



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

# Molecular diagnosis of viral diseases, present trends and future aspects A view from the OIE Collaborating Centre for the Application of Polymerase Chain Reaction Methods for Diagnosis of Viral Diseases in Veterinary Medicine

Sándor Belák\*

*Joint R&D Division, Departments of Virology, The National Veterinary Institute and the Swedish University of Agricultural Sciences, OIE Collaborating Centre for the Application of Polymerase Chain Reaction Methods for Diagnosis of Viral Diseases in Veterinary Medicine, Uppsala, Sweden*

Received 11 August 2006; accepted 27 November 2006  
Available online 28 December 2006

## Abstract

The emergence and re-emergence of transboundary animal diseases (TADs), e.g., foot-and-mouth disease, classical swine fever and the highly pathogenic avian influenza strongly indicate the need for the development of powerful and robust new diagnostic methods. The experiences of an OIE-Collaborating Centre and of two EU project consortia are summarised on the diagnostic application of gel-based PCR, general PCR systems, phylogeny, molecular epidemiology, real-time PCR (TaqMan, Molecular Beacons, Primer-Probe Energy Transfer), amplification without thermocycling (Invader), multiplex PCR, nucleic acid extraction and pipetting robotics, automation and quality control, including internal controls. By following the steps of OIE validation, the diagnostic assays are nationally and internationally standardised. The development of padlock probes and microarrays, as well as ultra rapid PCR and sequencing methods is further improving the arsenal of nucleic acid based molecular diagnosis. Further trends of diagnostic development are also mentioned, in order to combat TADs and other viral infections more effectively in the future.

© 2006 Elsevier Ltd. All rights reserved.

*Keywords:* Molecular diagnosis; PCR; Automation; Validation; Padlock probes; Microarrays

## 1. Introduction

The recent incidences of emerging and re-emerging transboundary animal diseases (TADs) lead to very heavy losses all over the world. The outbreaks are associated with huge economic and social impact in many countries. During such a short time as the last 18 months, the Office International des Epizooties (OIE, World Organisation for Animal Health) reported the occurrences of a high number of TAD outbreaks, e.g., foot-and-mouth disease on three continents (Africa, Asia and South America), classical swine fever (Africa, Asia, and Europe), rinderpest (Africa, Asia), or highly pathogenic avian influenza (Africa, Asia, and Europe). The costs should be

viewed both in terms of efforts to bring the disease under control and the consequent loss of livelihoods. Concerning the EU, during the last decade the member states have suffered from a rather high number of devastating outbreaks of diseases notifiable to OIE. The European Commission Scientific Committee on Animal Health and Animal Welfare report (adopted 24–25th April 2003) has stated: “Recent outbreaks of foot-and-mouth disease (FMD), classical swine fever (CSF) and avian influenza (AI) have occurred in several member states and resulted in the slaughter of large numbers of animals as well as severe economic consequences”. As an example, with regard to the UK FMD outbreak in 2001, the cost to the public sector was estimated at over 4.5 billion euro, and the cost to the private sector at over 7.5 billion euro. The ethical problem risen under the current eradication strategy and the social consequences of the slaughter

\* Tel.: +46 18 674 135; fax: +46 18 674 669.  
E-mail address: [sandor.belak@sva.se](mailto:sandor.belak@sva.se).

of large numbers of animals must also be taken into account when considering the effects of the diseases notifiable to OIE: ([http://www.oie.int/eng/info/hebdo/A\\_DSUM.htm](http://www.oie.int/eng/info/hebdo/A_DSUM.htm)).

The national and international organisations, veterinary health authorities, research institutes, diagnostic laboratories and field services make large efforts worldwide to prevent and combat TADs. The early warning systems and the rapid and highly specific detection of the agents are major tasks, considering that the timely recognition of such viral infections would prevent the spread of the diseases to large animal populations in huge geographic areas. Thus, the development of novel, powerful diagnostic assays is a basic issue today in veterinary research and animal health care. Molecular virology offers a range of new methods, which are able to accelerate and improve the diagnosis of infectious diseases in animals and in man. The new assays provide possibilities for a very rapid diagnosis, since the detection of viruses can be completed within several hours or minutes. Concerning the direct detection of viruses or viral components, various molecular approaches are introduced, like conventional gel-based PCR, real-time PCR, multiplex PCR, improved *in situ* hybridisation and *in situ* PCR. In addition, PCR-robotics, portable PCR machines, improved sample enrichment, amplification without thermocycling, macro- and micro-arrays are under development. As far as the indirect detection of the various viruses is concerned, antibody-detecting molecular methods are under construction, like improved ELISA systems, PCR for protein detection (DNA tags, proximity ligation), pen-side tests (e.g., dip-stick assays). In order to standardise the new methods worldwide, the steps of OIE validation are followed and introduced [1].

This article is illustrating new trends in the field of molecular diagnosis of viral diseases, summarising the recent achievements of an OIE Collaborating Centre and its EU project partner laboratories. The results of previous reviews are updated; new achievements are shown and discussed with special regard to modern trends in molecular detection of TADs and other viral infections in domestic or wild animals and in food safety.

## 2. Materials and methods and results

### 2.1. Conventional gel-based PCR assays

By introducing single and nested PCR assays as early as 1987–1988, two years after the description of the PCR principle, our laboratory was among the first ones to use this technique for diagnostic purposes [2,3]. Between 1987 and 2000 more than 50 diagnostic PCR assays have been developed and applied at our laboratory, to diagnose a wide range of diseases in various animal species. These assays, including precautions and laboratory practices to avoid false positive and false negative results, are summarised in previous general articles and reviews [1,4,5].

