Implementation of molecular detection techniques in veterinary virology

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Introduction

This article summarizes the research work of a recent PhD programme, which was a part of the international PhD training activities of the Veterinary Faculty of the SLU, the Collaborating Centre of the OIE. This PhD programme was focusing on the development and implementation of novel biotechnology-based techniques for the improved molecular diagnosis of infectious diseases of animals and man. In particular, it addressed diseases notifiable to the OIE, like the vesicular diseases complex and avian influenza, as well as Hepatitis E, an emerging zoonosis. With the worldwide introduction and use of polymerase chain reaction (PCR) methodologies, the detection of pathogens improved significantly - however, these systems have their weak points. Thus the simultaneous screening of multiple pathogens has not been satisfactorily solved, and the multiplexing capacity of most variants of the method is in general insufficient. The PhD programme did not intend to review the entire spectrum of molecular diagnostics or multiplex DNA detection; it was rather intended to highlight a specific area. The entire text can be downloaded from http://diss-epsilon.slu.se/archive/00001885/. Herewith, the results of this successful PhD programme are summarised by the new PhD holder (PG) and by the main supervisor (SB), in order to provide a brief review on this international training programme, focusing on new trends in molecular diagnostic virology, with special regards to the improved detection of zoonotic pathogens and/or viruses in food safety.

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 the long run, gives information about the epidemiological behaviour of the microbe. 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 many are already used for routine diagnosis [5]. The following section will list the methods relevant for this article.

DNA and RNA detection

In recent years, there has been considerable development of techniques, which allow 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 a more stable conformation than an imperfect match, which means that under ideal reaction conditions the perfectly matched oligonucleotides (oligos) will more often be found complexed with their targets than the ones with mismatches. In molecular diagnostics, the major determinants of the methods are their specificity and sensitivity. The specificity is a statistical measure in a binary classification that shows how good the system is to correctly identify and distinguish the negative cases, or cases that do not meet the given criteria. The sensitivity is defined as the minimum input signal required to produce a specified output signal (at a defined signal-to-noise ratio). In a biological context, the specificity shows the ability of a method to identify the target sequence in the abundance of other sequences, e.g. to identify 20-40 nucleotide (nt) long targets on a 13 kb influenza virus genome. The sensitivity, on the other hand, determines the lower limit of detection, the minimum number of target pathogen sequences necessary to obtain reproducible results.

Other important aspects of a method are its reliability (the quality of the measurement), reproducibility (consistent results if the assay is repeated; accurate reproduction of the experiment by other laboratories), scalability (the capability of the system to handle increasing sample throughput), and the throughput itself (the minimum/maximum number of samples that can be tested at the same time).

Other parameters are the cost (price for the customer), flexibility (rapid adaptation to emerging pathogens, and to different instruments) and the possibility of automation (using electronic controlling).

The Polymerase chain reaction (PCR)

The PCR is widely used in molecular biology because of its high sensitivity, specificity and user-friendly nature. The idea to replicate short DNA fragments using nucleotide primers in vitro was first published by Kjell Kleppe [22], but the invention of the complete assay is credited to Kary Mullis [31] [36]. Thanks to the input from other disciplines (enzymology, oligonucleotide synthesis, electromagnetic (dealing with different fluorophores) studies etc.), there is a large variety of PCR technologies available nowadays [16] [29]. In general terms, 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 oligos (primers), following the principle of Watson-Crick base pairing. It is a cycling reaction, where each cycle contains phases of denaturation, annealing and extension that result in an exponential amplification of the target sequence, producing vast amounts of DNA [16]. To identify the PCR product according to the length of the amplicon, gel electrophoresis with ethidium bromide (or other intercalating agent) staining is used. As the reaction progresses, the used primers and dNTPs will be built into the newly synthesized DNA strands, which will compete with the primers in the later stages of the reaction, finally reaching a plateau, and the amplification ceases or continues with low efficiency. If the available chemicals are used up, amplification also stops [20].

Although the sensitivity and specificity of the PCR are high, its multiplexing capability is limited: though more primer pairs can be used in a single reaction - to result in the simultaneous amplification of several target sequences - the accumulation of by-products (and consequently, the risk of false results) is then much higher [24]. Multiplex PCR systems usually have a lower sensitivity, and development of the assay needs longer optimization. The different length and melting temperatures of the primers, the tendency of the oligos to form secondary structures, the salt concentration used in the reaction all affect the efficiency.

Real-time PCR

To identify the amplified products, gel electrophoresis was used, but it proved to be time-consuming and cumbersome, with a high risk of contamination and carryover and consequently of false-positive results. In view of these weaknesses, applications were developed to monitor the amplification in real time. By the improvement of fluorescent probe chemistry, various real-time PCR methodologies were developed. They proved to be very useful because of increased speed, the possibility of obtaining quantitative measurements, the wide dynamic range and the reduced risk for false positive results caused by contamination [7] [29]. Apart from the TaqMan and PriProET variants (described in details below), several real-time PCR chemistries exist [5] [8]. The multiplexing capability of the real-time PCR assays suffers from the same problem as other PCR applications: fluorophores with different excitation/emission wavelengths can be used in one reaction, but the accumulation of undesired products and the overlapping fluorescent spectra can decrease the sensitivity and extend the optimization time.

