Implementation of Molecular Detection Techniques in the Field of Veterinary Virology

With Special Reference to the Ligation - Based Methodologies

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2008

Acta Universitatis Agriculturae Sueciae
2008:83

Cover: Microarray picture of influenza samples
(photo: Péter Gyarmati)

ISSN 1652-6880
ISBN 978-91-86195-16-8

Tryck: SLU Service/Repro, Uppsala 2008

Abstract

Gyarmati, Péter, 2008. Implementation of molecular detection techniques in the field of veterinary virology, with special reference to the ligation-based methodologies. Doctor`s dissertation.
ISSN 1652-6880, ISBN 978-91-86195-16-8

This thesis deals with the molecular diagnosis of infectious diseases concerning animal and human health: in particular, with diseases notifiable to the World Organization for Animal Health, OIE (the vesicular complex and avian influenza), as well as with Hepatitis E, representing emerging zoonotic aspects. With the worldwide introduction and use of the polymerase chain reaction (PCR) methodologies, the detection of different pathogens improved significantly - however, these systems have weak points as well. The parallel screening of more than a few pathogens is not resolved and, in general, the multiplexing capacity of most of the methods used in this area is insufficient.

In the case of the detection of pathogens causing similar symptoms (like the vesicular complex, involving Foot-and-Mouth Disease (FMD), Swine Vesicular Disease (SVD) and Vesicular Stomatitis (VS), the immediate differential diagnosis is essential, not only regarding the multiplexing, but also because of the high economic risks and the strict legislations.

Subtyping of the avian influenza viruses is a broad and extensive task because it needs to differentiate 16 hemagglutinin and 9 neuraminidase types and their variants. The padlock probes, as used in these studies, seem to be optimal to fulfill the multiplexing requirements and to provide novel, high-throughput tools for the improved diagnosis of the vesicular complex and of avian influenza.

The general detection and subtyping of Hepatitis E Virus (HEV) is an important and complicated task today, as the virus shows zoonotic potential by causing endemics in humans and persisting infections in different animal species. Thus, there is a high need for sensitive and specific methods and identification of HEV variants. In the frame of this work, a highly specific and sensitive diagnostic assay was developed, based on two types of real-time PCR methods. In addition, a genotyping system was constructed using a simple and quick ligation-based technique.

Keywords: padlock probes, PCR, microarray, vesicular complex, HEV, avian influenza
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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Banér, J., Gyarmati, P., Yacoub, A., Hakhverdyan, M., Stenberg, J., Ericsson, O., Nilsson, M., Landegren, U. & Belák, S. (2007). Microarray-based molecular detection of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses, using padlock probes. *J Virol Methods*. 143(2):200-6.

II Gyarmati, P., Conze, T., Zohari, S., LeBlanc, N., Nilsson, M., Landegren, U., Banér, J. & Belák, S. (2008). Simultaneous genotyping of all hemagglutinin and neuraminidase subtypes of avian influenza viruses using padlock probes. *J Clin Microbiol* 46(5):1747-51.

III Gyarmati, P., Mohammed N., Norder H., Blomberg J., Belák, S. & Widén, F. (2007). Universal detection of hepatitis E virus by two real-time PCR assays: TaqMan and Primer-Probe Energy Transfer. *J Virol Methods* 146(1-2):226-35.

IV Gyarmati, P., Belák, S. & Widén, F. (2008). Genotyping of Hepatitis E virus using ligation-dependent probe amplification. (Manuscript)

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Related Publications

1. Zohari, S., Gyarmati, P., Thorén, P., Czifra, G., Bröjer, C., Belák, S., & Berg, M. (2008). Genetic characterization of the NS gene indicates co-circulation of two sub-lineages of highly pathogenic avian influenza virus of H5N1 subtype in Northern Europe in 2006. *Virus Genes* 36(1):117-25.
2. Kiss, I., Gyarmati, P., Zohari, S., Wilbe Ramsay, K., Metreveli, G., Weiss, E., Brytting, M., Stivers, M., Lindström, M., Lundkvist, A., Nemirov, K., Thorén, P., Berg, M., Czifra, G. & Belák, S. (2008). Molecular characterization of highly pathogenic H5N1 avian influenza viruses isolated in Sweden in 2006. *Virology Journal* 5:113.
3. Zohari, S., Gyarmati, P., Ejdersund, A., Berglöf, U., Thorén, P., Ehrenberg, M., Czifra, G., Belák, S., Olsen, B. & Berg, M. Genetic analysis of the non-structural (NS) genes of influenza A viruses isolated in mallards in Northern Europe in 2005. *Submitted*.

Abbreviations

AIV	Avian Influenza Virus
bp	Base pair
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
FMDV	Foot-and-mouth Disease Virus
FRET	Förster resonance energy transfer
HA	Hemagglutinin
HEV	Hepatitis E Virus
HI	Hemagglutination inhibition
kb	Kilobase
MLPA	Multiplex ligation-dependent probe amplification
NA	Neuraminidase
nt	Nucleotide
OIE	Office International des Epizoties (World Organization for Animal Health)
PCR	Polymerase chain reaction
RCA	Rolling circle amplification
RNA	Ribonucleic acid
SD	Standard deviation
SNP	Single nucleotide polymorphism
SVDV	Swine Vesicular Disease Virus
Tm	Melting temperature
VSV	Vesicular Stomatitis Virus

1 Introduction

The recognition of the causative agent(s) behind infections has high importance for many reasons: it makes possible to choose the most effective therapy to recuperate the infected organism, eases the decision making for preventive actions to impede or reduce the speed of the spreading of the disease, helps to develop vaccines and, in a long run, it gives information about the epidemiological behavior of the microbe.