### 2.2. Determination of genetic relatedness of viruses, tracing the spread of virus variants by molecular epidemiology

A great diagnostic power of the PCR techniques is that the nucleotide sequencing and the comparative sequence analysis of the PCR products provide possibilities to determine the genetic relationship(s) of the detected viruses or other microbial agents. By this way phylogenetic trees are constructed, illustrating the genetic relatedness and background of the viruses. The genetic variants are rapidly identified and the routes of virus spreading are promptly traced and recorded in a country or even on much larger geographic areas. This approach, termed molecular epidemiology, provides novel tools to the veterinarians and to the animal health authorities in combating viral diseases. For performing studies of molecular epidemiology, variable genomic regions of the viral genomes are selected, amplified and sequenced. We developed such “phylogeny PCR assays” for the amplification of members of various virus families, like the *Flaviviridae* and the *Coronaviridae*. In the *Flaviviridae* family pestiviruses bovine viral diarrhoea virus (BVDV), classical swine fever virus (CSFV) and border disease virus (BDV) were amplified and grouped by amplifying and sequencing various regions of viral genomes, such as 3′NCR, 5′NCR, Npro, E2 and NS2 [6–11]. The assays of molecular epidemiology contributed to the detection and identification of BVDV variants in Scandinavia and determination of genetic clustering of the virus [12,13]. Furthermore, the PCR-based molecular detection and identification of BVDV variants proved to be a useful tool to study the biology and pathogenesis of the virus [14].

An interesting and practical approach is when the retrospective genetic analysis is applied to determine the identity of virus strains used for vaccine production. In one of our recent studies a virus “pick up” was observed in a commercially produced live attenuated BVDV vaccine. The results of this work emphasize that the contamination of commercially available live vaccines with exogenous BVDV strains is a real risk factor, and unequivocal analysis, including molecular methods, is needed to verify their authenticity [15].

Molecular epidemiology and viral phylogeny provide new possibilities to combat and eradicate infectious diseases. National and international tracing of virus variants during outbreaks helps the work of the veterinary health authorities, allowing to trace the ways of the spread of infection and to identify the various virus variants in the outbreaks [16–18].

### 2.3. Wide detection range “general” PCR systems

The majority of the diagnostic PCR systems is designed to be highly specific for the detection of the selected target virus(es). As a complement to these highly specific systems, “general”, or “universal”, PCR systems are also developed at our laboratories. The “general” systems are constructed to detect a wide range of related viruses, e.g., members of an entire virus family, or virus genus. To obtain a wide

range of target amplification, the primers are selected from well-conserved regions of the genome. These PCR systems provide very useful tools for the diagnostic virologists. Such systems are practical when more members of the same virus family or genus cause the same or very similar clinical picture(s). For example, cattle can be infected with BVDV and with border disease virus (BDV, alias ovine pestivirus), while swine can develop infection with any of the three pestiviruses, classical swine fever virus, BVDV and BDV, frequently showing only mild or inapparent symptoms. In such cases if a “general” pestivirus PCR is available, the diagnostician can directly screen the herds, in order to demonstrate or exclude the presence of any pestivirus, in a single PCR assay (instead of running three separate assays). Thus, the positive samples, pre-selected by the “general” PCR, can then be further tested with the virus specific PCR assays, in order to identify the pestivirus, which is causing the infection. The parallel use of “general” and specific PCR assays provides a rapid and effective diagnosis. Considering this requirement, a “general” pestivirus TaqMan assay is used at our laboratory for the simultaneous detection of the three pestiviruses in domestic and in wild animal populations [1,4,5]. Recently a SYBR green real-time PCR system was developed here for the generic detection of coronaviruses [19].

#### 2.4. The diagnostic application of real-time PCR assays

The real-time PCR assays provide novel rapid means of virus detection in the diagnostic laboratories. Several variants of real-time PCR methods and chemistries are used today, e.g., TaqMan, molecular beacons (MB), scorpion primers, dual probe systems as utilized in the LightCycler®, dye-labelled oligonucleotide ligation (DOL), Primer-Probe Energy Transfer System (PriProET). Compared to the “classical” single or nested PCR methods, the diagnostic application of the real-time PCR assays has certain advantages, such as: (a) faster and higher throughput assays; (b) post-PCR handling of the products is not needed; (c) despite of a non-nested set-up, the real-time-PCR is providing sensitivity close to or equal to traditional nested PCR; (d) the amplified products are detected by measuring fluorescence in the reaction vessel without having to open the system, thus, the risk of contamination is minimised; (e) the result of the PCR is not only “positive” or “negative”, but the real-time PCR assays allow quantitative estimation; (f) real-time quantitative PCR is more accurate and less labour-intensive than current quantitative PCR methods; (g) the hands-on time is greatly reduced, compared to traditional detection in agarose gels followed by ethidium bromide staining; (h) the principle of the real-time PCR allows automation of the procedure, and the use of a 96-well microplate format, without the need for nested PCR, makes it very practical to automate it; (i) diagnosis can be further automated by using robots for DNA/RNA extractions and pipetting; (j) probes for real-time PCR can be labelled with a number of different fluorophores, which function as individual reporter dyes for different primer sets. Thus,

