (ed.) PCR Technology: Principles and applications for DNA amplification. Stockton Press, New York, pp. 61-70.

Hiltunen, T., Raja-Honkala, M., Nikkari, T., and Ylä-Herttuala, S. (1994): A PCR artifact under low-stringency conditions due to amplification by only one primer. BioTechniques 17, 240-242.

Hinnisdaels, S., Del-Favero, J., and Vauterin, M. (1996): Direct cloning of PCR products amplified with Pwo DNA polymerase. BioTechniques 20, 186-188.

Hirahara, T., Yashura, H., Matsui, O., Yamanaka, M., Tanaka, M., Fukuyama, S., Izumida, A., Yoshiki, K., Kodama, K., Nakai, M., and Sasaki, N. (1990): Isolation of porcine adenovirus from the respiratory tract of pigs in Japan. Jpn. J. Vet. Sci. 52, 407-409.

Hofmann, M. A., Brechtbühl, K., and Stäuber, N. (1994): Rapid characterization of new pestivirus strains by direct sequencing of PCR-amplified cDNA from the 5' noncoding region. Arch. Virol. 139, 217-229.

Horner, G. W., Hunter, R., Bartha, A., and Benkő, M. (1989): A new subgroup 2 bovine adenovirus proposed as the prototype strain 10. Arch. Virol. 109, 121-124.

Horner, G. W., Tham, K.-M., Orr, D., Ralston, J., Rowe, S., and Houghton, T. (1995): Comparison of an antigen capture enzyme-linked assay with reverse transcription - polymerase chain reaction and cell culture immunoperoxidase tests for the diagnosis of ruminant pestivirus infections. Vet. Microbiol. 43, 75-84.

Hu, S.-L., Battles, J. K., and Potts, D. E. (1984b): Restriction analysis and homology studies of bovine adenovirus 7 genome. J. Virol. 51, 880-883.

Hu, S.-L., Hays, W. W., and Potts, D. E. (1984a): Sequence homology between bovine and human adenoviruses. J. Virol. 49, 604-608.

Innis, M. A. and Gelfand, D. H., (1990): Optimization of PCRs. In: PCR protocols: A guide to methods and applications. Innis, M. a., Gelfand, D. H., Sninsky, J. J., and White, T. J. Ed., Academic Press, San Diego, CA. pp. 13-20.

Jefferey, S. B., Good, R. T., and Morrow, C. J. (1995): Detection of the turkey pathogens *Mycoplasma meleagridis* and *M. iowae* by amplification of genes coding for rRNA. J. Clin. Microbiol. 33, 1335-1338.

Johansson, K.-E. (1993): Detection and identification of mycoplasmas with diagnostic DNA probes complementary to ribosomal RNA. In, I. Kahane and A. Adoni (Editors), Rapid Diagnosis of Mycoplasmas, Plenum Press, New York, pp. 139-154.

Jordan, F. T. W. (1983): Recovery and identification of avian mycoplasmas. In, S. Razin and J. G. Tully (Editors), Methods in Mycoplasmology, Vol. 2., Academic Press, pp. 69-79.

Kaszanyitzky, É., Czifra, G., and Stipkovits, L. (1994): Detection of *Mycoplasma gallisepticum* antibodies in turkey blood samples by ELISA and by the slide agglutination and haemagglutination inhibition tests. Acta Vet. Hung. 42, 69-78.

Katz, J. B., Ridpath, J., and Bolin, S. R. (1993): Presumptive diagnostic differentiation of hog cholera virus from bovine viral diarrhea and border disease viruses by using a cDNA nested-amplification approach. J. Clin. Microbiol. 31, 565-568.

Kawasaki, E. S. (1990): Amplification of RNA. In: PCR protocols: A guide to methods and applications. Innis, M. a., Gelfand, D. H., Sninsky, J. J., and White, T. J. (eds.) Academic Press, San Diego, CA. pp. 21-27.

Kecskeméti, S., Kisary, J., Pálfi, V., Bacsó, T., Bistyák, A., Hortobágyi, E., and Kiss, I., (1996): The diagnosis of Derzsy's disease. Derzsy's day, Budapest, September 19.

Kelly, W. R. (1993): The liver and biliary system. In: Jubb, P., Kennedy, C. and Palmer, N. (eds.) Pathology of Domestic Animals. 4th Ed. Vol 2. K. V. F. Academic Press, San Diego, pp. 364-366.

Khan, M. I., Lam, K. M., and Yamamoto, R. (1987): *Mycoplasma gallisepticum* strain variations detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Avian Dis. 31, 315-320.