The laboratory diagnosis of a pathogen can be done in various ways and the major options can be grouped into the following variants (according to the Merck Manual of Diagnosis and Therapy, 2006) as the diseases can be identified by:

- Culture-based
- Immunologic tests
- Microscopy
- Non-nucleic acid-based identification
- Nucleic acid-based identification methods
- Susceptibility testing

These variants are strongly related and built on each other, and none of them is a standalone procedure, since all of them have benefits and weaknesses, therefore they are supporting each other rather than competing. Depending on the target of the assay, the molecular diagnostic tools can be divided into nucleic acid or protein detection techniques. Large numbers of methods for nucleic acid detection are under development and a large variety of them are already used for routine diagnosis (Belák, 2007). The following section will list the methods relevant for this thesis.

2 DNA- and RNA-detection

In recent years, there has been considerable development of techniques, which can assist the identification of target molecules. In the case of nucleic acid detection, the target recognition and identification is usually based on the specificity of hybridization formed by Watson-Crick base pairs between oligonucleotide chains. A perfect match between the pair of sequences offers more stable conformation than an imperfect, which means that under ideal reaction conditions the perfectly matched oligonucleotides will more often be found in complex with their targets than the ones with one or more mismatches.

In the molecular diagnostics, the major determinants of the methods are the specificity and sensitivity – the specificity as a statistical measure in a binary classification shows how good the system is to correctly identify and separate the negative cases, or cases that do not meet the given criteria; and the sensitivity as a minimum of input signal that is required to produce a specified output signal (having a defined signal-to-noise ratio). In biological context, the specificity shows the ability of the method to specifically identify the target molecules in an abundance of others, e.g., identify 20-40 nt long targets on a 13 kb long influenza genome; while the sensitivity determines the lower limit of the detection, the minimum number of target pathogens necessary to obtain reproducible results.
Both of these parameters are important prerequisites for the proper diagnosis: to only detect the targets which are aimed to detect and detect these targets in a very few copies are fundamental requirements.

Other important aspects are the reliability (the quality of the measurement), reproducibility (the assay should give consistent results if it is repeated and any other laboratories should be able to accurately reproduce the experiment), scalability of the method (either vertical or horizontal, the scalability shows the capability of the system to handle an increasing

throughput), and the throughput itself (what determines the minimum/maximum number of samples can be tested in the same time); cost (the availability is heavily affected by the price), flexibility (in the case of emerging pathogens, there might be a need to widen the assay, and it should be adaptable to different instruments) and the possibility of automation (using electronic controlling) is also relevant.

2.1 Polymerase chain reaction (PCR)

The PCR is a widely used technique in molecular biology because of its high sensitivity, specificity and user-friendly nature.

In 1971, the idea by Kjell Kleppe (Kleppe *et al.*, 1971) was described to replicate short DNA fragments by nucleotide primers *in vitro*, but the invention of the complete assay is credited to Kary Mullis (Saiki *et al.*, 1985; Mullis *et al.*, 1986). By the development of related disciplines (enzymology, oligosynthesis, electromagnetism, etc.), there is a large variety of PCR technologies available nowadays (Heid *et al.*, 1996; Mackay *et al.*, 2002).

At the present time, the PCR is based on a thermostable DNA polymerase, which amplifies a specific region of the target DNA initiated by short, 15-30 bp long oligonucleotides (primers), following the principle of Watson-Crick base pairing. It is a cycling reaction where each cycle contains denaturation, annealing and extension that results in an exponential amplification, producing vast amount of DNA at the end of the procedure (Heid *et al.*, 1996). To identify the PCR product according to the specific length of the amplicon, gel electrophoresis with ethidium bromide (or other intercalating agent) staining is used for size separation.

As the reaction is going on, the used primers and dNTPs will be built in to the newly synthesized DNA strands, which will compete with the primers on the later stages of the reaction, finally reaching a plateau phase and the amplification ceases or continues with very low efficiency. If the reaction consumes the available chemicals, it also stops the amplification (Kainz, 2000).

Although the sensitivity and specificity of the PCR are high, the multiplexing capability of the method is limited: it is possible to use more primer pairs in one reaction resulting in simultaneous amplification of many targets, but the accumulation of by-products (and consequently, the risk of false diagnosis) is much higher (Figure 1). The multiplex PCR systems usually have lower sensitivity and the development of the assay needs longer optimization. The different length and melting temperatures of the primers,

the susceptibility of the oligos forming secondary structures, salt concentration used in the reaction also affect the efficiency.

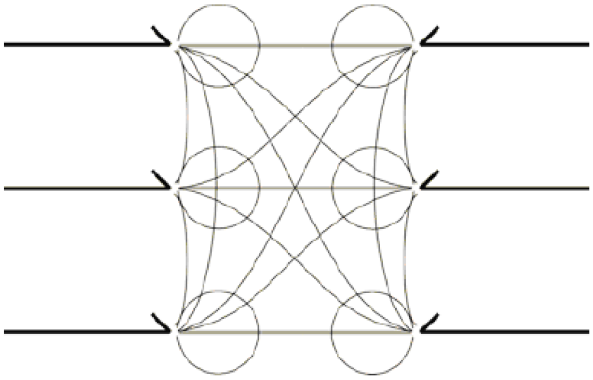

Figure 1. Possible primer combinations in a multiplex PCR using three primer pairs. Thick arrows indicate the primers, straight grey lines show the designed products, and waving grey lines show the possibilities for cross-reactions. The total number of possibilities is expressed with the $2n^2+n$ formula where n is the number of primer pairs, and it gives 21 combinations in this example (Landegren & Nilsson, 1997).