Table 1

Examples for diagnostic real-time PCR assays developed at the OIE Collaborating Centre (SVA, Uppsala)

Assay	Targeted region
Equine herpesvirus 1	Glycoprotein C
Equine herpesvirus 4	Thymidine kinase
Equine influenza A	Non-structural protein (NS1)
Equine rhinovirus B	Polyprotein
Parvovirus, canine/feline	VP2
Feline leukaemia virus	Envelope protein
Bovine respiratory syncytial virus	Fusion protein (F)
Bovine coronavirus	Haemagglutinin-esterase (HE)
Bovine rotavirus	VP6
Bovine parainfluenza 3 virus	Fusion protein (F)
Bovine viral diarrhoea virus	5'-non coding region
Swine vesicular disease virus	Polymerase protein (3D)
Deformed wing virus, honey bee	Polyprotein
Mouse hepatitis virus	E1 (M) protein
Lactate dehydrogenase elevating virus	ORF 7
Mink astrovirus	RNA polymerase

These systems are regularly used in routine diagnostic work [26, Belák and Thorén, OIE Collaborating Centre Report 2005].

real-time PCR technique is very suitable for the development of multiplex PCR systems; (k) lower diagnostic costs.

The advantages of diagnostic real-time PCR assays and more details are summarised in previous reviews and reported in recent articles [1,4,5,20–23].

During the last years we have developed a wide range of real-time PCR assays for diagnostic purposes. The various assays were developed at the OIE Collaborating Centre or in collaboration with our partner laboratories in large diagnostic projects of the European Commission (<http://www.multiplex-eu.org/>, <http://www.labonsite.com/>). By comparing different approaches, the TaqMan, but especially the PriProET techniques were found robust and highly reliable for diagnostic purposes. This fluorescence resonance energy transfer detection system combines probe-based real-time monitoring of PCR amplification with confirmation of probe hybridization from the melting temperature ( $T_m$ ) curve. The PriProET system allows quantification of the specific amplicon, as the fluorescence emitted from the reporter depends directly on the amount of amplicon formed [23]. In contrast to PriProET, the performance of other methods, like Molecular Beacons, was rather strongly influenced by the point mutations in the target sequences. We have found that even the TaqMan and the Scorpion primers assays can be fragile to single point mutations, or they demand longer conserved areas for hybridisation. Since many of our targets, especially the RNA viruses show a high mutation rate, we suggest using first of all the PriProET principle (and with some considerations the TaqMan) for the development of diagnostic real-time PCR assays [4,5,23].

For the detection of various viruses a range of real-time PCR assays has been developed in the last years at our laboratory and is used in routine diagnosis (Table 1). The majority of these methods is based on TaqMan or PriProET principles. In addition, isothermal amplification methods, like the

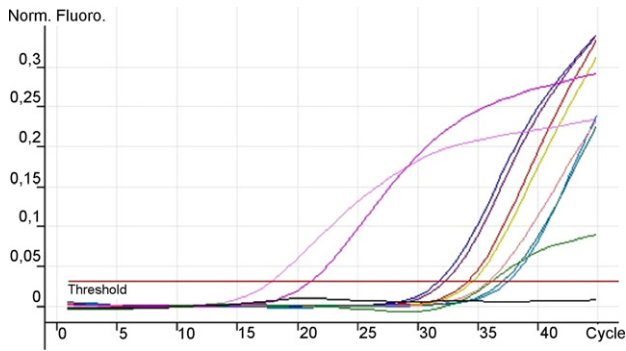


Fig. 1. Real-time PCR system (TaqMan) developed for the detection of hepatitis E virus. The first two curves are positive controls and the rest are clinical samples from pigs.

Invader technology, were developed for the rapid detection of viruses in the frame of our EU projects [21]. Some of the very recently developed assays target even viruses, which are important in zoonoses and in food safety, such as noroviruses and Hepatitis E virus ([22] and, Fig. 1).

### 2.5. Multiplex diagnostic PCR assays

In general, the multiplex PCR methods (using multiple primers to allow amplification of multiple templates within a single reaction) are useful for diagnostic purposes, providing the diagnostician the ability to detect more than one infectious agent(s) in a single assay. For example, we analyse a single nasal or rectal swab collected from an animal suffering from a respiratory disease, or from enteritis/diarrhoea syndrome, respectively. By performing multiplex PCR, we seek to diagnose all possible pathogens, which can be considered in this disease. The real-time PCR is very suitable for multiplexing, since the individual probes for the component assays can be labelled with different fluorophores, each of which functions as a specific colour reporter dye for one set of primers. Since the fluorescent probes emit at different colour wavelengths, it enables an easy multiplexing of the assays. Although the “classical” PCR techniques are also suitable for the development of multiplex systems, the use of “classical” nested PCR for the construction of multiplex assay would be rather complicated, considering the large number of primers required. These might “compete” with each other, as they have to be placed in the same reaction mix of the classical nested PCR. In general, the real-time PCR assays (using only single primer pairs) provide better possibilities for the construction of multiplex systems with multiple target components. Considering the diagnostic advantages of this principle, various multiplex PCR assays were developed at our laboratory, partly as own development or in collaboration with EU project partner laboratories (<http://www.multiplex-eu.org/> and <http://www.labonsite.com/>). For example, a multiplex (duplex) real-time PCR assay was developed and is used in routine diagnosis for the simultaneous detection of bovine respiratory syncytial virus and bovine respiratory coronavirus, two pathogens important in the respiratory disease

complexes of young calves (Fig. 2). We have found that multiplex real-time PCR has the potential to produce considerable savings in time and effort, without compromising the robustness and sensitivity of the virus detection assays [24].