TaqMan PCR

The TaqMan format is one of the most popular PCR methods, where a dual-labeled fluorogenic probe and non-labeled primers are used. 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 as DNA synthesis commences, the polymerase degrades the annealed proportion of the TaqMan probe. When quencher and reporter molecules are no longer in close proximity, the fluorophore emits the fluorescent signal. Hence, the detected fluorescence is proportional to the amount of DNA present in the reaction [16].

Primer Probe Energy Transfer (PriProET)

The PriProET PCR is a fluorescence resonance energy transfer (FRET)-based application. It uses 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, excited by light, reaches a higher energy level. In its excited state, the donor molecule emits a photon that can be accepted by a nearby receiver molecule (if the distance between the two molecules are ≤10 nm), causing the acceptor to emit light, which is measurable by a CCD camera connected to a PCR instrument. The fluorescent signal is proportional

to the amount of DNA present in the reaction [35].

DNA microarrays

DNA microarrays are high-throughput devices using the principle of base pairing during hybridization. The concept of microarrays was developed in the 1980s, with the objective to direct the hybridization on a solid surface, like the Southern blot, dot blot, reverse dot blot or comparative genomic hybridization (CGH) - and to solve their limited resolution. The different types of arrays can be grouped according to the way of fixation, their synthesis and the type of oligos used. With respect to depositing the oligos, two major methods are distinguished. There are the spotting techniques, which may use ink-jet and piezoarray technology, where the pre-synthesized probes are placed onto the solid surface by a robotic printer (either by direct contact or ejection from a small distance), and there are techniques where oligos are synthesized in situ, directly onto the solid surface. This group can be further subdivided according to the technology used: the traditional phosphoramidite chemistry [4] or the photolithography [14] [27] – both are cycled reactions, where the specific oligos are bound with the help of protective groups. A phosphoramidite is a normal nucleotide with blocked reactive amine, hydroxyl and phosphate groups: during the synthesis, these protective groups are sequentially removed by chemicals to make the addition of a new nucleotide possible. The photolithographic technique uses photolabile reagents to block the reactive parts of the nucleotides and applies light for their removal.

According to the types of oligos deposited on the surface, two groups are distinguished: printed cDNAs/PCR-products (the length can be extended to a few thousand base pairs) or printed, synthesized oligos, which are generally <70 base pairs. Higher specificity was reported for the latter [38]. Coating of the array surface depends upon the binding moiety: silane for glass surfaces, thiols for gold surfaces and polyelectrolytes for glass and dielectric materials [39].

The DNA segments to be examined are usually labeled with fluorophores and hybridized to the immobilized probes (also called microarray tags: oligos with characterized sequences in 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 arbitrary fluorescent units [38]. Because of the high density of the spots on the support, cross-hybridization may occur, which is minimized by careful probe design, optimized hybridization time, temperature and salt concentration. Although equipment necessary for constructing and reading microarrays is still uncommon in diagnostic laboratories, similar methodologies are becoming widely accepted [43] [44].

Ligation-based techniques

Ligase-based detection techniques are built upon the high fidelity of DNA ligases, when differentiating between a perfect match and mismatches. Ligases are enzymes that catalyze 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 is a very specific reaction; it requires perfectly matched DNA fragments directly next to each other on a template molecule. Ligases can be grouped according to their cofactors that can be either ATP or NAD^+: the eukaryotic, archaebacterial and viral ligases all require ATP as cofactor, whereas the eubacterial ligases require NAD^+ [9] [10] [17]. Ligases have been isolated from thermophilic bacteria (*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 [47]. At the end of the 1980s, the ligation reaction was a common method used for genetic analyses [18] [19] [45]. The oligonucleotide ligation assay (OLA) and the ligase chain reaction (LCR) were developed as diagnostic applications. Due to their high precision, both methodologies were used for the detection of single nucleotide polymorphisms, predominantly in human genetics. The OLA may need further amplification, where the ligation serves for differentiation; the LCR is an amplification reaction in itself, as the ligated pairs of probes act as templates for further ligation events [3] [23].

Padlock probes

A variant of the OLA technique uses so called padlock probes (PLPs), where an oligonucleotide with target-complementary ends is circularized by ligation, in the presence of the appropriate target. The

ligation event serves for differentiation, because the unreacted probes remain linear. PLPs contain – apart from the target complementary ends that are unique in each probe – two common primer regions that allow amplification of the PLPs, and microarray tags unique for each probe are used to identify the positive probes on a solid surface [32]. Due to the design of the probes, only their circularized forms are amplified, the non-reacted (linear) probes cannot serve as a template for polymerization [2] [32]. The padlock probes are generally oligos 80-110 bp in length and are usually synthesized using phosphoramidite chemistry, which may cause impurities at that size. The photolithographic synthesis – production of oligos on a solid surface using photomasks and photolabile protection groups - is a viable, but more costly alternative. Probes can also be synthesized enzymatically, or shorter fragments can be ligated to form the PLPs [2].