2.1.1 Real-time PCR

To identify the amplified products, the gel-based PCR methodology proved to be a long and cumbersome procedure, with high risk of contamination and carryover and consequently false positive results. Considering the weaknesses, applications were arising to monitor the amplification in real time. By the improvement of fluorescent chemistries, various real-time PCR methodologies were developed. It has proven to be very useful because of the increased speed, the quantitative measurements, the extensive dynamic range and the reduced risks for false positive results caused by contamination (Bustin, 2004, Mackay *et al.*, 2002).

Apart from the TaqMan and PriProET (described in details below), several real-time PCR chemistries exist (Belák, 2007, Didenko, 2001). For example, the SYBR Green chemistry uses a non-specific fluorescent dye what binds to double-stranded DNA; the molecular beacons are probes what form a hairpin in their native state, having a quencher and a reporter in a close proximity – in the case of hybridization, this proximity ceases and the fluorescent signal from the reporter molecule is detectable.

The multiplexing ability of the real-time PCRs suffers the same problem as other PCR-based applications: it is possible to apply fluorophores with different excitation/emission wavelengths in one reaction, but the accumulation of undesired products and the overlapping fluorescent spectra can decrease the sensitivity and elongate the optimization time.

TaqMan PCR

The TaqMan chemistry is one of the most popular PCR methods, using a dual-labeled fluorogenic probe and non-labeled primers. The principle is based on the exonuclease activity of the polymerase: the intact probe cannot emit fluorescent signals because of the close proximity of the reporter and quencher molecules, but during the PCR, as DNA synthesis commences, the polymerase degrades the annealed proportion of the TaqMan probe. When the close proximity of the quencher and the reporter molecules ceases, the fluorophore emits fluorescent signal. Hence, the detected fluorescence is proportional to the amount of DNA present in the reaction.

Primer Probe Energy Transfer (PriProET)

The PriProET PCR is a FRET-based application, using a labeled probe, one labeled and one non-labeled primer. When the polymerization commences, there is a close proximity between the donor (the labeled primer/probe) and the acceptor (the labeled probe/primer, respectively), the donor, in its excited state, can transfer energy to the acceptor. The fluorescence, emitted by the acceptor, is measured. The fluorescent signal is proportional to the amount of DNA present in the reaction.

2.2 DNA microarrays

Considering the platforms, these tools can be grouped into solid and liquid phase (e.g., Luminex) arrays; this thesis will focus only on the former.

DNA microarrays are high-throughput devices using the principle of base pairing during hybridization. To direct the hybridization on a solid surface, like the Southern blot, dot blot, reverse dot blot or comparative genomic hybridization (CGH) - and their limited resolution - were leading to the concept of microarrays in the 1980s.

There are several types of arrays and they can be grouped depending on the way of fixation, synthesis and type of the oligonucleotides used in the assay. According to the positioning of the oligos, two major groups are separated: with the spotting techniques, which may use ink-jet and piezoarray technology, the presynthesized probes are placed onto the solid

surface of the array by a robotic printer (either by direct contact or injection from a small distance), or the oligos can be synthesized *in situ,* directly on the surface. This group can be further divided according to the technology used: the traditional phosphoramidite chemistry (Beaucage & Caruthers, 1981) or the photolithography (Fodor *et al.*, 1991; Lipshutz *et al.*, 1999) – both are cycled reactions and the specific oligos are bound with the help of protective groups, but chemicals are used in the first case, and photolabile reagents in the latter.

According to the types of oligos positioned on the surface, two groups are separated: printed cDNAs/PCR-products (the length can be extended up to few thousand base pairs) or printed, artificially synthesized oligos, which are generally short, up to 70 base pairs. Higher specificity was reported in the latter case (Seela & Budow, 2008).

The coating of the array surface is fundamentally divided into categories according to the binding moiety: silane for glass surfaces, thiols on gold surfaces or polyelectrolytes to glass and dielectric materials (Sobek *et al.*, 2006).

The examined DNA segments (usually labeled with fluorophores) hybridize to the immobilized probes also called microarray tags (oligonucleotides with characterized sequences and pre-determined positions). The evaluation is based on the measured intensity of the fluorescent signals.

The solid surface arrays have different kinetics than the applications using liquid phase, usually requiring longer hybridization time. The dynamic range of the microarrays is generally 10^3-10^4 (Seela & Budow, 2008).

Because of the high density, cross-hybridization may occur but can be minimized by careful probe design, optimized hybridization time, temperature and salt concentration. Although equipments necessary for constructing and reading microarray devices are not common in diagnostic laboratories currently, similar methodologies are becoming widely accepted (Wang *et al.*, 2002; Wilson *et al.*, 2002).

2.3 Ligation-based techniques

The ligase-based detection techniques are built upon the high fidelity of DNA ligases, when differentiating between a perfect match and mismatches. (Ribozyme ligases, because of their substantially lower efficiency, were not the topic of the thesis work).