### 2.6. A simple way of complex diagnosis: development of “multi PCR” assays

As mentioned above, “primer-competition” may occur during the construction of multiplex PCR systems. The development of multiplex PCR assays in such cases might be very difficult and time consuming. The levels of specificity and sensitivity may strongly drop. Our experience is that in such cases it is more advisable not to “force” the co-amplification in the same reaction vessel, but rather to amplify the various viruses side-by-side on a microplate. This system is also “multiplex”, regarding that the various viruses are simultaneously detected from a tested clinical sample. We term this approach “multi” PCR. By using automated systems, the multiplex and rapid detection of the various pathogens is achieved very rapidly not only in multiplex PCR, but also in multi PCR [4,5].

### 2.7. Application of the primer-probe energy transfer (PriProET) system for the rapid detection of foot-and-mouth disease virus (FMDV)

In frame of a recent EU project (<http://www.multiplex-eu.org/>) we developed a quantitative real-time PCR assay for the simultaneous detection of all the seven serotypes of FMDV. This method is based on the PriProET principle and is targeting the 3D gene of the virus. The real-time PCR assay was validated for the efficacy to detect all known FMDV serotypes. The test method was linear over a range of at least seven orders of magnitude and the detection limit was below the equivalent of 10 genomic copies of the virus. Analysing African clinical samples the method was able to detect FMDV in materials from both cattle and buffalo. A considerable diagnostic advantage is that the PriProET method provides a laboratory result much faster than virus cultivation. The rapid detection and diagnosis of a new foot-and-mouth-disease outbreak, including the determination of the serotype of the virus, is accomplished within several hours [23].

### 2.8. Development and comparison of various real-time PCR diagnostic assays to diagnose the viral vesicular complex of swine

Since FMDV, swine vesicular disease virus (SVDV) and vesicular stomatitis virus (VSV) cause very similar or clinically identical vesicular lesions in swine, it is a crucial requirement to develop methods for the rapid detection and identification of these three viruses, in order to assure the differential diagnosis. For this purpose, the above mentioned PriProET diagnostic PCR was completed by a range of other

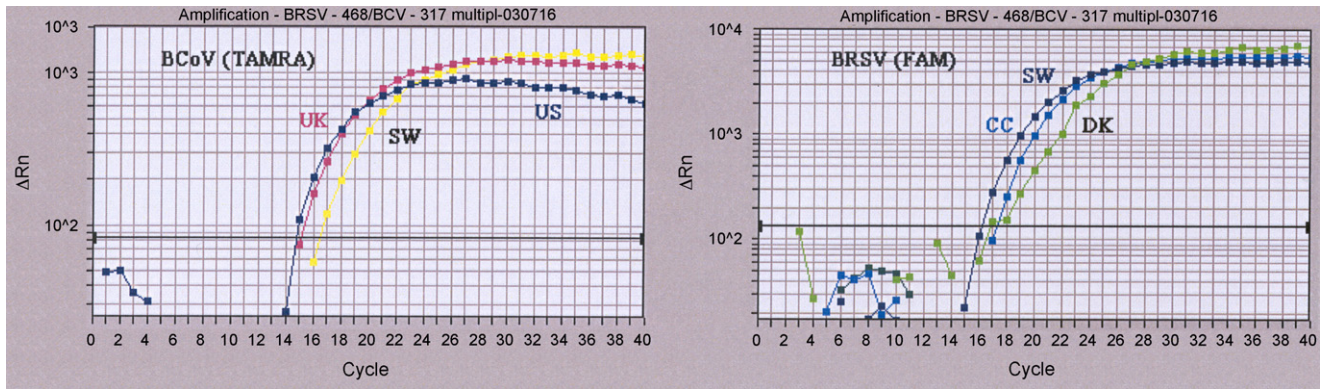


Fig. 2. Multiplex (duplex) real-time PCR for the simultaneous detection of bovine respiratory syncytial virus (BRSV) and bovine respiratory coronavirus (BCoV) in the respiratory disease complex of calves. BRSV strains SW (Sweden), DK (Denmark) and “cell culture” (CC) were detected by the FAM probe, while BCoV strains SW, UK and US were amplified by the TAMRA-probe, as shown by various colours.

real-time PCR assays, to detect all the three viruses. By comparing the detection range and applicability of the PriProET assays and molecular beacons we found the PriProET as a superior tool to detect various variants of SVDV. Results with different SVDV strains and especially those with mutations in the probe region demonstrated the robustness of the PriProET system compared to the other real-time PCR assays (molecular beacon, TaqMan) that usually require perfect probe match with a target of interest. With the PriProET system there is a chance to identify phylogenetically divergent strains of SVDV, which may appear as negative in other real-time PCR assays. Initially the primers and probe used in these experiments were intended for detection by molecular beacons. In spite of the theoretical fitness of the probe we discovered a reduced sensitivity using molecular beacon compared to PriProET. This discrepancy in the sensitivity is explained by the FRET system. For the PriProET system even a low efficient hybridising probe will bring the reporter fluorophore in proximity of the donor enabling release of reporter fluorescence. There is no competition with the stem-loop structure (as in molecular beacons) and there is no need for probe degradation to release fluorescence (as in TaqMan), which impairs detection of strains with mutations in the probe target region. In addition, the PriProET system gives a specific  $T_m$  for each of the strains, which can reveal mutations in the target. In conclusion, the observation that PriProET detects more SVDV strains than molecular beacons makes the former superior for analysis of unknown diagnostic specimens. The high sensitivity and specificity of the SVDV PriProET assay may improve the early and rapid detection of viral nucleic acids of a wide range of SVDV strains, allowing reduced turnaround time and use of high-throughput, automated technology [25].

