EXPERT REVIEWS

# Advances in viral disease diagnostic and molecular epidemiological technologies

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The early and rapid detection and characterization of specific nucleic acids of medico–veterinary pathogens have proven invaluable for diagnostic purposes. The integration of amplification and signal detection systems, including online real-time devices, have increased speed and sensitivity and greatly facilitated the quantification of target nucleic acids. They have also allowed for sequence characterization using melting or hybridization curves. The newer-generation molecular diagnostic technologies offer, hitherto, unparalleled detection and discrimination methodologies, which are vital for the positive detection and identification of pathogenic agents, as well as the effects of the pathogens on the production of antibodies. The development phase of the novel technologies entails a thorough understanding of accurate diagnosis and discrimination of present and emerging diseases. The development of novel technologies can only be successful if they are transferred and used in the field with a sustainable quality-assured application to allow for the optimal detection and effective control of diseases. The aim of these new tools is to detect the presence of a pathogen agent before the onset of disease. This manuscript focuses mainly on the experiences of two World Organisation for Animal Health collaborating centers in context to molecular diagnosis and molecular epidemiology of transboundary and endemic animal diseases of viral origin, food safety and zoonoses.

**Keywords:** amplification chemistry • detection chemistry • early and rapid diagnosis • label • microarray • molecular epidemiology • PCR • phylogeny • probe • transboundary animal disease • viral disease • viral metagenomic

Secure and safe food, an increasing human population, an increase of human and animal movement, intensification of animal husbandry, globalizing trade of live animals and/or animal products, bedding and feeds, are all factors that are leading to the increasing threat of emerging and re-emerging animal diseases (including unknown diseases) and those that affect humans. Food security has a high public priority, and the biggest threat is seen as the outbreak of a pandemic event. Despite our best efforts with regards to intensive disease control and progressive vaccination programmes, the implementation of early-warning tools and systems, the use of diagnostic programs and tools, the utilization of marker and tracer vaccines [1,2], the unpopular employment of stamping-out policies and the regulation of animal transfer or movements, animal diseases still have a very high impact on animal health and human welfare. Globalization and the opening of borders between countries (e.g., the European continent) have contributed to new and challenging high-risk situations. The recent worldwide incidences of foot-and-mouth disease, the extension of bluetongue in the European region and Rift Valley fever into the Arabian Peninsula, as well as the recent reoccurrence and rapid spread of influenza viruses, demonstrates the serious economic and social impact of highly contagious transboundary animal diseases (TADs).

Global climatic changes or variations are predicted to have a direct effect on the occurrence, emergence and spread of viral diseases, especially those that are transmitted by insect vectors. This predicted trend is clearly illustrated by the northward spread of bluetongue virus [3] and African horse sickness virus in Europe – regions of the world where these diseases have previously not been reported. This phenomenon is a consequence of insect vectors (*Culicoides*) expanding into new territories that were previously unsuitable for their lifecycles. Other vector-borne viral diseases, such as African swine fever, also show

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a similar tendency of expansion to new, previously noninfected territories. In addition to TADs, there is a range of other animal diseases with a 'more restricted' or endemic geographic character, but with an equally high economic and socioeconomic impact. These disease pathogens (e.g., bovine herpesviruses, adenoviruses, bovine viral diarrhea virus [BVDV], bovine respiratory syncitial virus and bovine coronaviruses) are frequent in cattle populations throughout the world, and their elimination or eradication are important ongoing activities [2,4,5]. Similarly, the swine populations of the world suffer from a wide variety of viral diseases – classical swine fever, African swine fever, Aujeszky's disease, foot-and-mouth disease and swine vesicular disease. For example, classical swine fever has been eradicated from domestic pig herds in the EU but wild boar populations are still infected in several EU countries, posing a permanent danger of reinfection for the whole continent; in addition, the spreading of African swine fever is complicated by the fact that there is no competent vaccine available and that new variants of the virus have emerged in Africa, while in Sardinia, there is an endemic form of the disease.

Porcine respiratory and reproductive syndrome is now a commonly occurring disease, caused by an emerging arterivirus, which was first detected in 1991 in The Netherlands and has since rapidly spread throughout the world. There are other diseases of growing importance, such as postweaning multisystemic wasting syndrome or porcine dermatitis and nephropathy syndrome, that are associated with porcine cirvovirus type 2; however, several questions are still unanswered in etiology, pathogenesis and many other important factors of these swine diseases.

In addition to the aforementioned examples given for livestock, there is a long list of emerging or re-emerging diseases in various host species, including humans, such as the diseases caused by hantaviruses, Japanese encephalitis virus, HIV, dengue viruses, menangle virus, Australian bat lyssavirus, ebola virus, avian influenza virus (AIV) variants, such as H5 and H7, severe acute respiratory syndrome, coronavirus, nipah virus and hendra virus, amongst others, which have recently caused concern. Several of these diseases have serious zoonotic concerns and global implications. This list clearly indicates that there is a need for the following:

- Early and confirmatory detection of harmful pathogens;
- Improved diagnostic technologies for both human and animal populations;
- Harmonization and validation of such technologies.

At this point, it is important to note that there exist many competent diagnostic assays that are fit for their intended purpose but might, in varying degrees, need to be validated and harmonized.

To facilitate the timely diagnosis and effective control of TADs and those of zoonotic nature, scientists and private enterprises have embarked on a research-and-development path in search of molecular tools that will be able to satisfy the need for the early and rapid detection of harmful pathogens. Critical technologic advances for the rapid detection and diagnosis of high-threat diseases are becoming increasingly available in research and industrial laboratories. Much emphasis has been placed on the development of amplification platforms, such as PCR; therefore, the biased focus of this article is toward thermal amplification platforms and their associated amplification and detection chemistries. However, these technologies have not yet been fully exploited and developed into applied tools. Furthermore, efficient, affordable and reliable devices that combine these amplification technologies with sample processing through analysis and reporting processes are needed for early warnings against many important livestock diseases. This article aims at providing an overview of some of these technological advances.

## Molecular diagnosis of viral diseases

The development of diagnostic and monitoring tools are experiencing an unprecedented growth phase. This is partly owing to the increased demand for secure and safe food and on the need by countries to detect and control plant and animal pests and diseases as part of their public obligation. The following sections will concentrate on the molecular diagnostic and characterization technologies that are showing promise.