The ligase is an enzyme, which catalyzes the joining of breaks in the sugar phosphate backbones of double-stranded DNA fragments. Ligation

results from the formation of a covalent phosphodiester bond between the 3` hydroxyl and the 5` phosphate ends in the case of their close proximity. The joining event (or the absence of it) is used as a measure of the presence of the targeted nucleic acid sequences. The ligation, by its nature, is a very specific reaction because it requires perfectly matched DNA fragments directly next to each other on a template molecule. Therefore, it is very unlikely that the fragments will be joined with mismatched DNA molecules. The ligases can be grouped depending on their cofactor, what can be either ATP or NAD^+: the eukaryotic, archaebacterial and viral ligases all require ATP as cofactor and the eubacterial ligases require NAD^+ (Doherty & Suh, 2000; Engler & Richardson, 1982; Higgins & Cozzarelli, 1979).
Ligases have been isolated from thermophilic organisms *(Th. thermophilus, Th. acquaticus, D. ambivalens)*, which possess high stability at high temperatures to make PCR-like cycling possible.

The first biochemical characterization of a ligase was described in 1967 (Zimmerman *et al.*, 1967). At the end of the 1980s, the ligation was a common mechanism used for genetic analyses (Hoog *et al.*, 1987, Howell & Gilbert, 1988, Zervos *et al.*, 1988). The OLA (oligonucleotide ligation assay) and the LCR (ligase chain reaction) were developed as diagnostic applications. Due to the high precision, both methodologies were used for SNP detection, predominantly in the field of human genetics. The OLA may need further amplification, where the ligation serves as differentiation; the LCR is an amplification reaction itself, as the ligated pairs of probes act as templates for further ligation events (Landegren *et al.*, 1988, Barany, 1991).

2.3.1 Padlock probes

A variant of the OLA technique, where an oligonucleotide, having target-complementary ends, becomes circular by ligation (in the presence of the appropriate target), forming so called padlock probes (PLPs). The ligation event serves as differentiation because the unreacted probes remain linear. PLPs contain – apart from the target complementary ends what are unique in each probe – two common primer regions what make the amplification of the PLPs possible and microarray tags unique for each probe that helps to identify the positive probes on a solid surface microarray (Figure 2). Due to the design of the probes, only the circularized probes are amplified, the non-reacted (linear) probes cannot serve as a template for polymerization (Banér *et al.*, 1998).

The padlock probes are generally 80-110 bp long oligonucleotides, usually synthesized by phosphoramidite chemistry, which may cause impurities in that size. The photolithographic synthesis – produce the oligos on a solid surface using photomasks and photo labile protection groups - is a viable, but more costly alternative. Probes can also be synthesized enzymatically or shorter fragments can be ligated forming PLPs (Banér *et al.*, 2001).

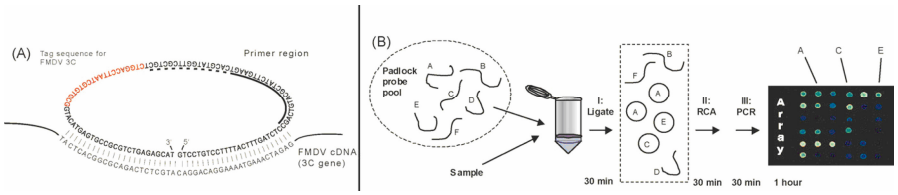

Figure 2. Schematic representation of the padlock probe structure (A) and the outline of the padlock probe assay (B) containing ligation, double amplification by RCA-PCR and visualization by microarray read-out.

Rolling circle amplification of padlock probes

When the probes become circular, their number is (depending on the ligation timing) approximately proportional to the amount of target molecules and – aiming for lower detection limit – needs to be amplified. Since circles are formed in the positive PLP reactions, the rolling circle amplification (RCA), which synthesizes multiple copies of circular molecules but let the linear ones untouched, fits into the padlock probe system perfectly (Banér *et al.* 1998).

The RCA mechanism is used by several viruses and bacteriophages to replicate their genetic material (Blanco *et al.*, 1989, Gilbert & Dressler, 1968) and was applied to the biotechnology field by Fire (Fire & Xu, 1995). Polymerization is initiated from the hybridized primer end and proceeds extensively, because – unlike the PCR – it is not self-inhibited. The final product of the RCA reaction is a long, single-stranded concatamer molecule. The RCA itself is a linear amplification, but by introduction of a second primer complementary to the RCA product, the resulting amplification (HRCA – hyperbranched RCA) is close to exponential (Lizardi *et al.*, 1998).

2.3.2 Multiplex ligation-dependent probe amplification (MLPA)

Another variation of the OLA assay, the MLPA uses two probes per target: both contain a common primer region and a unique target-complementary region. Occasionally - and due to the design of the probes – some probes should contain spacer sequences to ensure unique length for each probe pair. The two corresponding probes, which belong to the same target, are designed to bind next to each other in the presence of the target DNA. During the ligation, the two probes are sealed together in the presence of the target or remain unreacted in its absence. By the primer sites, the successful ligation event results in PCR-amplifiable products. Between the primer and target complementary sites, there are spacer oligonucleotides that make the separation of the products possible by their unique length (Figure 3). The differentiation can be done by gel-electrophoresis (either by high concentration agarose gels or polyacrylamide gels – the latter allows the use of automated sequencers).

The assay permits multiplexing of up to 40 targets (Schouten *et al.*, 2002) without requiring specific equipments, what exceeds the multiplexing capacity of the PCRs but is far behind the microarray-based techniques.