### 2.9. The use of robots in molecular diagnosis

The use of nucleic acid extraction robots is further accelerating the diagnostic procedures worldwide. The labo-

ratories are using various types of the nucleic acid purifying robots. For example, the GenoVision M48 extraction robots (Biorobot M48 station, Qiagen, Norway), utilize magnetic separation of the target molecules. By comparing the results of nucleic acid preparations of the robot with manual procedures, we found the robot more efficient and reliable. In this robot the viral nucleic acids are purified simultaneously from 48 samples and the procedure is finished within 2.5 h. The products are clean enough to be amplified directly in the PCR. In addition to high speed, robustness and low labour-input, a further advantage of the robots is the reduced risk of cross-contamination between specimens. By the introduction of special tools laboratory practices and internal controls (mimics) it was possible to reduce the danger of false positivity and false negativity rather soon in the history of the diagnostic PCR, as it was previously reported and summarised [1,4,5]. The introduction of the closed and automated systems of the robots provides further insurance today for the safe diagnostic reliability of the PCR-based diagnostic assays. The simultaneous use of the nucleic acid extraction and pipetting robots with the real-time PCR machines provides an automated diagnostic chain. Such automated diagnostic chains have been established at our laboratory for the detection of several viruses. By this way high throughput and robust diagnostic assays have been established, with reduced manipulation requirement, less contamination risk and a very rapid diagnosis time, which is shortened from hours to minutes [5].

### 2.10. OIE rules for international standardisation and validation of the PCR-based diagnostic assays

The international validation and standardisation of the diagnostic assays is very important today. National and international authorities require rigorous proof that the assays, used in various laboratories, are as reliable as possible and give identical results. International agencies like the OIE, the Joint FAO/IAEA Division, national research institutions and commercial companies make large efforts to

agree on international standardisation. The OIE regularly publishes standards for the validation of diagnostic assays (see [http://www.oie.int/eng/publicat/en\\_standards.htm](http://www.oie.int/eng/publicat/en_standards.htm)). Validation and international standardisation of nucleic acid amplification-based diagnostic methods (like PCR) is the major task for these authorities. The usual practice is that the specificity and the sensitivity of the newly developed PCR assays are compared to conventional assays, like virus isolation. The “in house” PCR assays will soon have to be replaced by validated and standardised procedures. The validation, standardisation and quality control of PCR-based diagnostic techniques, which are now in progress, are a major task. Our laboratory, in collaboration with other international partners has been actively involved in the validation processes, by following the stages of assay validation as suggested by the OIE [1,4,5,26]. Considering these efforts and achievements, the OIE designated in 2005 our laboratory as “OIE Collaborating Centre for the Application of Polymerase Chain Reaction Methods for Diagnosis of Viral Diseases in Veterinary Medicine”. By acknowledging this important title, we put large efforts in development of molecular diagnostics and to continue the international standardisation and validation. Furthermore, we provide international training programmes in molecular diagnostics.

### 3. Further methods under development in molecular diagnostics, future trends

As summarised above, the various real-time PCR assays provide powerful novel means for the very rapid detection and quantitation of targeted viral nucleic acids in clinical specimens. The real-time PCR assays have opened a new area of molecular diagnosis. However, although it has many advantages, a vulnerable side of the PCR-based diagnostic assays is that the detection efficiency is decreased by the high nucleotide sequence variability (mismatches) in the genomes of the various variants of the targeted virus(es). The increasing numbers of mismatches between target and primer sequences result in decreased amplification or even in negative PCR results. The variability of the target nucleotide sequences might be associated with various phenomena, such as mutations, deletions or duplications in the genomes of the sought viruses. Similarly, weaker or negative PCR results may occur when trying to amplify the genomes of newly emerging genomic variants of a virus genus. The PCR analysis of emerging new viruses may yield negative results, due to the novel nucleotide sequences in these viral genomes. Considering these vulnerable sides of the PCR-based diagnostic assays, there is a high need to develop further approaches of molecular diagnosis. There is a strong tendency today to further increase the number of various methods, in order to strengthen the molecular diagnosis, to reduce the diagnosis time and to improve the diagnostic complexity. We list several examples here, including the development processes of our group and its partners.

#### 3.1. *Microarray based complex and simultaneous molecular detection of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses by using padlock probes*

The padlock probes, adapted to microarray formats, provide novel means of powerful and very complex novel molecular diagnosis. Padlock probes are circularizable oligonucleotides useful for highly multiplex genetic studies [27,28]. These probes have the capacity to detect simultaneously thousands of different target sequences in a single multiplex array system. Each target serves as template for a padlock probe equipped with a unique sequence (tag) associated to that specific target. Circularized probes are amplified with a single universal primer pair and the fluorescently labelled products are then sorted, using the tag sequences, on a microarray [27]. The application of padlock probes for detection of pathogens is a very recent trend in molecular diagnosis [29]. We developed a padlock probe package for the simultaneous and rapid detection of multiple viruses in the vesicular disease complex of swine, using a microarray-based read-out. The assay principle was straightforward comprising a few internally controlled reaction steps in a single vessel. Run-times were comparable to real-time PCR, but with the benefit that the presence of several viruses and their various serotypes can be analyzed within the same reaction (Fig. 3a and b). In conclusion, our multiplex detection system could have immediate implications in more effective screening for viruses causing similar vesicular symptoms in the swine populations [30].