King, A. A. and Harkness, J. W. (1975): Viral contamination of fetal bovine serum. Vet. Rec. 97, 16.

Kisary, J., Derzsy, D., and Mészáros, J. (1978): Attenuation of the goose parvovirus strain B. Laboratory and field trials of the attenuated mutant for vaccination against Derzsy's disease. Avian Pathol. 7, 397-406.

Kisary, J. (1993): Derzsy's disease of geese. In: J. B. McFerran and M. S. McNulty (Eds.), Virus infection of birds, Elsevier Science Publishers, Amsterdam, pp. 157-162.

Kiss, I., Matiz, K., Allard, A., Wadell, G., and Benkő, M. (1996a): Detection of homologous DNA sequences in animal adenoviruses by polymerase chain reaction. Acta Vet. Hung. 44, 243-251.

Kiss, I., Matiz, K., Bajmóci, E., Rusvai, M., and Harrach, B. (1996b): Infectious canine hepatitis: detection of canine adenovirus type 1 by polymerase chain reaction. Acta Vet. Hung. 44, 253-258.

Kiss, I., Matiz, K., Kaszanyitzky, E., Chavez, Y., and Johansson, K.-E. (1997): Detection and identification of avian pathogen mycoplasmas by polymerase chain reaction and restriction fragment length polymorphism assay. Vet. Microbiol. (in press)

Kiss, I., Matiz, K., Kecskeméti, S., and Tanyi, J. (1997): Experiences of using the PCR at the Veterinary Institute of Debrecen. Hung. Vet. J. 119, 556-558.

Kleiboeker, S. B., Seal, B. S., and Mengeling W. L. (1993): Genomic cloning and restriction site mapping of a porcine adenovirus isolate: demonstration of genetic stability in porcine adenovirus. Arch. Virol. 133, 357-368.

Kleven, S. H., Morrow, C. J., and Whithear, K. J. (1988): Comparison of *Mycoplasma gallisepticum* strains by hemagglutination-inhibition and restriction endonuclease analysis. Avian Dis. 32, 731-741.

Kobs, G. (1997): Cloning blunt-end DNA fragments into the pGEM-T vector systems. Promega Notes 62, 15-19.

Kurokawa, T., Igarashi, K., and Sugino, Y. (1978): Biochemical studies on bovine adenovirus type 3 III. Cleavage maps of viral DNA by restriction endonucleases *Eco*RI, *Bam*HI and *Hin*dIII. J. Virol. 28, 212-218.

Lakatos, B., Farkas, J., Egberink, H. F., Vennema, H., Horzinek, M. C., van Vliet, A., Rossen, J., Benkő, M., and Ongrádi, J. (1997): PCR detection of adenovirus in a cat. Hung. Vet. J. 119, 517-519.

LØken, T., Krogsrud. J., and Bjerkas, I. (1991): Outbreaks of border disease in goats induced by a pestivirus-contaminated orf vaccine, with virus transmission to sheep and cattle. J. Comp. Path. 104, 195-209.

Lauerman, L. H., Hoerr, F. J., Sharpton, A. R., Shah, M. S., and van Santen, V. L. (1993a): Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. Avian Dis. 37, 829-834.

Lauerman, L. H., Shah, M. S., Williams, J. C., Corsiglia, C. M., and Herring, R. J. (1993b): Immunoglobulin receptors used in avian mycoplasma identification. Avian Dis. 37, 1080-1084.

Lazar, J. G. (1994): Advanced methods in PCR product detection. PCR Methods and Applications. Manual Supplement. 4, 1-14.

Le, S.-Y., Sonenberg., N., and Maizel, J.V. jr. (1995): Unusual folding regions and ribosome landing pad within hepatitis C virus and pestivirus RNAs. Gene 154, 137-143.

Ley, D. H. and Avakian, A. P. (1992): An outbreak of *Mycoplasma synoviae* infection in North Carolina turkeys, comparison of isolates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and restriction endonuclese analysis. Avian Dis. 36, 672-678.

Liedtke, W., Battistini, L., Brosnan, C. F., and Raine, C. S. (1994): A comparison of methods for RNA extraction from lymphocytes for RT-PCR. PCR Methods and Applications 4: 185-187.

Limn, C., Yamada, T., Nakamura, M., and Takehara, K. (1996): Detection of Goose parvovirus genome by polymerase chain reaction: distribution of Goose parvovirus in Muscovy ducklings. Virus Res. 42, 162-172.

Lowe, T., Sharefkin, J., Yang, S. Q., and Dieffenbach, C. W. (1990): A computer program for selection of oligonucleotide primers for polymerase chain reactions. Nucleic Acids Res 18,1757–1761.