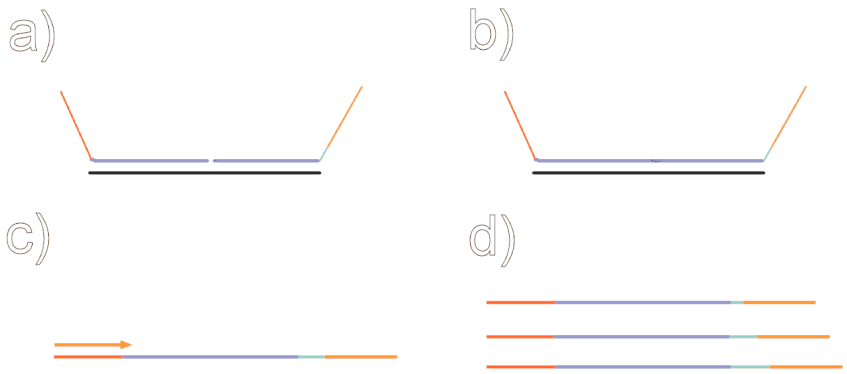

Figure 3. Schematic representation of the MLPA assay (black color indicates the target DNA, blue the target-complementary regions of the MLPA probes, red shows the primer regions and the green symbolizes the spacer region). The two corresponding probes hybridize to their target. According to the design, it ensures their close proximity (a). The ligase seals the two ends with a phosphodiester bond (b). Since the primer regions are common in every probe, all of them can be amplified by the same primer pair in one PCR (c) and because of the different length of the spacer fragment, the products can be separated by their length (d).

Compared to the padlock probe methodology, the MLPA has the advantage because of the shorter oligos used in the reaction (allowing more precise synthesis) but has the drawback because two independent hybridization events are necessary to ensure the ligation of the corresponding probes, while the padlock probes are unimolecular and therefore the two target-complementary ends are close to each other.

3 Aims of the study

3.1 **Paper I** Microarray-based molecular detection of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses, using padlock probes.

Banér J., Gyarmati P., Yacoub A., Hakhverdyan M., Stenberg J., Ericsson O., Nilsson M., Landegren U. & Belák S. (2007). J Virol Methods 143(2):200-6.
This paper describes a novel method based on two consecutive amplification procedures using padlock probes for virus detection. It demonstrates the usefulness of RCA-PCR of the PLPs in the diagnostic field, detecting three different viruses (FMDV, SVDV, VSV) causing similar symptoms. Referring to their importance, the diseases caused by these pathogens are listed as "Diseases notifiable to the OIE".

FMD virus belongs to the *Picornaviridae* family, Apthovirus genus. The Foot-and-mouth disease is one of the most contagious animal diseases, causing important economic losses.
As it stands on the OIE web site: "Foot and mouth disease (FMD) is the most contagious disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus, namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Infection with one serotype does not confer immunity against another. FMD cannot be differentiated clinically from other vesicular diseases, including swine vesicular disease, vesicular stomatitis and vesicular exanthema. Laboratory diagnosis of any suspected FMD case is therefore a matter of urgency."
(http://www.oie.int/eng/normes/mmanual/2008/pdf/2.01.05_FMD.pdf)

The laboratory diagnosis is performed by various approaches, i.e., ELISA, PCR, complement fixation and virus isolation (http://www.oie.int/eng/normes/mmanual/A_00024.htm).

SVD virus belongs to the *Picornaviridae* family, Enterovirus genus. In general, the morbidity is high in grouped pigs.

The OIE web site is summarizing: "Swine vesicular disease (SVD) is a contagious disease of pigs, caused by an enterovirus and characterised by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout and teats. Strains of SVD virus may vary in virulence, and the disease may be subclinical, mild or severe, the latter usually only being seen when pigs are housed on abrasive floors in damp conditions. The main importance of SVD is that it is clinically indistinguishable from foot and mouth disease (FMD), and any outbreaks of vesicular disease in pigs must be assumed to be FMD until investigated by laboratory tests and proven otherwise. However, subclinical infection has been the most frequent condition observed during recent years."

(http://www.oie.int/eng/normes/mmanual/2008/pdf/2.08.09_SVD.pdf)

The laboratory diagnosis is performed by various approaches, i.e., ELISA, PCR, complement fixation and virus isolation (http://www.oie.int/eng/normes/MMANUAL/A_00026.htm).

VS virus belongs to the *Rhabdoviridae* family, Vesiculovirus genus.

As OIE states: "Vesicular stomatitis (VS) is a vesicular disease of horses, cattle and pigs caused by vesiculoviruses of the family Rhabdoviridae. This disease is clinically indistinguishable from foot and mouth disease (FMD), vesicular exanthema of swine (VES), or swine vesicular disease (SVD) when horses are not involved. Sheep, goats and many other wild species can be infected. Humans are also susceptible.

The disease is limited to the Americas; however, it was previously described in France and in South Africa. Although virus is transmitted directly by the transcutaneous or transmucosal route, it has been isolated from sandflies and mosquitoes, suggesting that it could be insect-borne. There is, therefore, seasonal variation in the occurrence of VS: it disappears at the end of the rainy season in tropical areas, and at the first frosts in temperate zones. There is also some evidence that it could be a plant virus and that animals are the end of the epidemiological chain. The pathogenesis of the disease is unclear, and it has been observed that the humoral-specific antibodies do not always prevent infection with VS serogroup viruses. Although VS may be suspected when horses are involved as well as pigs and cattle, prompt differential diagnosis is essential because the clinical signs of VS are

indistinguishable from FMD when cattle and pigs are affected, and from SVD or VES when only pigs are affected."
(http://www.oie.int/eng/normes/mmanual/2008/pdf/2.01.19_VESICULAR_STOMITIS.pdf)

The laboratory diagnosis is performed by various approaches, i.e., ELISA, PCR, complement fixation and virus isolation (http://www.oie.int/eng/normes/MMANUAL/A_00025.htm).