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 a lower detection limit – needs to be increased. Since circles are formed in positive PLP reactions, the rolling circle amplification (RCA), which synthesizes multiple copies of circular molecules but leaves the linear ones untouched, fits perfectly into the padlock probe system [1]. The RCA mechanism is used by several viruses and bacteriophages to replicate their genetic material [6] [15] and was introduced into biotechnology by Fire [13]. Polymerization is initiated from the hybridized primer end and proceeds continuously, because – unlike the PCR – it is not self-inhibited; its final product is a long, single-stranded concatemer molecule. The RCA has a linear dynamic, but by introduction of a second primer complementary to the RCA product, the resulting amplification is close to exponential (termed HRCA – hyperbranched RCA; [28].

Multiplex ligation-dependent probe amplification (MLPA)

The MLPA, another variant of the OLA assay, uses two probes per target, both containing 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 belonging to the same target are designed to bind next to each other, in the presence of the target DNA. During ligation, the two probes are joined in the presence of the target or remain unreacted in its absence. Successful ligation results in PCR-amplifiable products. Between the primer and target complementary sites, there are spacer oligos that allow the separation of the products due to their unique length [37]. The differentiation can be done by gel electrophoresis (either by high concentration agarose or polyacrylamide gels – the latter allows the use of automated sequencers). The assay permits multiplexing of up to 40 targets [37] without specific equipment, which exceeds the multiplexing capacity of the PCR variants but lags far behind the microarray-based techniques. Compared to the padlock probe methodology, the MLPA has the advantage that shorter oligos (which can be more precisely synthesized) are used in the reaction but the drawback of two independent hybridization events. These are necessary to ensure the ligation of the corresponding probes - in contrast, the padlock probes are unimolecular and the two target-complementary ends in close proximity of each other.

Further reading

Rather than giving experimental details for the above-mentioned methodologies, we quote the abstracts of several articles from our group, which show the power of molecular detection techniques:

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.: Microarray-based molecular detection of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses, using padlock probes.

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”. 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 [12], 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.

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

This paper describes a clinical utilization of padlock probes. The determination of all different subtypes of avian influenza viruses (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 hemagglutinin and 9 neuraminidase subtypes. In order to ensure a safe diagnosis - despite of the high variability of the influenza genome -, the surface antigens were targeted in more than one position. 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.

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

This paper describes diagnostic methods, which allow the detection of all recently known variants of hepatitis E virus. 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 [35].

Gyarmati P., Belák S. & Widén F., 2008. Genotyping of hepatitis E virus using ligation-dependent probe amplification (Manuscript, available upon request)

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 [33]. 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 [46].

Outlook

The techniques discussed in this review enable precise and rapid data acquisition about the presence and quantitation of different viral pathogens. However, this is a snapshot of the present state of the art - the development of molecular diagnostics is a never-ending process until the one-and-only perfect method does not exist. Considering this dilemma, a number of methodologies can be envisioned and developed. Padlock probes combined with the microarray technique offer a great range of multiplexing and scalability options in diagnostics [41] - but compared to the PCR, they are weaker in sensitivity and dynamic range of the assay. The PCR is of a high sensitivity, but the simultaneous detection of several pathogens cannot be performed in one reaction. There are efforts to combine the advantages of these two methodologies, either by initiating polymerization on the microarray surface on locally ligated padlock probes [11], or by porting this platform to liquid-phase amplification and/or read-out systems that would allow different kinetics [25] [42].

Following developments in related areas, the microarray technique is becoming more and more advanced, with higher spot density and precision. Oligonucleotide synthesis is now performed on different platforms, even for long molecules, avoiding contamination by incomplete oligos, which cause constantly high backgrounds. Real-time PCR machines and microarray scanners offer a wide range of detectable fluorescent signals. Large-scale sequencers are entering diagnostic fields and offer unique possibilities to identify and classify pathogens by their entire genomic contents [30]. Using random amplification and microarray detection strategies, pan-virus methodologies emerge that allow getting an overview of the pathogen pool contained in a sample [34].

Molecular diagnostic applications can be carried even further by exploiting synergies between different fields, like the HPLC-detection of pathogens, nucleotide identification and/or measurements by electro-chemical detection schemes [21] [26], and by using nanobeads that detect the Brownian relaxation frequency [40]. A multidisciplinary approach may open up new dimensions for clinical

diagnostics. Efforts should be made to transfer the newly developed methods from the research community to the diagnostic laboratories and to make them suitable for routine operation.

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