### *Gel-based & real-time PCR platforms*

A wide range of PCR [6] and real-time PCR assays [7] have been developed since the late 1980s and applied to the diagnosis of TADs and other infectious diseases, as well as for the improved detection of pathogens in human food and animal feed. Real-time PCR has truly changed the molecular diagnostic scene and must be considered as one of the more important developments during the last few years. The various real-time PCR platforms and their respective chemistries, such as Taqman®, molecular beacons, primer probe energy transfer (PriProET), scorpion primers, dual probe systems, such as those utilized in the LightCycler® (Roche, NJ, USA), dye-labeled oligonucleotide ligation and SYBR Green, have showed high sensitivity and specificity in the detection of viral and bacterial pathogens [6–12]. Compared with the 'classical' single, nested or ELISA PCR platforms, real-time PCR assays offer a number of important, well-known advantages:

- Faster and higher throughput assays;
- One-step amplification set-up that provides sensitivity and specificity close or equal to traditional nested PCR;
- Detection of amplified products measured online and in real-time through the lid, side or bottom of the reaction vessel without opening the system, thus minimizing the risk of contaminating the laboratory environment, or carryover into nearby samples;
- Post-PCR handling of the amplicons/products is not required (i.e., the contamination risk posed by postamplification products are minimized);
- Output is not only positive or negative but enables a quantitative estimation of target or input nucleic acid in the sample;
- Hands-on time is greatly reduced, compared with traditional detection using agarose gels followed by ethidium bromide or GelRed™ staining;

- Practical to automate with the use of a 96-well microtiter plate format, without the need for nested PCR;
- High throughput potential due to robotic automation on both the sample extraction and the sample processing/amplification sides;
- Probes can be labeled with a number of different fluorophores that function as individual reporter dyes for different primer sets. Thus, it is suitable for the development of multiplex PCR systems;
- Lower costs per detected agent, if the equipment can be used for a large number of samples. For further information see [7,8].

The advantage of real-time PCR techniques in allowing the quantitative assessment of the targeted genomes can be very important in disease diagnosis, such as in the case of diagnosis of postweaning multisystemic wasting syndrome, where the number of viral particles or the viral loads of porcine circovirus type 2 have to be determined [13]. Currently, TaqMan and molecular beacons are the most commonly used real-time PCR chemistries in routine diagnostic laboratories [10] and, therefore, this article will focus on these, but will include other exciting approaches, such as the PriProET and light-upon-extension (LUX) systems [14–16].

New real-time PCR applications, such as the linear-after-the-exponential (LATE)-PCR[6] , are now considered as robust, reliable assays for improved detection of various pathogens, especially when adapted to simple tools, such as portable PCR machines, that will allow onsite disease diagnosis [17]. These platforms will increasingly be commercialized as early, rapid, onsite and high-throughput systems. The specification of the real-time PCR platform, such as TaqMan or molecular beacons, is important, considering that the various platforms and chemistries have different strengths and weaknesses, such as detection range, sensitivity, specificity and sensitivity to mismatch, which influence the platforms results. It is important to note that, even within the same platform of real-time PCR or similar real-time PCR chemistries, differences can, and will, occur within and between laboratories [7]. This variation has been highlighted with various TaqMan assays developed for the detection of the same virus that can differ with regards to diagnostic specificity, sensitivity and other important assay parameters [17,18]. Therefore, it is important to clearly specify the exact real-time PCR platform, chemistry and assay concerned and to provide very exact specifications for the applied method in order to avoid confusion and misunderstanding between results.

On the other hand, a molecular diagnostic laboratory generally has a number of assays to analyze, and it is not practical if all assays used are of different design or utilize different real-time PCR machines. Thus, it is not feasible to believe that molecular diagnostic laboratories will set up the recommended assays in exactly the same way. Some adaptation to various nucleic acid extractions, real-time PCR machines or PCR kits will always be the case, further underlying the importance of community reference laboratories and annual proficiency testing. It is also important to note that reference samples are available to laboratories in need to adapt assays to their own work processes. Not all laboratories are going to use the exact same reaction conditions but it is very important that the resulting varieties of assays will result in comparable assay characteristics in each diagnostic laboratory.

### *Sensitivity & specificity of real-time PCR platforms*

Some real-time PCR platforms are able to detect as few as ten genome copies of the targeted viruses. This indicates very high analytical sensitivity and specificity, with the majority of the platforms being able to exclusively detect and amplify the selected target nucleic acids where crossreactivity could be a factor in the diagnosis. However, wide-spectrum, pan- or general PCR assays, for example, the pan-pesti PCR assays, which amplify very conservative regions of the pestivirus genomes (e.g., selected regions from the 5′NCR) and are able to amplify all tested pestiviruses, such as BVDV, classical swine fever virus (CSFV) and border disease virus [11], are useful where a wider range of targets can be expected or the exact causative agent is not known [18]. One strategy of laboratory diagnostic analysis could be to use an arsenal of wide-range (general) real-time PCR assays for preliminary screening of the samples [19] as a first line of detection, while the exact identification/characterization of the detected pathogens is made by narrower-range, highly specific PCR assays. These assays not only allow for the exact identification of the virus variant(s) but also allow for its molecular epidemiological characterization [9,10].

### *Multiplex PCR in routine diagnosis*

Multiplex PCR platforms are based on the use of multiple primers to allow amplification of multiple templates within a single reaction. For example, this approach can be used to analyze a single nasal swab for a respiratory disease or rectal swab collected from an animal suffering enteritis/diarrhea syndrome. By performing multiplex PCR, the detection specificity is broadened to diagnose all possible pathogens present that can be considered as agents for a particular disease. These platforms are useful for diagnostic purposes, providing the laboratory has the capability to analyze and interpret the more-complex multiplex results.

The gel-based PCR platforms facilitated the development of multiplex PCR assays but the advent of real-time PCR platforms provided an important high-throughput tool in a diagnostic laboratory's arsenal. This development was possible as all the individual and specific probes used for the component assays can be labeled with a different fluorophores, each of which function as a specific-color reporter dye for one set of primers. Since the fluorescent probes emit at different color wavelengths, it enables multiplexing of the assays [10,20–23]. Compared with single-target PCR platforms, construction of multiplex platforms can be rather complex, considering the large number of oligonucleotides required to hybridize to different targets with different empiric values [6]. The multiplex real-time PCR platform has the potential to produce considerable savings in time and effort, without compromising the robustness and sensitivity of virus detection assays [10]. However, the competition of 'in-test-tube' oligonucleotides frequently hinders the development of potent multiplex PCR platforms and, therefore, necessitates an