Since these viruses cause diseases with similar symptoms, differential diagnosis is required – because the parallel detection of these pathogens is not resolved, the aim of the study was to develop a PLP-based system that can detect all three viruses in one reaction with high sensitivity and specificity in a short time.

3.2 **Paper II** Simultaneous genotyping of all hemagglutinin and neuraminidase subtypes of avian influenza viruses using padlock probes.

Gyarmati P., Conze T., Zohari S., Leblanc N., Nilsson M., Landegren U., Banér J. & Belák S. (2008). J Clin Microbiol 46(5):1747-51.

This paper describes a clinical utilization of padlock probes. Influenza viruses cause huge economic losses to the poultry industry and are a continuous risk to human health. The importance is reflected as AIV is listed as a notifiable disease by the OIE.

Avian influenza viruses belong to the *Orthomyxoviridae* family, Influenza A genus. Influenza A viruses are further divided into subtypes according to their surface proteins, hemagglutinin (HA) and neuraminidase (NA). By now, 16 different HA and 9 different NA types are known. In theory, all (144) possible combinations can occur, but approximately 100 were observed naturally. The seriousness of the symptoms depends on the pathogenicity of the virus: the low-pathogenic variants usually cause milder infections, but the infections caused by highly pathogenic strains have high mortality rate (van Reeth, 2007).

Subtyping of the influenza viruses is essential to broaden our knowledge about the spreading of the virus, can help to reveal the phenomenon of the yearly endemics, acquire more epidemiological data about the distribution of the low-pathogenic strains and supports the investigation of possible double infections, reassortants. The routine diagnosis usually does not include the determination of the subtypes unless it is H5 or H7 in which cases there is a chance for high pathogenicity. Therefore the impacts of

other low pathogenic strains are difficult to assess but should not be ignored - for example, low pathogenic H9N2 viruses, which were introduced into humans, are considered to be enzootic in Asia (Alexander, D.J., 2006).

The golden standard used for AIV subtyping (http://www.oie.int/eng/normes/mmanual/A_00037.htm) is a serological test (hemagglutinin inhibition), but it is a laborious procedure, takes a long time to complete and its sensitivity is reported to be around 10^6 virus particles, so the preceding virus cultivation is unavoidable (Donald & Isaacs, 1954).

The aim of the study was to develop a specific and sensitive method, which allows the diagnosis of AI and the identification of all known HA and NA subtypes and their variants in one reaction, within a short time.

3.3 **Paper III** Universal detection of hepatitis E virus by two real-time PCR assays: TaqMan and Primer-Probe Energy Transfer.

Gyarmati P., Mohammed N., Norder H., Blomberg J., Belák S. & Widén F. (2007). J Virol Methods 146(1-2):226-35.

This paper describes diagnostic methods, which allow the detection of all recently known variants of hepatitis E virus. The virus is the sole member of the *Hepeviridae* family, Hepevirus genus, causing epidemics in humans in territories with poor sanitation, but can cause sporadic cases worldwide. The transmission is mainly food- or waterborne, but bloodborne and parental transmission was also reported.

Because of the high variability of HEV genome, a universal detection was proved to be difficult for years. To target the virus with ligation-based probes needed a stable identification system in advance. For this purpose, two real-time assays were developed, both targeting the same ORF2-3 regions. The TaqMan assay was chosen because of its reliability, prevailing and widespread nature; the PriProET assay was chosen because of its reported higher tolerance for mismatches (Rasmussen *et al.*, 2003).

The aim of the study was to have a stable diagnostic system to detect hepatitis E virus and to compare the efficiency, reliability and sensitivity of the two different diagnostic applications. Besides, this study serves as a basis to allow further investigations of the virus.

3.4 **Paper IV** Genotyping of hepatitis E virus using ligation-dependent probe amplification.

Gyarmati P., Belák S. & Widén F. (2008). Manuscript

This paper describes a ligation-based application used to separate and identify all known genotypes of hepatitis E virus. Hepatitis E viruses have one serotype and the recent taxonomy classifies them into four subtypes according to their genetic context what seem to be showing relations to their geographical position (Okamoto, 2007). Because of the serotype identity, the ELISA-based methodologies are not able to offer any epidemiological data about the distribution of the different variants of the virus. The determination of subtypes is usually performed by analyzing the nucleic acid sequence, namely by sequencing either the whole genome or a region that is informative for subtyping (Zhai *et al.,* 2006).

The aim of the study was to simplify and shorten the HEV genotyping procedure by a ligation-based detection technique, and, at the same time, introduce the MLPA technology in the field of veterinary virology.

4 Results and discussion

4.1 Paper I

In order to establish the padlock probe-based system in the clinical diagnostics, a novel virus detection method, using padlock probes with double amplification, was developed to identify and differentiate three vesicular viruses (FMDV, SVDV, VSV) causing similar symptoms. The method also allows the serotyping of VSV.

Forty-one cDNAs were tested to investigate the assay performance, 39 were detected and identified correctly according to the identity previously determined by PCR. Most likely, the two false negative samples had low viral titers, under the detection level of the assay. The diagnosis is based on the SD-ratio threshold and the cut-off value of the microarray read-out that was defined experimentally by well-characterized samples. The sensitivity was determined in comparison with the level of background signals generated by misamplification and was defined by dilution of samples with known $TCID_{50}$-value.