#### 3.2. *Broadly targeted triplex real-time PCR detection of influenza A, B and C viruses based on the amplification of genomic sequences from the nucleoprotein gene*

A simple multiplex real time PCR system was designed recently for the general and simultaneous detection of influenza A–C viruses, i.e., many members of the *Orthomyxoviridae* family, originating from animals and humans [31]. The assay is performed in a single tube, using broadly targeted primers and probes. The “ConSort”<sup>®</sup> computer program (developed by J. Blomberg, Uppsala University) indicated conserved regions in the nucleoprotein genes, based on comparative sequence analysis of several thousands of influenza virus genome sequences. These regions were used to design a triplex reverse transcription real-time PCR (“3QPCR”). It was evaluated with eight influenza A reference strains (H5N1<sup>Scotland</sup>, H5N1<sup>Turkey</sup>, H5N2, H7N1, H7N7, H9N2, H1N1 and H3N2), serial dilutions of influenza A–C, and synthetic DNA targets. The real time PCR assays were able to detect 1–10 viral RNA copies per PCR assay for influenza A–C, when testing virus isolates and clinical specimens. Our study shows that 3QPCR is a reliable, sensitive, and rational method for detecting and identification of influenza viruses. Its generic nature should enable the detection not only of most human influenza viruses, but also of avian and other

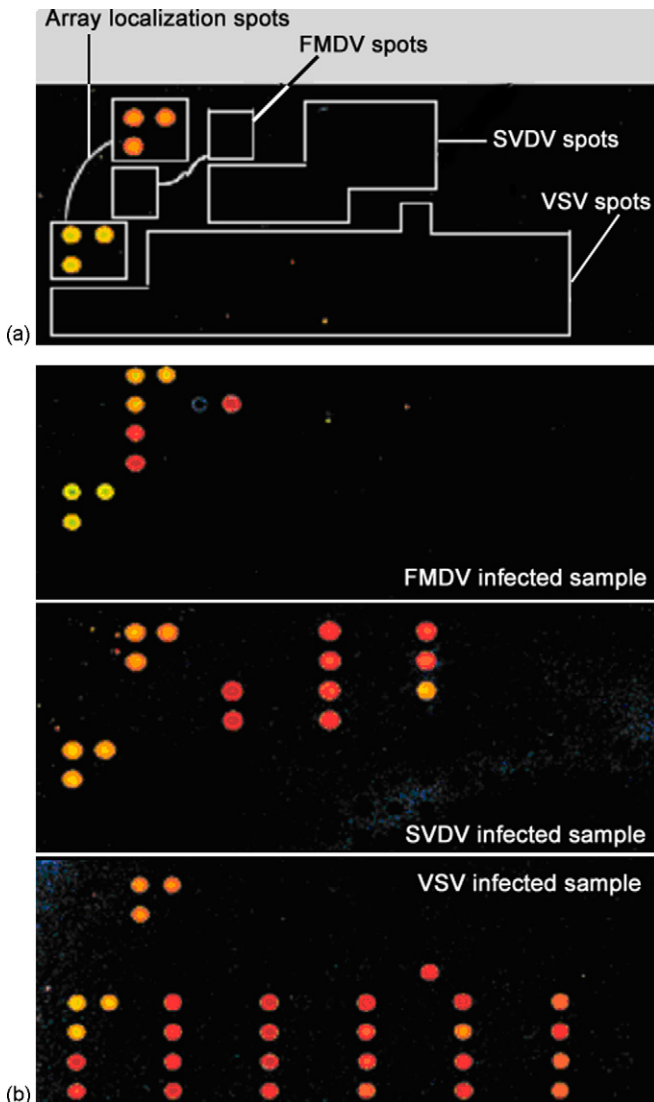


Fig. 3. (a) The arrangement and localisation of the spots of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses in the microarray system. (b) Reading the signals on the microarray system, indicating positive spots for foot-and mouth disease, vesicular stomatitis and swine vesicular disease viruses, respectively.

influenza strains. This wide-range assay provides a powerful novel detection tool for diagnostic laboratories. The broad detection range ensures that the 3QPCR provides powerful means for the discovery of newly emerging variants of influenza viruses [31].

### 3.3. Very rapid PCR and nucleotide sequencing analysis – diagnosis of influenza virus infections within hours (minutes)

We developed a one-step real-time PCR assay, based on the amplification of genomic sequences from the HA gene, for the rapid and simultaneous detection of a broad spectrum of influenza viruses, including highly pathogenic avian influenza viruses. A prototype of real-time PCR system,



Fig. 4. QuanTyper instrument for performing ultra rapid PCR and sequencing (Alpha Helix). Features of the instrument: (a) high speed and sensitivity: 100  $\mu$ L real time Q-PCR in <11 min; (b) cycle sequencing reaction: 500 bases in <15 min; (c) high convenience in combination with Capillette. Sample volume ranges 20–200  $\mu$ L.

which uses the superconvection principle (“Superconvection QPCR”; AlphaHelix, Uppsala, Sweden), was used both for amplification and for cycle sequencing reactions (Fig. 4). Identification of pathogenicity of AIV (HPAI or LPAI) including sequence information of HA gene was obtained in less than 2 h: RNA purification – 30 min, superconvection QPCR – 30 min, cycle sequencing reactions with superconvection – 20 min and sequencing by capillary electrophoresis (up to 700 b) – 20 min. The wide screening of different subtypes of avian influenza in a single QPCR, followed by rapid sequencing that covers cleavage site of HA gene, will allow monitoring the viral load of influenza strains in wild birds and in farmed poultry. Furthermore, the method could provide a very rapid and highly reliable molecular diagnosis in a possible pandemic influenza A scenario [32].