Lowings, J. P., Paton, D. J., Sands., J. J., De Mia, G. M., and Rutili, D. (1994): Classical swine fever: genetic detection and analysis of differences between virus isolates. J. Gen. Virol. 75, 3461-3468.

Luo, G., An, G., and Wu, R. (1994): A PCR differential screening method for rapid isolation of clones from a cDNA library. BioTechniques 16, 670-675.

Macháty, Z., Páldi, A., Csáki, T., Varga, Zs., Kiss, I., Bárándi, Zs., and Vajta, G. (1993): Biopsy and sex determination by PCR of IVF bovine embryos. J. Reprod. Fertil. 98, 467-470.

Matiz, K. (1997): A new pathogen adenovirus serotype in cattle: BAV-10? Hung. Vet. J. 119, 140-142.

Matiz, K., Ursu, K., Harrach, B., Zádori, Z., and Benkő, M.: Sequencing and phylogenetic analysis of the protease gene, and genetic mapping of bovine adenovirus type 10 define its relatedness to other bovine adenoviruses. submitted to Virus Research.

Meinkoth, J. and Wahl, G. (1984): Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. 138, 267-284.

Mészáros, J. (1985): A liba Derzsy-féle betegsége. In: Szent-Iványi, T. and Mészáros, J. (eds.) A háziállatok fertőző betegségei. Mezőgazdasági Kiadó, Budapest, pp. 317-321.

Meyers, G., Tautz, N., Dubovi, E. J., and Thiel, H.-J. (1991): Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. Virology, 180, 602-616.

Miller, K. and Storts, D. R. (1995): A sensitive single-tube, two-enzyme system for RT-PCR. Promega Notes 53, 2 -5.

Mittal, S. K., Prevec, L., Babiuk, L. A., and Graham, F. L. (1992): Sequence analysis of bovine adenovirus type 3 early regions and fibre protein genes. J. Gen. Virol. 73, 3295-3300.

Montpetit, M. L., Cassol, S., Salas, T., and O'Shaughnessy, M. V. (1992): OLIGSCAN: a computer program to assist in the design of PCR primers homologous to multiple DNA sequences. J. Virol. Methods. 36, 119–128.

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., 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.

Nascimento, E. R., Yamamoto, R., Herrick, K. R., and Tait, R. C. (1991): Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. Avian Dis. 35, 62-69.

Nuovo, G. J. (1995): In situ PCR: protocols and applications. PCR Methods and Applications, Manual Supplement. 4, 151-167.

Oefner, P. J., Huber, C. G., Puchhammer-Stöckl, E., Umlauft, F., Grünewald, K., Bonn, G. K., and Kunz, C. (1994): High-performance liquid chromatography for routine analysis of hepatitis C virus cDNA/PCR products. BioTechniques 16, 898-908.

Paton, D. J. (1995): Pestivirus diversity. J. Comp. Pathol. 112, 215-236.

Paton, D. J., Sands, J. J., Lowings, J. P., Smith, J. E., Ibata, G., and Edwards, S. (1995): A proposed division of the pestivirus genus using monoclonal antibodies, supported by cross-neutralisation assays and genetic sequencing. Vet. Res. 26, 92-109.

Paton, D. J., Simpson, V., and Done, S. H. (1992): Infections of pigs and cattle with bovine viral diarrhoea virus on a farm in England. Vet. Rec. 131, 185 -188.

Pellerin, C., Van Den Hurk, J., Lecomte, J., and Tijssen, P. (1994): Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203, 260-268.

Pettersson, B., Johansson, K.-E., and Uhlén, M. (1994): Sequence analysis of 16S rRNA from mycoplasmas by direct solid-phase DNA sequencing. Appl. Environ. Microbiol. 60, 2456-2461.

Pfeffer, U., Fecarotta, E., and Vidali, G. (1995): Efficient one-tube RT-PCR amplification of rare transcripts using short sequence-specific reverse transcription primers. BioTechniques, 18, 204-206.

Poole, T. L., Wang, C., Popp, R. A., Potgieter, L. N. D., Siddiqui, A., and Collett, M. S. (1995): Pestivirus translation initiation occurs by internal ribosome entry. Virology 206, 750-754.

Radwan, G. S., Brock, K. V., Hogan, J. S., and Smith, K. L. (1995): Development of a PCR amplification assay as a screening test using bulk milk samples for identifying dairy herds infected with bovine viral diarrhea virus. Vet. Microbiol. 44, 77-92.

Rashtchian, A. (1994): Amplification of RNA. PCR Methods and Applications, Manual Supplement. 4, 83-91.