The assay takes approximately three hours to perform, which is comparable to real-time PCR methods. Although other PCR-methodologies were described for the same purpose (Fernandez *et al.*, 2008), the usefulness of the padlock probe-based system in the veterinary diagnostics has been verified by the current work that not only fulfilled the primary aim – to detect and identify these three viruses in one reaction – but also opened possibilities for further improvements of the virus detection techniques.

4.2 Paper II

The utilization of the padlock probe-based system combined with microarray output was carried out with the purpose to detect avian influenza viruses based on the probes designed for the M (matrix) gene, the most conservative part of the influenza genome, and to identify their subtypes according to the hemagglutinin and neuraminidase genes.

The determination of all different subtypes of AIV was a suitable aim to exploit the multiplexing capacity of the PLP assay because of the large number of possible targets: the method was aimed to differentiate 16 HA and 9 NA subtypes and all types were targeted by at least two individual probes (except the rare variants where few sequence data were available for the design) in order to ensure a safe diagnosis despite of the high variability of the influenza genome. To avoid the false negative results, the surface antigens were targeted in more than one positions – and in the case of the most variable variants, 6-7 probes were designed to target different parts of the genes, which included all genomic variants currently available in databanks.

The genetic change of AIV is a continuous process, but because the different genes were targeted in different positions, the chance for simultaneous mutations is very low and the failure or success of certain probes can indicate the region with mutation and the assay - because of the redundant structure of the probe design – is still capable of the correct diagnosis. The flexibility of the system can be proven by following the yearly epidemics and - in the case of new emerging variants - the probe set can be extended by addition of PLPs designed for the variants what the existing system may miss.

Seventy-seven samples (allantoic fluids containing virus isolates) were used to test the assay performance. All of the samples were diagnosed as AIV according to the matrix gene-based probes and 75 were subtyped correctly according to the identities previously determined by hemagglutination inhibition reaction. None of the samples were falsely subtyped. The two failed samples contained rare subtypes (H10N8 and H12N5) and most likely the design did not include them due to their low prevalence.

The assay takes approximately four hours to perform, which is comparable with real-time PCR methods. The sensitivity was tested in two ways: by 10-fold dilutions of three samples with known HI-titer and by artificially synthesized oligos with known concentration that covered the target region of the H_13 and H_14 probes.

The lowest sample dilution resulted interpretable signals was 10^{-4} in all three cases where the samples had HI-titer 1:32. For comparison, the

dilutions were tested with HI also but they gave barely readable signals even after the first dilution step. The sample dilutions were also examined with the OIE-recommended routine diagnostic real-time PCR assay (Spackman *et al.*, 2002) where the lowest detectable dilution was 10^{-6}. The referred real-time PCR system was capable of detecting only H5 and H7 subtypes, which could not serve as a complete evaluation but there was no comparable assay present for the same purpose. The smallest detectable dilution of artificial target regions was 600 copies.

In conclusion, the sensitivity of the padlock probe system did not reach the sensitivity of the real-time PCR assays but proved to be much more sensitive than the traditional technique (HI) and offers advantages in multiplexing and target region selection that the PCRs cannot. The cost of the PLP assay is approximately the same as for the HI-test, mainly because of the high density of the microarray.

4.3 Paper III

In order to study hepatitis E viruses at a more detailed level, the primary aim was to develop a stable and quick diagnostic procedure that can detect all variants of the virus.

To reach this purpose, two real-time chemistries were compared sharing the same primer-probe distribution. The TaqMan method was chosen because of its simplicity and widespread nature. Considering that HEV is a variable virus, the PriProET approach was also applied, because of its reported higher tolerance to mutations in the targeted genome sequences (Hakhverdyan *et al.*, 2006). Fifty-four samples were tested and both methods gave the same positivity as the nested, gel-based control PCR. The positive samples represented all four genotypes. Both the TaqMan and the PriProET assays were performing with high reliability and sensitivity. The Ct-values obtained by the PriProET assay were slightly higher indicating lower efficiency. It is likely that it was caused by the less than optimal positioning of the labeled donor primer and the acceptor probe but the HEV contains very few conservative regions, hence there were limited alternatives to vary the arrangement. Because the PriProET is a FRET-based reaction, the distance between the two fluorophores can be decisive (Ha *et al.*, 1996).

In summary, the two examined assays proved to be very sensitive with having 1-20 viral copies as the lower detection limit and both were able to detect all variants of HEV. Given the present difficulties in HEV detection, which are mainly due to the high genetic diversity of the virus and the

possible asymptomatic occurrence of infection, these methods are particularly appealing in view of favoring both surveillance of farmed animals and possibly evaluate risks for the human population either in sporadic or endemic cases, related to contacts with animals, tissues and food.

4.4 Paper IV

The detection of HEV is a fundamental issue to track foodborne infections. However, to obtain more epidemiological knowledge, the data about the genotypes is also required. Although the HEV genotypes show geographical arrangement, it is not very strict. The virus is often referred as "the traveler's disease", which indicates the loosening of the distribution of the virus because of its quick spreading. By knowing more about the occurrence of given genotypes under well-defined circumstances, more information can be unraveled about the epidemiology (e.g. the zoonotic behavior, different transmission routes) of the virus (Lu *et al.*, 2006, Zheng *et al.*, 2006).

The MLPA is a popular technique in the human genetics (Reedeker *et al.*, 2008, Schouten *et al.*, 2002) but, according to our knowledge, it has not been used in the field of veterinary virology so far. The benefits of this approach were reported in the human diagnostics (Reijans *et al.*, 2008). The multiplexing capacity of the technique is higher than the PCR assays` but far below the capability of microarrays. For medium-scale (1-40-fold) multiplexing it is optimal to use and its simplicity can make the method widespread.