### 3.4. Proximity ligation: novel means of protein detection by nucleic acid amplification

We participate in the work of a joint research group, which is developing proximity ligation reactions to detect proteins of bacteria and of virus particles via nucleic acid amplification. The principle is that antibodies recognizing viral or bacterial surface proteins are coupled to DNA strands that could be joined by ligation when several antibodies were bound in proximity to surface proteins of individual infectious agents. This method enables the detection of target proteins by ligated DNA strands, which are then amplified by real-time PCR. Our partner laboratories (Olink and SVANOVA, Uppsala) reported that detection sensitivities similar to those of nucleic acid-based detection reactions were achieved directly in infected samples for a parvovirus and an intracellular bacterium (M. Merza, personal communication). Compared to AgELISA, the sensitivity of proximity



ligation proved to be higher. Thus, proximity ligation of proteins can be of great value for early diagnosis of infectious disease and in biodefense [33]. At present, we are adapting the proximity ligation technique to the detection of surface antigens of various viruses causing TADs, such as FMDV and CSFV. The combination of nucleic acid and antigen detection approaches will hopefully yield a more complex, multilateral diagnosis of TADs.

In summary, the goal of this article was to illustrate some of the recent achievements in the development of molecular diagnosis, with special regard to the achievements and experiences gained at our institute and at our partner laboratories. As it is shown by the examples, the illustrated methods provide novel means for detecting and identifying the viruses and for combating the TADs and other viral diseases in a more effective way.

### Acknowledgements

Thanks are due to all my colleagues at the National Veterinary Institute (SVA), the Swedish University of Agricultural Sciences (SLU), Uppsala University (UU) and at AlphaHelix AB in Uppsala. The scientific input of the collaborating partners in the LAB-ON-SITE EU project (as partly listed in the references) is highly appreciated. Mikhail Hakhverdyan, Jonas Blomberg, Peter Thorén, Johan Banér, Péter Gyarmati, Frederik Widén and Alia Yacoub are specially thanked for their important contribution. This work was supported by EU project grants QLK2-CT-2000-00486-MULTIPLEX PCR, SSPE-CT-2004-513645-LAB-ON-SITE, and PATHOGEN COMBAT, as well as by grants provided by Formas, Sida/Sarec, SLF and by SVA.