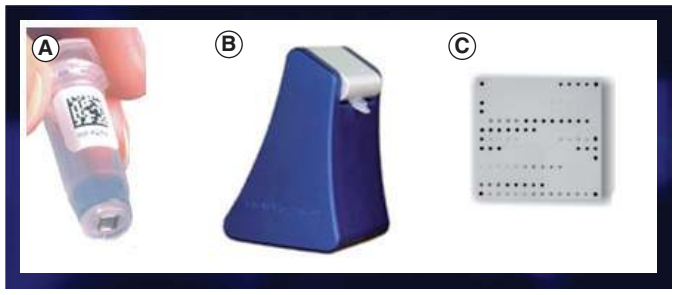


**Figure 1. Clondiag Chip technolgies ArrayTube® platform.** **(A)** ArrayTube (with probe array chip at the bottom of the tube), **(B)** the ATR 03 reader (connected to a PC or laptop to run image acquisition and analysis) and **(C)** magnification of probe array chip. The detection reaction consists of horseradish peroxidase (HRP) and a HRP substrate, tetramethylbenzidine, which react to form a blue precipitate.

optimal primer/probe design with as few crossreaction possibilities as possible. 'Primer competition' could lead to a decrease in the levels of specificity and sensitivity of the PCR platform and, in certain cases, the system will simply not work at all in a multiplex arrangement.

A novel multiplex detection system for bacterial genomes using zinc-finger proteins was developed recently [24]. Zinc-finger proteins are DNA-binding proteins that can bind to dsDNA with high affinity and specificity. Since zinc-finger proteins can directly detect PCR products and can double-check the specific PCR amplification and sequence specificity of the PCR products, this novel method has great potential and has enabled the detection for three pathogens, *Legionella pneumophila*, *Salmonella* spp. and influenza A virus, in a multiplex format. Many such advances have been published recently, and it takes a matrix of scientific and commercial entities to work together to ensure the sustainable development of these platforms into the diagnostic market. Advances in both hardware and molecular chemistries have allowed single-well multiplex assays to become a reality in routine laboratories, with assays that are both US FDA approved and Conformité Européene (CE) marked.

### *Microarrays & other novel technologies*

The advancement in microarray technology created a breakthrough in diagnostics allowing many specific sequences to be analyzed simultaneously. Microarrays function in a number of different ways but the common characteristic is that many specific pieces of nucleic acid can be identified through the use of complementary probes that make up the array. In diagnostics, the first microarray-based kit approved by the FDA [101], and for use in the EU, was in 2004. The test is called the AmpliChip® Cytochrome P450 Genotyping Test (Roche Molecular Systems Inc., CA, USA) and was cleared for use with the GeneChip® Microarray Instrumentation System (Affymetrix Inc., CA, USA). The assay uses a solid-phase planar microarray format and was designed to identify dozens of variants of the genes *CYP2D6* and *CYP2C19* in patients to help determine their proficiency in drug metabolism. Later on, multiplex and microarray-based diagnostics expanded into the field of viral diseases; however, the traditional microarray format using spotted glass slides has not been well accepted in diagnostics laboratories and will be replaced with more user-friendly planar formats.

The company, CLONDIAG Chip Technologies GmbH (Jena, Germany) has produced the ArrayTube® and ArrayStrips™ systems, which use a planar array printed on the bottom of custom sample tubes or strips and read in their specialized workstations. The ArrayTube is a single tube with an array printed on the bottom of the tube. The ArrayStrips use the same principle but are designed for use in 96-well format, suitable for automation and high throughput (Figure 1) [102]. This system is in the mid-plex range, with a capacity of 400 probes per tube. The UK's Veterinary Laboratories Agency (Surrey, UK) has launched a commercial product, known as Identibac®, for the analysis of antimicrobial resistance or virulence in a range of different bacteria [103]. In The Netherlands, the Institute for Food Safety ([RIKILT], Wageningen, The Netherlands) is also developing kits for routine pathogen detection in combination with padlock probe chemistry. Examples of this technology applied to viral diagnostics include the detection and subtyping of avian influenza [25] and the detection of herpesvirus and adenovirus coinfections [26]. Aside from planar arrays, other multiplex technologies are also available. Seegene Inc. (MD, USA; Seoul, Korea) has 16 CE marked products, most of which include viral detection. One such assay is the Seeplex® RV12 Affinity Capture Elution detection assay, designed to detect 12 major respiratory viruses, 11 respiratory RNA viruses and one DNA virus from patients' samples, including nasopharyngeal aspirates, nasopharyngeal swabs and bronchoalveolar lavage. This technology has also been applied to the veterinary field with the Seeplex® Porcine Diarr-V Detection Kit and Seeplex Porcine Diarr-B Detection Kit, which are designed to detect five viruses and bacteria playing roles in swine gastrointestinal diseases, including porcine epidemic diarrhea. The general system used for these assays combines a novel chemistry using bipartite primers connected with a polyinosine linker and conventional gel-based detection using amplicon size [27–31].

Another multiplex system that has entered the field of viral diagnostics, uses an analogous approach to the conventional microarray for detection, where nucleic acid probes are covalently linked to microspheres instead of attached to a solid surface. This technology was brought to the market by the Luminex Corporation (TX, USA) and has since been licensed to many other commercial partners (Figure 2). In this system, nucleic acids can be coupled to coloured microspheres creating up to a 100-plex assay on the Luminex® 200™. Luminex has announced a 500-plex instrument for release in 2009. This technology runs on an open architecture platform so assays can be custom designed by researchers using 'naked beads' and oligonucleotides of their own design [32]. As an aside, it is noteworthy that this technology can also be used to detect proteins [33]. In viral diagnostics, the xTAG Respiratory Viral Panel [34] received 510(k) clearance from the FDA in 2008 (also CE marked for use in EU). This test was FDA cleared for 12 viruses and subtypes and CE marked for 19 viruses. The xTAG® Respiratory Viral Panel is the first multiplexed nucleic acid test for respiratory viruses cleared for *in vitro* diagnostic use by FDA. It

also is the first test of any kind the FDA has cleared to detect human metapneumovirus, the first test cleared for influenza A subtyping and the first ever molecular test cleared for adenovirus detection. The molecular chemistry used in this assay involves several primer pairs, bipartite primers that contain complementary sequences to those on the microspheres and two rounds of PCR.

Technologies different from the probe-based methods mentioned exist and are being applied in viral diagnostics; these include surface plasmon resonance and mass spectrometry. One, in particular, that has been applied to viral diagnostics is the IBIS™ system, which uses mass spectrometry on amplified PCR products to derive base compositions [35–39]. This was used, for example, to detect an unknown contaminant (found by the IBIS system to be bluetongue virus) in a large virus-culture library (Bulte R, Pers. Comm.). Furthermore, low-volume, multiwell, PCR-based systems are reaching the point where automated microfludic platforms and nanovolume reaction mixes offer a variety of systematic options in terms of designing fit-for-purpose molecular detection systems that generate massive amounts of PCR data.