Reddy, S. P., Nagy, É., and Derbyshire, B. J. (1993): Restriction endonuclease analysis and molecular cloning of porcine adenovirus type 3. Intervirology 36, 161-168.

Reddy, S. P., Tuboly, T., Nagy, É., and Derbyshire, B. J. (1995): Molecular cloning and physical mapping of porcine adenovirus types 1 and 2. Arch. Virol. 140, 195-200.

Reeben, M. and Prydz, H. (1994): An improved method for detection of 5-methyl-cytosine by PCR-based genomic sequencing. BioTechniques 16, 416-417.

Rhoades, K. R., Phillips, M., and Yoder, Jr. H. W. (1974): Comparison of strains of *Mycoplasma gallisepticum* by polyacrylamide gel electrophoresis. Avian Dis. 18, 91-96.

Ridpath, J. F., Bolin, S. R., and Katz, J. (1993): Comparison of nucleic acid hybridization and nucleic acid amplification using conserved sequences from the 5' noncoding region for detection of bovine viral diarrhea virus. J. Clin. Microbiol. 31, 986-989.

Roberts, M. M., White, J. L.,Grütter, M. G., and Burnett, R. M. (1986): Three-dimensional structure of the adenovirus major coat protein hexon. Science 232, 1148-1151.

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. J. Bacteriol. 176, 2577-2586.

Roszkowski, J., Gazdzinski, PCR., Kozaczynski, W., and Bartoszcze, M. (1982): Application of the immunoperoxidase technique for the detection of Derzsy's disease virus antigen in cell culture and goslings. Avian Pathol. 11, 571-578.

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.

Saiki, R. K., (1990): Amplification of genomic DNA. In: PCR protocols: A guide to methods and applications. Innis, M. a., Gelfand, D. H., Sninsky, J. J., and White, T. J. (eds.) Academic Press, San Diego, CA. pp. 13-20.

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

Saiki, R.K. (1989): The design and optimization of the PCR. In: Erlich, H., A. (ed.) PCR Technology: Principles and Applications for DNA Amplification. Stockton Press, New York, pp. 7-16.

Salmon, K. and Haj-Ahmad, Y. (1994): Sequence analysis bovine adenovirus type 2 early region and pIX gene. Intervirology 37, 298-305.

Sarkar, G., Kapelner, S., and Sommer, S. S., (1990): Formamide can dramatically improve the specificity of PCR. Nucleic Acids Res. 18, 7465.

Scamrov, A. and Beabealashvilli, R. (1991): *Mycoplasma gallisepticum* strain S6 genome contains three regions hybridizing with 16S rRNA and two regions hybridizing with 23S and 5S rRNA. FEBS Lett. 291, 71-74.

Schlegel, J., Vogt, T., Münkel, K., and Rüschoff, J. (1996): DNA fingerprinting of mammalian cell lines using nonradioactive arbitrarily primed PCR (AP-PCR). BioTechniques 20, 178-180.

Schmitt, B. J., Lopez, O. J., Ridpath, J. F., Galeota-Wheeler, J., and Osorio, F. A. (1994): Evaluation of PCR for diagnosis of bovine viral diarrhea virus in tissue homogenates. J. Vet. Diagn. Invest. 6, 44-47.

Schneeberger, C., Speiser, P., Kury, F., and Zeillinger, R. (1995): Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain. PCR Methods and Applications 4, 234-238.

Selvakumar, N., Ding, B.-C., and Wilson, S. M. (1997): Separation of DNA strands facilitates detection of point mutations by PCR-SSCP. BioTechniques 22, 604-606.

Slavik, M. F., Wang, R.-F., and Cao, W.-W. (1993): Development and evaluation of the polymerase chain reaction method for diagnosis of *Mycoplasma gallisepticum* infection in chickens. Mol. Cell. Probes 7, 459-463.

Smith, L. S., Lewis, T. L., and Matsui, S. M. (1995): Increased yield of small DNA fragments purified by silica binding. BioTechniques 18, 970-975.

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

Stipkovits, L., Czifra, Gy., and Sundquist, B. G. (1993): Indirect ELISA for detection of specific antibody response against *Mycoplasma gallisepticum*. Avian Pathol. 22, 481-494.

Stock, C., White, D., and Golumbeski, G. (1995): Properties of Taq DNA polymerase purifird by varios methods. Promega Notes 51, 2-9.

Sugden, B. (1993): How some retroviruses got their oncogenes. Trends in Biochemical Sciences 18, 233-235.

Sullivan, D. G. and Akkina, R. K. (1995): A nested polymerase chain reaction assay to differentiate pestiviruses. Virus Res. 38, 231-239.