Eighteen – previously partially sequenced and genotyped - samples were tested to verify the reliability of the assay and all were identified in a complete agreement with the data obtained by sequencing. The samples represented all four genotypes. The sensitivity of the method was tested by 10-fold dilutions of a genotype 1 plasmid with known concentration; the lowest detectable amount was approximately 200 copies. It was possible to further enhance the sensitivity by preamplification, where the lowest detectable amount was around 20 copies.

5 Implications for future research, concluding remarks

The techniques listed in this thesis enable precise and rapid data acquisition about the presence or absence of different pathogens and their quantities. However, the diagnostic development is a never-ending process since we lack the one-and-only perfect method. Considering this scenario, a number of methodologies can be envisioned and realized. Padlock probes, combined with microarray, offer a great range of multiplexing and scalability in the diagnostics (Szemes *et al.*, 2005) but compared to PCR, they are weaker from the points of sensitivity and dynamic range of the assay. The PCR offers a highly sensitive approach, but the parallel detection of a large number of pathogens cannot be performed in one reaction. There are efforts to combine the advantages of these two methodologies, initiating polymerization on the microarray surface on the locally ligated padlock probes (Ericsson *et al.*, 2008), or placing this platform to liquid-phase amplification and/or read-out systems that would allow different kinetics (Leamon *et al.*, 2003; Vignali, D.A., 2000).

By the developments in related areas, the microarray technique is becoming more and more advanced with higher density and precision. The synthesis of oligonucleotides now has different widespread platforms even for long molecules avoiding incomplete oligonucleotides causing constantly high backgrounds. Real-time PCR machines and microarray scanners offer a wide range of detectable fluorescent signals. Large-scale sequencers are targeting diagnostic fields offering a unique possibility to identify and classify pathogens by its complete genomic contents (Margulies *et al.*, 2005); or with random amplification strategies and microarray detection, pan-virus methodologies appear to obtain a complete overview of the pathogen pool contained in a sample (Quan *et al.*, 2007).

This thesis is focusing on nucleic acid detection but if the central dogma of the molecular biology is considered, the efforts on protein detection and of their amplification will be further emphasized - the proximity ligation with tag-encoded amplification is aiming for such purposes (Fredriksson *et al.*, 2002). The capacity of protein microarrays are far behind the DNA-based arrays but this tendency can change (Zhu *et al.*, 2001).

The diagnostic applications can be improved not only by development of certain areas but by synergies between different fields, like HPLC-detection of pathogens, nucleotide identification and/or measurements by electro-chemical detection schemes (Kerman *et al.*, 2004; Liepold *et al.*, 2007) or using nanobeads, detecting the Brownian relaxation frequency (Stromberg *et al,* 2008). These fusions may reveal new dimensions of the clinical diagnostics. Significant efforts should be put not only on further investments, but also to make the newly developed methods available to the research community and suitable for routine applications.

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7 Acknowledgements

Yes, you just have come to the right page, the important list of important people, The ACKNOWLEDGEMENTS! Here it comes.
I would like to express my sincere gratitude to:

Sándor Belák, my main supervisor for giving me the opportunity to complete these works and for all the constant support, providing me the resources and possibilities to chase my projects and for the confidence for letting me roam free in the labs.

György Berencsi, for always being a mentor and an endless source of ideas.

My co-supervisors, Frederik Widén, Mikael Berg and Jonas Blomberg for always having time for discussions in your related areas.

Johan Banér, my distant supervisor, for teaching me constantly for years and answering even the most pointless questions.

Ulf Landegren and Mats Nilsson, for providing the opportunity to work in your lab under your admirable guidance.

Former and present head of the department: Berndt Klingeborn and Jens Matsson.

Anna Lunden, for answering patiently at all times and for taking care of the PhD-students.

Siamak, for being not only a critical co-author and an excellent workmate but also a friend on whom I always can reckon.

Anne-Lie, for being who you are and for revealing the hidden secrets of ~~alchemy~~ scraped pizzas. I ought to write a separate book to acknowledge you properly.

Lihong, for giving an insight to the Chinese lifestyle more closely and for showing your favorite fishing place.

Jonas, for struggling through in the jungle of the PhD-studies, -courses at the same time and for all the fun in the lab courses.

Former, present and future members of the department: Anna-Malin, Claudia, Alia, Misha, Hongyan, Linda, Karl, Karin, Mikael, Neil, Ádám, Ákos, Attila, Miklós, Mehdi, Femi, Munir, George, Yaruwan, Jessica, Nora, Sara, Sophie, Bonnie for maintaining a nice place to work at and for the O`Connors-excursions.

The Rudbeck people, particularly Tim, for the endless attempts to print a perfect slide.

People at the department in the administrative part: Anna and Madde, for making the life a lot easier.

Berka, for the excellent technical assistance and for the postcard.

Former students: Anna-Malin, Hanna, Frida and Stefan for enthusiastically probing my pedagogic trials.

Anna and István, forming a little cosy Hungarian colony and bringing me home-made food. I am grateful for your friendship and for the coffee breaks. Special acknowledgement goes to Moha.

My parents, for constant encouragement and keeping the postal service busy, my brother and his family – hope Rita will be a great scientist!

For those who feel they are missing: ~~life is tough, huh?~~ please do not feel offended, I am just simply scatterbrained.