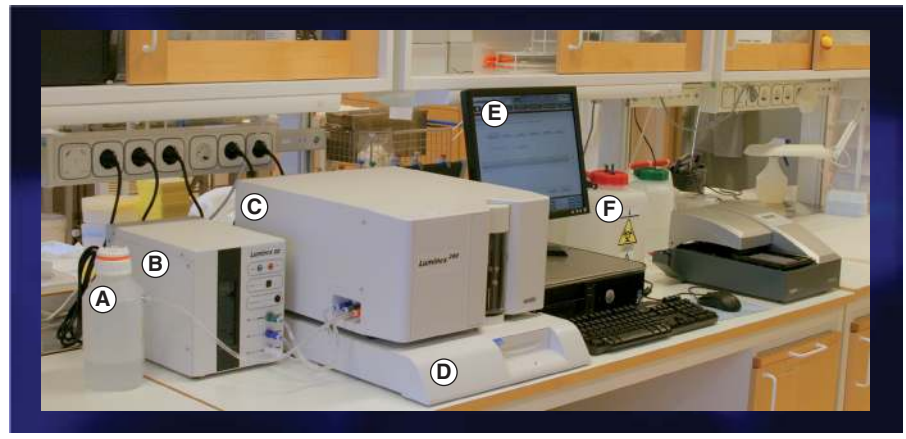


**Figure 2. Luminex laboratory at the National Veterinary Institute and the Swedish University of Agricultural Sciences in Uppsala, Sweden.** Luminex 200™ system, including **(A)** waste bottle, **(B)** the Luminex SD sheath fluid-delivery system the **(C)** Luminex 200 instrument, the **(D)** Luminex XY plate-handling platform and **(E)** Luminex xPONENT 3.0 software and a PC. The microsphere-based detection system allows for 100 different analytes to de detected in each sample (100-plex). Also pictured, is **(F)** the Tecan Hydroflex wash-station with buffer and waste bottles. This instrument performs automated microplate washing and vacuum filtration for use with both bead types (magnetic and polystyrene) used in the Luminex system.

The BioTrove OpenArray™ system is an example of such a technology, using a standard PCR cycling protocol designed to function with the system. It can produce 3072 simultaneous PCRs in 33-nl reaction volumes in one plate (approximate dimensions of conventional microarray slide). The plate composition consists of 48 subarrays, each with 64 through-holes or wells (Figure 3). To perform the PCR, the OpenArray plate can be constituted in two ways; either the wells can be spotted with primers or the wells can be empty with primers being added as part of the reaction mix. Sample application to the wells can be configured in a variety of ways with the assistance of the AutoLoader equipment. The NT cycler can run three plates simultaneously (9216 individual PCR assays) and monitors the reaction progress in real-time.

### *Robots accelerate molecular diagnosis & improve safety*

An important aspect of the molecular diagnostic process is the extraction of nucleic acid [6]. This step has a large impact on the downstream applications and can be very difficult depending on the origin of the sample. Several sample types are prone to inhibit PCR and could have a profound impact on the outcome of the diagnostic procedure. In addition, RNA/DNA extraction could be a real bottleneck when large numbers of samples are processed. The use of nucleic acid-extraction robots is now common in the preparation and purification of the targeted viral nucleic acids that are crucial steps of molecular diagnostic procedures. The use of these extraction robots has accelerated the diagnostic procedures, improved repeatable and increased safety. Realizing the high demand for these equipments, industry is producing a wide range of various types of the nucleic acid-purifying robots. As examples, the Magnatrix 8000™ and the recent Bullet (Nordiag, Norway), the Qiagen Symphony™ and others, utilize magnetic separation of the target molecules and nucleic acid preparations, and the results are more efficient and reliable than manual procedures. The robots simultaneously purify the nucleic acids from 96 samples within 3–4 h, and the products are clean enough to be amplified directly in the PCR. In addition to high speed, robustness and low labor input, robots also can reduce the risk of cross contamination between specimens.

The introduction of special tools, laboratory practices and internal controls (mimics) made it possible to reduce the danger of false-positive and -negative results in the protocols of diagnostic PCR assays [7,9,10]. Closed and automated systems of extraction robots provide internal security for the PCR-based diagnostic assays, as human manipulations are kept to a minimum within the assay. It is possible to automate most steps in diagnostic procedures using nucleic acid extraction and pipetting robots, followed by PCR in real-time PCR machines, thus establishing an automated

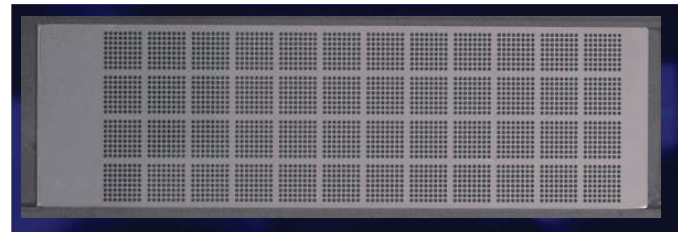

**Figure 3. BioTrove OpenArray™ platform plate.** A plate consists of 48 subarrays each containing 8 × 8 through-holes (wells) for a total of 3072 reaction chambers per plate. Three plates can be read in the NT instrument (not shown) for a total of 9216 simultaneous real-time quantitive PCR results.


**Figure 4. BioSeeq Portable Veterinary Diagnostic System.** **(A)** Portable PCR instrument with five independent thermocyclers (running assay in far left thermocycler) with built in communications, including global positioning system, WiFi and Bluetooth technology. **(B)** Disposable automated sample-preparation unit processes raw sample into PCR mixture allowing test to be conducted at pen-side.

diagnostic chain. The introduction of robots has led to the establishment of high-throughput and robust diagnostic assays with less contamination risk and shortened diagnosis time from hours to minutes [9]. Nucleic acid-based diagnosis has developed along similar lines to that of ELISA-based diagnostic chains, using automated systems that provide rapidity, robustness, low costs, reduced labor-requirement and an increased reliability of diagnosis.

### *Isothermal amplification using simple thermoblocks*

Alternative methods, such as Invader® or loop-mediated isothermal amplification (LAMP) technologies, utilizing the isothermal characterization of the amplifying mechanism instead of varying temperature amplification methods (our commonly used PCR technology) are becoming more accepted. In short, LAMP is very simple and rapid wherein the amplification can be completed in less than 1 h under isothermal conditions employing a set of six specially designed primers spanning eight distinct sequences of a target gene by incubating all the reagents in a single tube. These alternative systems do not need the costly investment of PCR-amplifying machines as isothermal amplification methods use simple thermoblocks that are more affordable [40,41].