Szent-Iványi, T. (1985): Adenovirusok okozta betegségek. In: Szent-Iványi, T. and Mészáros, J. (eds.) A háziállatok fertőző betegségei. Mezőgazdasági Kiadó, Budapest, pp. 324-333.

Takehara, K., Hyakutake, K., Imamura, T., Mutoh, K., and Yoshimura, M. (1994): Isolation, identification and plaque titration of parvovirus from Muscovy ducks in Japan. Avian Dis. 38, 810-815.

Talkington, F. D. and Kleven, S. H. (1983): A classification of laboratory strains of avian mycoplasma serotypes by direct immunofluorescence. Avian Dis. 27, 422-429.

Temesgen, B. and Eschrich, K. (1996): Simplified method for ligase-free cloning of PCR products. BioTechniques 21, 828-832.

Terpstra, C. and Wensvoort, G. (I988): Natural infections of pigs with bovine viral diarrhoea virus associated with signs resembling swine fever. Res. Vet. Sci. 45, 137-142.

Thomas, C. B. and Sharp, P. (1988): Detection of antigenic variation among strains of *Mycoplasma gallisepticum* by enzyme-linked immunosorbent inhibition assay (ELISIA) and Western blot analysis. Avian Dis. 32, 748-756.

Timme, T. L. and Thompson, T. C. (1994): Rapid allalotype analysis of p53 knockout mice. BioTechniques 17, 460-463.

Toogood, C. I. A. and Hay, R. T. (1988): DNA sequence of the adenovirus type 41 hexon gene and predicted structure of the protein. J. Gen. Virol. 69, 2291-2301.

Tuboly, T., Reddy, S. P., Nagy, É., and Derbyshire, B. J. (1995): Restriction endonuclease analysis and physical mapping of the genome of porcine adenovirus type 5. Virus Res. 37, 49-54.

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.

Van Oirschot, J.T. (1988) Description of the virus infection. In: B. Liess (ed.), Classical swine fever and related viral infections. Martinus Nijhoff, Boston, pp. 1-25.

Vilček, S., Herring, A. J., Herring, J. A., Nettleton, P. F., Lowings, J. P., and Paton, D. J. (1994): Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch. Virol. 136, 309-323.

von Beroldingen, C. H., Blake, E. T., Higuchi, R., Sensabaugh, G. F., and Erlich, H. (1989): Applications of PCR to the analysis of biological evidence. In: Erlich, H., A. (ed.) PCR Technology: Principles and applications for DNA amplification. Stockton Press, New York, pp. 61-70.

Weisburg, W. G., Tully, J. G., Rose, D. L., Petzel, J. P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T. G., van Etten, J. L., Maniloff, J., and Woese, C. R. (1989): A phylogenetic analysis of the mycoplasmas, basis for their classification. J. Bacteriol. 171, 6455-6467.

Welch, H. M., Bridges, C. G., Lyon, A. M., Griffiths, L., and Edington, N. (1992): Latent equid herpesvirus 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. J. Gen. Virol. 73, 261-268.

Wensvoort, G. and Terpstra, C. (1988): Bovine viral diarrhoea infections in piglets born to sows vaccinated against swine fever with contaminated vaccine. Res. Vet. Sci. 45, 143-148.

Wensvoort, G., Bloemraad, M., and Terpstra, C. (1988): An enzyme immunoassay employing monoclonal antibodies and detecting specifically antibodies to classical swine fever virus. Vet. Microbiol. 17, 129-140.

Westermeier, R. (ed., 1993): Electrophoresis in practice. VCH, Weinheim, pp. 5-33.

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

Yogev, D., Levisohn, S., Kleven, S. H., Halachmi, D., and Razin, S. (1988): Ribosomal RNA gene probes to detect intraspecies heterogeneity in *Mycoplasma gallisepticum* and *M. synoviae*. Avian Dis. 32, 220-231.

Zádori, Z., Stefancsik, R., Rauch, T., and Kisary, J. (1995): Analysis of the complete nucleotide sequences of goose and Muscovy duck parvoviruses indicates common ancestral origin with adeno-associated virus 2. Virology 212, 562-573.

Zhao, S. and Yamamoto, R. (1993): Detection of *Mycoplasma meleagridis* by polymerase chain reaction. Vet. Microbiol. 36, 91-97.

Zimmermann, K, Schögl, D., Plaimauer, B., and Mannhalter, J. W. (1996): Quantitative multiple competitive PCR of HIV-1 DNA in a single reaction tube. BioTechniques 21, 52-56.

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