### References

- [1] Belák S, Thorén P. Molecular diagnosis of animal diseases. *Expert Rev Mol Diag* 2001;1:434–44.
- [2] Belák S, Ballagi-Pordány A, Flensburg J, Virtanen A. Detection of pseudorabies virus DNA sequences by the polymerase chain reaction. *Arch Virol* 1989;108:279–86.
- [3] Ballagi-Pordány A, Klingeborn B, Flensburg J, Belák S. Equine herpesvirus type 1: detection of viral DNA sequences in aborted fetuses with the polymerase chain reaction. *Vet Microbiol* 1990;22:373–81.
- [4] Belák S. The molecular diagnosis of porcine viral diseases. *Acta Vet Hung* 2005;53:113–24.
- [5] Belák S, Hakhverdyan M. Recent achievements and trends in the molecular diagnosis of bovine viral diseases—a view from the OIE Collaborating Centre for the Application of Polymerase Chain Reaction Methods for the Diagnosis of Viral Diseases in Veterinary Medicine. *Dtsch Tierärztl Wochenschr* 2006;113:129–33.
- [6] Vilcek S, Belák S. Genetic identification of pestivirus strain Frijters, isolated from pigs, as a border disease virus. *J Virol Meth* 1996;60:103–8.
- [7] Vilcek S, Stadejek T, Ballagi-Pordány A, Lowings JP, Paton DJ, Belák S. Genetic variability of classical swine fever virus. *Virus Res* 1996;43:137–47.
- [8] Vilcek S, Nettleton P, Paton D, Belák S. Molecular characterization of ovine pestiviruses. *J Gen Virol* 1997;78:725–35.
- [9] Vilcek S, Belák S. Organization and diversity of the 3′-noncoding region of classical swine fever virus genome. *Virus Genes* 1997;15:181–6.
- [10] Stadejek T, Vilcek S, Lowings P, Ballagi-Pordány A, Paton D, Belák S. Genetic heterogeneity of classical swine fever virus in Europe. *Virus Res* 1998;52:195–204.
- [11] Vilcek S, Paton D, Lowings P, Björklund H, Nettleton P, Belák S. Genetic analysis of pestiviruses at the 3′ end of the genome. *Virus Genes* 1999;18:107–14.
- [12] Vilcek S, Alenius S, Paton D, Mittelholzer C, Belák S. Genetic clustering of bovine viral diarrhoea viruses in cattle farms: genetic identification and analysis of viruses directly from cattle sera. *Vet J* 1999;158:33–8.
- [13] Ståhl K, Kampa J, Baule C, Isaksson M, Moreno-Lopez J, Belák S, et al. Molecular epidemiology of bovine viral diarrhoea during the final phase of the Swedish BVD-eradication programme. *Prev Vet Med* 2005;72:103–8.
- [14] Lindberg A, Niskanen R, Gustafsson H, Bengtsson B, Baule C, Belák S, et al. Prenatal diagnosis of persistent bovine viral diarrhoea virus (BVDV) infection by detection of viral RNA in fetal fluids. *Vet J* 2002;164:151–5.
- [15] Bálint A, Baule C, Pálfi V, Belák S. Retrospective genome analysis of a live vaccine strain of bovine viral diarrhoea virus. *Vet Res* 2005;36:89–99.
- [16] Björklund H, Lowings P, Stadejek T, Vilcek S, Greiser-Wilke I, Paton D, et al. Phylogenetic comparison and molecular epidemiology of classical swine fever virus. *Virus Genes* 1999;19:189–95.
- [17] Farsang A, Ros C, Renström L, Baule C, Soós T, Belák S. Molecular epidemiology of infectious bronchitis virus in Sweden, indicating involvement of a vaccine strain. *Avian Pathol* 2002;31:229–36.
- [18] Stadejek T, Stankevicius A, Storgaard T, Oleksiewicz MB, Belák S, Drew TW, et al. Identification of radically different variants of porcine reproductive and respiratory syndrome virus (PRRSV) in Eastern Europe: towards a common ancestor for European and American viruses. *J Gen Virol* 2002;83:1861–73.
- [19] Escutenaire S, Mohamed N, Isaksson M, Thorén P, Klingeborn B, Belák S, et al. SYBR green real-time reverse transcription-polymerase chain reaction method for the generic detection of coronaviruses. *Arch Virol* 2007;52:41–58.
- [20] Hakhverdyan M, Hagglund S, Larsen LE, Belák S. Evaluation of a single-tube fluorogenic RT-PCR assay for detection of bovine respiratory syncytial virus in clinical samples. *J Virol Meth* 2005;123:195–202.
- [21] Hjertner B, Meehan B, McKillen J, McNeilly F, Belák S. Development of invader squared assay for the detection of African swine fever DNA and comparison to PCR based assays. *J Virol Meth* 2005;124:1–10.
- [22] Mohamed N, Belák S, Hedlund KO, Blomberg J. Development of a rational diagnostic single tube real time PCR for human caliciviruses, *Norovirus* genogroup I and II. *J Virol Meth* 2006;132:69–76.
- [23] Rasmussen T, Uttenthal Å, de Stricker K, Belák S, Storgaard T. Development of a novel quantitative real-time PCR assay for the simultaneous detection of all serotypes of foot-and-mouth disease virus. *Arch Virol* 2003;148:2005–21.
- [24] Agüero M, Fernández J, Romero LJ, Zamora J, Sánchez, Belák S, et al. A highly sensitive and specific gel-based multiplex PCR assay for the simultaneous and differential diagnosis of African swine fever and classical swine fever in clinical samples. *Vet Res* 2004;35:551–63.
- [25] Hakhverdyan M, Rasmussen T, Thorén P, Uttenthal Å, Belák S. Development of a real-time PCR assay based on primer-probe energy transfer for the detection of swine vesicular disease virus. *Arch Virol* 2006;151:2365–76.
- [26] Belák S, Thorén P. Validation and quality control of polymerase chain methods used for the diagnosis of infectious diseases. Office International des Epizooties (OIE), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). Fifth Edition 2004; Chapter I.1.4: 30–6.

- [27] Banér J, Isaksson A, Waldenstrom E, Jarvius J, Landegren U, Nilsson M. Parallel gene analysis with allele-specific padlock probes and tag microarrays. *Nucleic Acids Res* 2003;31(17):e103.
- [28] Hardenbol P, Banér J, Jain M, Nilsson M, Namsaraev EA, Karlin-Neumann GA, et al. Multiplexed genotyping with sequence-tagged molecular inversion probes. *Nat Biotechnol* 2003;21(6):673–8.
- [29] Szemes M, Bonants P, de Weerd M, Banér J, Landegren U, Schoen CD. Diagnostic application of padlock probes—multiplex detection of plant pathogens using universal microarrays. *Nucleic Acids Res* 2005;33(8):e70.
- [30] Banér, J, Gyarmati, P, Yacoub, A, Hakhverdyan, M, Stenberg, J, Ericsson, et al. Microarray-based molecular detection of foot-and-mouth disease virus, vesicular stomatitis virus and swine vesicular disease virus, using padlock probes. Submitted for publication.
- [31] Mohamed N, Escutenaire S, Belák S, Czifra G, Herrmann B, Berencsi G, et al. Broadly targeted triplex real-time PCR detection of influenza A, B and C viruses based on the nucleoprotein gene. Submitted for publication.
- [32] Yacoub A, Hakhverdyan M, Thorén P, Mohamed N, Gyarmati P, Belák S, et al. Very rapid detection of highly pathogenic avian influenza viruses. Submitted for publication.
- [33] Gustafsdottir SM, Nordengrahn A, Fredriksson S, Wallgren P, Rivera E, Schallmeiner E, et al. Detection of individual microbial pathogens by proximity ligation. *Clin Chem* 2006;52(6):1152–60.