### *Portable PCR machines*

Portable PCR machines are constructed to bring laboratory facilities closer to field cases and disease outbreaks [17]. Several companies are producing and optimizing battery-powered machines that can be used easily under field conditions, which allow for complete disinfection of the equipment, require simple sample preparation and produce rapid results without the need for specific training. For example, Smiths Detection's Portable Veterinary Diagnostic Laboratory takes the laboratory to the field. It has been designed with field veterinarians in mind, comprising of a portable briefcase-sized PCR instrument and a disposable sample preparation unit. This integrated system provides rapid onsite identification under a wide range of weather conditions. The operators require no technical understanding of the PCR methodologies but the equipment provides an outcome concerning the suspected diagnosis of a viral disease in the field (Figure 4) [104]. Detection is based on advanced PCR chemistry known as LATE PCR. This is an advanced form of asymmetric PCR that efficiently generates single-stranded amplicons under controlled conditions. This technology has also improved sample preparation, suppressed amplification errors, improved probe design for rapid high-resolution analysis of the amplified product to make multiplexing easier, which allows for rapid DNA sequencing, and enhance data analysis [42,105]. This combination of a simple, portable PCR machines and LATE PCR technology provides simple facilities for the onsite diagnosis of viral diseases.

### *Molecular epidemiology*

PCR assays yield specific DNA products or amplicons that can be analyzed, sequenced and characterized by several means. Nucleic acid sequences can be analyzed and compared with each other and with previously described sequences obtained from large international databases, such as GenBank. The rapid nucleic acid phylogenetic identification, classification and tracing of DNA and RNA targets (in this case, viruses) is termed 'molecular epidemiology'. Studies on molecular epidemiological were conducted, for example, when genetic variants of CSFV were identified in several countries of Central Europe. It was hypothesized that European and US genotypes of the porcine respiratory and reproductive syndrome virus evolved from a common ancestor that was suspected to have originated from Eastern Europe [43–46].

PCR amplification and comparative nucleotide sequence analysis not only allows for the direct detection of the viruses but also retrospective genetic analysis of biological products and clinical samples. Such approaches can also be applied to determine the identity of virus strains used in vaccine production. Recent studies have found that a virus 'pick up' had occurred in a commercially produced, live-attenuated BVDV vaccine. These results emphasize that the contamination of commercially available live vaccines with exogenous virus strains (e.g., the BVDV strain originating from fetal calf serum or bovine cells) is a real risk factor in bio-industries. Unequivocal analysis, including molecular methods, is needed to verify the authenticity of biological products, such as vaccines, fetal calf serum batches and cell lines, to reduce the risk of viral contamination [47]. In addition, molecular epidemiology can be a useful tool for animal-health authorities when combating various viral diseases and when doing their risk assessment. The occurrence of virus variants can be detected and the spread of various variants traced, allowing epidemiological analysis and provide the necessary support to combat infection and control disease spread.

### *World Organisation for Animal Health: standards for international standardization & validation of PCR-based diagnostic assays*

Considering the frequent occurrence of viral diseases worldwide and the intensive research and development in the field of molecular diagnosis, there is an ongoing need for international

standardization and validation of developed assays. It is critical that veterinary diagnostic laboratories use validated techniques fit for their intended purpose to provide comparable results that allow the same conclusions and actions to be taken in different regions of the world. This consolidated and integrated approach is seen as having the best chance of success to combat TADs and endemic diseases effectively on a wider or global scale while adhering to the 'one world, one health' principle.

National and international authorities require rigorous proof that assays used in laboratories are as reliable as possible and provide identical results [48]. International agencies, such as the World Organisation for Animal Health (OIE), the joint Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) Division, national research institutions and commercial companies take considerable effort to provide guidelines on international standardization of protocols. The OIE regularly publishes standards for the validation of diagnostic assays [106]. Validation and international standardization of nucleic acid-amplification-based diagnostic methods (e.g., PCR) is a major task for the animal-health authorities. The in-house PCR assays will soon have to be replaced by validated and standardized international procedures or be properly validated themselves (please see [107]).

## Recent developments in the field of molecular diagnostics

### *Improved detection of foot-and-mouth disease virus*

It is important to mention foot-and-mouth disease, as a trade-related disease, emerges and re-emerges in areas where it is considered eliminated or where it did not occur before. A characteristic of this virus is its genetic and antigenic drift. It is, therefore, important to be able to detect and differentiate the appropriate serotype in the field if possible. For this purpose, a robust quantitative multiplex real-time PCR platform for the simultaneous detection of all the seven serotypes of foot-and-mouth disease virus (FMDV) has been developed. This platform is based on the proven PriProET principle and is targeting the coding region of the 3D gene of the viruses. The authors provided sufficient data to conclude that a complex diagnosis of a foot-and-mouth disease outbreak can be accomplished within a short period of time [14].

### *Solid-phase microarrays in veterinary diagnostic virology based on padlock probes*

A solid-phase microarray system has been developed for the simultaneous detection of FMDV, vesicular stomatitis and swine vesicular disease viruses (SVDVs) using padlock probes [49]. The application of padlock probes to the detection of pathogens is a recent development in molecular disease diagnosis. Padlock probes have the capacity to detect many different nucleic acid target sequences in a single multiplex-array platform system. Each viral nucleic acid template is a target for a specific and unique padlock probe [49,50]. The more available specific probes, the more 'different' targets can be detected. The circularized target/probes are then amplified with a single universal primer set. The high-throughput sorting of fluorescently labeled amplicons is then conducted using the tag sequences on a microarray platform. Although the cost for oligonucleotides and array slides is higher than that for conventional PCR assays, it is reduced in proportion to the number of assays in comparison with conventional PCR assay. In this format, multiplex detection using padlock probes and microarrays could have implications in more-effective screening for viruses causing similar vesicular symptoms and, in turn, facilitate rapid counteractions, especially in case of foot-and-mouth outbreaks [49].

### *Padlock probes for AIVs*

An assay has been developed for the simultaneous detection and subtyping of AIV using padlock probe chemistry for multiplexed preamplification and microarray detection. The assay has the ability to identify both the hemagglutinin and neuraminidase surface antigens of AIV in a single reaction. Gyarmati *et al.* analyzed 77 influenza strains, representing the entire assortment of hemagglutinin and neuraminidase, and all (77 out of 77) of the samples were identified as AIV and 97% (75 out of 77) were correctly subtyped [50]. The specificity of the assay was determined testing heterologous pathogens. The results indicated that the assay is a useful and robust tool with a high throughput for the rapid detection and typing of AIVs compared with conventional methods. In summary, the padlock probes, adapted to microarray formats, provide a novel means for powerful and very complex novel molecular diagnosis. Compared to real-time PCR assays, the padlock probe-based microarrays are inferior in sensitivity, but they allow for a highly multiplex diagnosis and simultaneous analysis of thousands of specimens in the same system.

### *Novel TaqMan & primer-probe energy-transfer assays for the detection of hepatitis E virus*

Hepatitis E virus is an important cause of food- and waterborne diseases in countries with poor sanitation but, recently, it has been occurring more frequently in regions of the world where health services are at a high standard. Previously, the disease cases were associated with traveling; however, recently zoonotic transmission has also been suspected (i.e., a direct route of infection from animals to humans). Two real-time PCR methods have been developed and compared: a TaqMan and PriProET assay to improve the detection of the virus. These robust, highly sensitive methods provide valuable diagnostic tools to investigate zoonotic transmission and to detect the virus in the food chain. They have also been used in research related to the potential of hepatitis E virus to cross the species barrier. The two novel PCR assays detected a broad range of viruses representing all the four genotypes of hepatitis E virus. The TaqMan assay showed higher fluorescence values for positive samples, while the PriProET assay better tolerated the point mutations in the target nucleic acids and, therefore, is a more powerful tool for the detection of new hepatitis E virus variants. These two real-time PCR assays are useful novel tools for virus detection and molecular epidemiology [51].

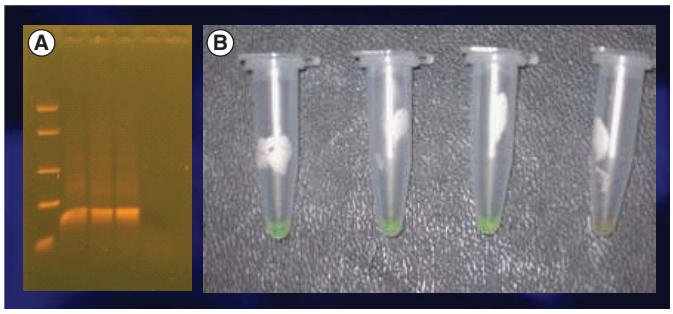


**Figure 5. Simple detection of swine vesicular disease virus by loop-mediated isothermal amplification assay with the application of isothermal amplification using only a simple thermoblock. (A)** The reading of the results is either by running a gel electrophoresis or **(B)** visual inspection through the addition of SYBR Green with positive results presenting as a green colour. Three positive samples are seen on the left and one negative on the right, on both panels **(A)** and **(B)**. In addition, **(A)** shows a size marker on the far left.

### *Real-time PCR assay based on primer-probe energy-transfer to detect SVDV*

A real-time PCR assay to detect SVDV based on PriProET has been developed. The assay is highly sensitive with a detection limit corresponding to five copies of viral genome equivalents, and has a high specificity demonstrated by testing with heterologous viruses. A major advantage of the PriProET chemistry is a tolerance of mutations in the probe region. Melting-curve analysis directly after PCR, with determination of probe melting point confirms the specific hybridization of SVDV strains. There is a high probability of identifying phylogenetically divergent strains of SVDV that may appear negative in other probe-based real-time PCR assays. Moreover, any difference in melting points may provide an indication of mutation divergence in the probe region [15].

### *Simple & rapid detection of SVDV with a one-step reverse transcriptase LAMP assay*

A one-step reverse transcriptase (RT)-LAMP assay was developed recently to improve the detection of SVDV [40]. The assay provided a wide SVDV strain-detection range, since all 28 tested isolates of this virus tested positive during the developmental phase. Simultaneously, RT-LAMP yielded high specificity, as all tested heterologous viruses gave negative results for viruses such as FMDV and vesicular stomatitis. Since SVDV, FMDV and vesicular stomatitis virus all cause similar symptoms, the high specificity of detection and identification of SVDV is very important in differentiating these diseases. RNA from clinical samples that included nasal swabs, serum and feces were used to evaluate the performance of the RT-LAMP with real-time PCR assays. When testing nasal swabs and serum, the sensitivity of the assays were not significantly different but, with fecal samples, the RT-LAMP assay performed significantly better than real-time PCR. The RT-LAMP assay has several features that supports its applicability as a powerful new tool for the detection of SVDV:

- It is an isothermal amplification method that requires a simple thermoblock
- It is rapid, with the result being obtained within 30–60 min
- It is highly specific and sensitive
- The test reading is simple as the results are visualized either by gel electrophoresis or visually through the addition of SYBR Green (Figure 5)

Reverse transcriptase-LAMP provides a novel tool for the 'front-line' diagnosis of swine vesicular disease, an important TAD since it can easily be performed in modestly equipped field laboratories and adapted to mobile diagnostic units.

### *Subtyping & pathotyping of AIVs with a one-step real-time SYBR Green RT-PCR assay*

For the rapid subtyping and pathotyping of AIVs, a one-step real-time SYBR Green RT-PCR assay was developed. Primers were selected to target highly conserved nucleotide stretches that flank the cleavage site of the *HA* gene of AIVs. Sequencing the amplified PCR products in AIV subtypes, including the H5 subtypes, allowed the rapid detection of the pathotype of AIV. The specificity of the assay was confirmed by testing 27 strains of AIV and nine heterologous pathogens, including influenza B and C, and various other avian viruses. Since the subtype and pathotype determinations were completed within approximately 6 h, the SYBR Green RT-PCR assay provides a powerful new tool in the arsenal of influenza diagnostics [52].

### *Proximity ligation: protein detection by nucleic acid amplification*

Proximity ligation is based on the recently established proximity-ligation mechanism that enables sensitive high-capacity protein detection, identification and measurement by converting detected specific proteins to an analysis of DNA sequences. Proximity ligation enables a specific and quantitative transformation of proteins present in a sample into nucleic acid sequences. As pairs of so-called proximity probes bind individual target protein molecules at distinct sites, these reagents are brought in close proximity. These probes consist of a protein-specific binding part coupled to an oligonucleotide with either a free 3′- or 5′-end capable of hybridizing to a common connector oligonucleotide. When the probes are in proximity, promoted by target binding, then the DNA strands can be joined by enzymatic ligation. The nucleic acid sequence that is formed can then be amplified and quantitatively detected in a real-time monitored PCR.

This convenient assay is simple to perform and allows highly sensitive protein detection. Nordengrahn *et al.* reported that detection sensitivities were similar to those for nucleic acid-based detection reactions for the rapid detection of FMDV [53]. All indications are that the sensitivity of proximity ligation is on a par with antigen ELISA (Belák S, Thorén P, LeBlanc N *et al.* Pers. Observ.) and, therefore, could prove to play a significant role in the early and rapid diagnosis of infectious diseases (including biodefense). This platform is presently being adapted to be able to detect the surface

antigens of viruses (e.g., AIV). The combination of nucleic acid and antigen detection approaches contribute to a complex, multilateral diagnosis of TADs. In addition, replacement of antigen ELISA with a more sensitive and robust assay will be a significant advancement in the diagnosis of infectious diseases.

### *Magnetic bead-based microarray for detection & discrimination of pestiviruses*

A novel assay was developed for the rapid detection and discrimination of pestiviruses (i.e., BVDV types 1 and 2, CSFV and border disease virus) using magnetic-bead detection of PCR products in microarrays. After amplification, the PCR products are hybridized onto an array, followed by visualization with streptavidin-coated magnetic beads for visual inspection or microscope examination, and this makes this novel assay suitable for use in modestly equipped laboratories. A panel of pestiviruses comprising members of all the four accepted species was used to evaluate the assay with other post-PCR detection methods (e.g., gel electrophoresis and suspension microarray) used as comparisons to determinate sensitivity of the assay. The results clearly demonstrated that the assay provided a novel, robust, sensitive and specific method for the improved detection and discrimination of viral pathogens. In view of the simplicity of the assay and the very simple detection procedure in particular, this magnetic bead-based assay offers a powerful and novel technology for molecular diagnostics in virology [54].

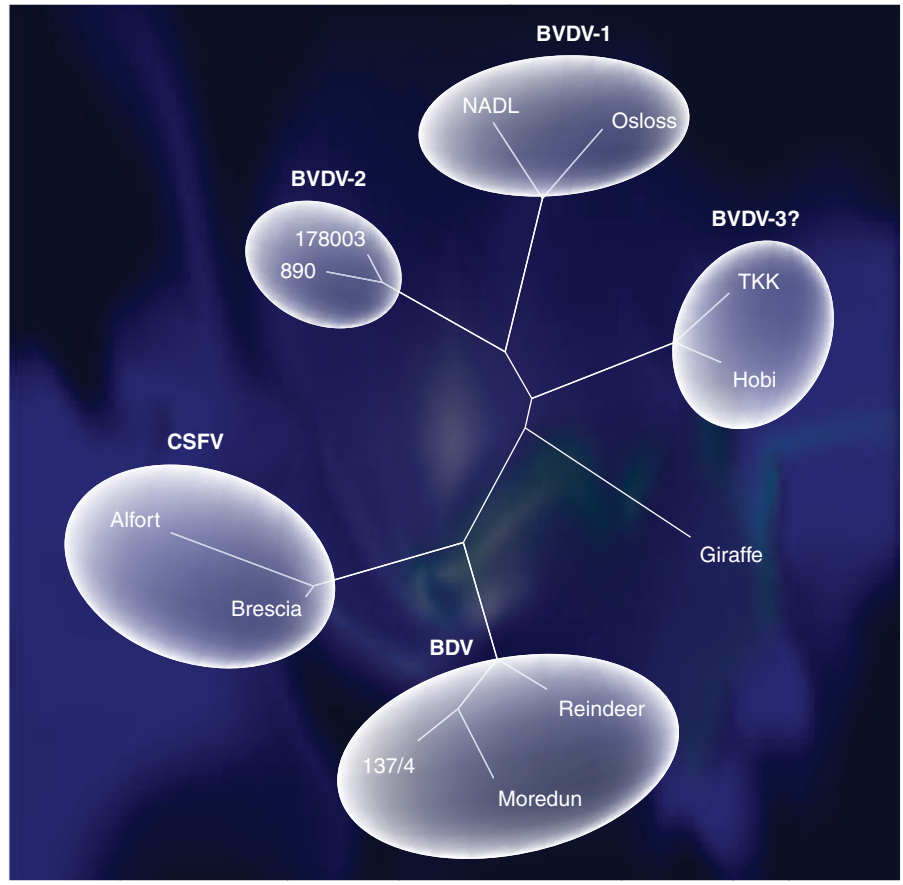


**Figure 6. Molecular epidemiology, identification of new pestiviruses, including Th/04_KhonKaen.** Unrooted phylogram generated from a fragment of the 5′NCR sequences of selected representatives of each known species within the genus *Pestivirus*, including the tentative pestivirus of giraffe, the D32/00_'HoBi' and Th/04_KhonKaen. The sequences of pestivirus reference strains and previously described strains were obtained from the GenBank. The phylogram shows the phylogenetic position of the newly detected pestiviruses HoBi and TKK as new variants of BVDV (BVDV-3 perhaps). BDV: Border disease virus; BVDV: Bovine viral diarrhea virus; CSFV: Classical swine fever virus.
For details see [55].

### *Detection of an emerging pestivirus by means of molecular diagnostics & reverse genetics*

During a study of bovine viral diarrhea epidemiology in Thailand using indirect antibody ELISA, an antigen ELISA and PCR, a pestivirus was detected in heat-inactivated serum of a calf. The PCR product was sequenced and comparative nucleotide sequence analysis showed that this virus was closely related to a recently described atypical pestivirus (D32/00 'HoBi') that had been first isolated from a batch of fetal calf serum collected in Brazil (Figure 6). It was also demonstrated that the Thailand virus (known as Th/04_KhonKaen) was circulating in the Thai herd [44]. The study was the first to report a natural infection in cattle with a virus from this group of atypical pestiviruses. The data suggest that these viruses had been spread to cattle populations in various regions of the world. If this global spread has occurred, then atypical bovine pestiviruses will have important implications for bovine viral diarrhea control. This spread also has implications for the biosafety of vaccines and other biological products, produced with fetal calf serum.

Virus isolation was not possible since the virus in the serum had been inactivated during processing; thus, reverse genetics methods were applied to 'reconstruct' the inactivated virus until it regained its capacity to grow in cell cultures by using transfection of viral nucleic acids. Full-genome characterization and phylogenetic analysis of the new virus were performed after recuperation of the inactivated virus and, based on this, it was demonstrated that the virus was closely related to BVDV, suggesting that Th/04_KhonKaen and other HoBi-like pestiviruses constitute a third genotype of BVDV (i.e., BVDV type 3) [55].

### *Microfluidic platforms & nanoscale reactions*

Droplet-based microfluidics is a recent technology for high-throughput parallel PCR analyses, which allows for millions of discrete picoliter reactions [56]. There are commercial enterprises,

such as Raindance Technologies, which develop and drive such platforms. The previously mentioned BioTrove is another exciting technology, which offers high-throughput parallel analyses. These technologies, with the amount of information they can provide, will find a place in both diagnostics and in molecular epidemiology sooner rather than later, as they bring huge advantages to the early and rapid detection of disease pathogens (even before the onset of disease).

### *New approaches to molecular epidemiology*

Considerable progress was made in the development of various nucleic acid-detection methods and molecular epidemiology. Traditionally, inferring molecular phylogeny was constructed by distance-based analysis (i.e., neighbor joining for single genes). In a recent study at the OIE Collaborating Centre for Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine, a Bayesian analysis was carried out to analyze five genetic regions of the BVDV genome (5-UTR, $N^{pro}$, E2a, E2b and NS3) for 68 taxa retrieved from GenBank. The best outcome for the analysis was achieved when analyzing genetic regions with appropriate proportions of conserved and variable sites or a combined dataset consisting of all five genetic regions. In the future, Bayesian analyses combined with other traditional tree-building methods can be used to estimate a more reliable viral phylogenetic tree and to study the emerging and/or occurrence new variants of BVDV [57].

### *Full-genome amplification of viral genomes*

To systematically identify and analyze viral genomes (e.g., the 15 hemagglutinin and nine neuraminidase subtypes of influenza A virus), reliable and simple methods that not only characterize partial sequences but also analyze the entire genome are needed. It is possible to generate full-length cDNAs to subtype viruses, sequence their DNA and construct expression plasmids for reverse genetics systems by the selection and construction of specific sets of primers [58].

Through the use of new sequencing machines, such as the 454 sequencers (Roche) [108], approximately 30–60 million nucleotides can be sequenced within several hours (the number of nucleotides is constantly increasing). The technique is extremely important in genetic research, including the investigation and comparison of the viral genomes. This approach will lead to exact information being obtained about the full-length sequences of the tested viral genomes and, therefore, they will provide more-reliable data for determination of evolutionary aspects, relationships of viruses, viral subpopulations and many other important aspects of molecular virology [59].

### *Using viral metagenomics to search for unknown viruses*

Since, so far, unknown and/or recently emerging or re-emerging viruses are very important factors in the emergence of various infectious diseases, it is important to work on methods that facilitate the detection of these unsuspected agents. In order to facilitate the detection of such viruses, a range of molecular methods have been developed to genetically characterize new viruses without prior *in vitro* replication or the use of virus-specific reagents.

Characterization of new, hitherto unknown, viruses is often complicated by:

- Inability or very poor capacity to grow in cell cultures
- Low copy number or unusual virion structure, limiting the detection and identification of the virions with electron microscopy
- Being a minor part of mixed viral infections, where the coinfecting viruses are dominant
- Limited antigenic/serological crossreactivity, affecting the detection by antigen–antibody assays, such as antigen-capture ELISA, immunofluorescence or immunohistochemistry
- Lack of nucleic acid identity or similarity to known viral sequences; thus, the virus remains undetected in nucleic acid hybridization assays
- Remaining undetected even by PCR assays, due to unique genome structures

In recent metagenomic studies, viral nucleic acids were detected in uncultured environmental and clinical samples by using random amplification of their nucleic acids prior to subcloning and sequencing. Already-known and novel viruses were then identified by comparing their translated sequence to those of viral proteins reported in public sequence databases. A wide range of specimens, such feces, serum, plasma, respiratory secretions and organ suspensions, seawater and near-shore sediments, were tested by viral metagenomic approach. Viral metagenomic approaches provide novel opportunities to generate an unbiased characterization of the viral populations in various organisms and environments. For example, such techniques have led to the detection of new parvoviruses, termed bocaviruses, in lower respiratory tract infections of children [60]. By detecting a range of new viruses recently, viral metagenomics has broadened known viral diversity and opened a very interesting new area in virological research and diagnosis [61]. It is worth mentioning that the aforementioned approaches to determine viral diversity are not completely different and independent of each other as they are frequently used together in a complex assay. For example, full-length amplification and full-length sequencing of viral genomes is a logical combination for this approach to viral identification.

## Expert commentary

Considering the serious impact and socioeconomic effect of infectious diseases in human and in animal health today, there is a need for the very rapid development of novel, highly sensitive and robust biotechnology-based diagnostic techniques, which facilitate the early detection and identification of pathogens and support the immediate implication of disease prevention and control measures. The globalization of international trade, the rapid and free movement of large groups of people, animals, food and feed products at a global scale has created a novel and epidemiologically highly vulnerable situation. In this novel scenario, both known infectious agents and/or newly emerging pathogens have the chance to spread all over the world within

several hours or days, causing serious epidemics. Regarding this epidemiological risk, it is extremely important to develop and apply powerful techniques, which are able to detect and identify the variants of the pathogens very rapidly and effectively. During the past several years, numerous developments have taken place in the field, including a variety of advances in the real-time PCR methods, such as PriProET and LATE-PCR. The purpose is to increase the diagnostic robustness of the PCR-based diagnosis and to introduce various simple technical platforms, such as portable PCR machines, which allow the application of PCR under field conditions for 'point-of-care' or onsite detection of the pathogens. The introduction of novel isothermal amplification techniques, such as Invader and LAMP, is further facilitating the adaptation of amplification-based techniques for the front-line diagnosis of infectious diseases. The onsite detection of pathogens is further facilitated by other simple diagnostic tools, such as 'dip-stick' tests, including lateral-flow technique-based simple devices, which are able to detect and identify the pathogens directly in the disease outbreaks, in human medical and in animal clinics, in animal farms and transport conditions or in the food chain, among others. The introduction of the onsite diagnosis provides novel facilities and strong support to the authorities in prevention and control of infectious diseases, since the rapid, first-line diagnosis is extremely important. However, it has to be considered and accepted that, in general, but especially in case of highly contagious and devastating infectious diseases, such as TADs, the onsite methods have to be confirmed by laboratory-based diagnosis and pathogen identification. The laboratory-based direct or indirect diagnosis is based on a wide range of confirmatory techniques of pathogen detection and pathotype identification.

To date, it is a positive tendency that a wide range of central and field laboratories can afford the purchase and use of various real-time PCR machines. By the wider introduction of isothermal amplification assays, the equipment facilities of the laboratories are further improved, considering that this diagnosis requires only simple low-cost thermoblocks. Since sample preparation, such as nucleic acid extraction, is another important step of molecular diagnosis, many laboratories purchase nucleic acid-extraction and -pipetting robots. By robotization the nucleic acid-based diagnosis is saving manpower, and the automated processes allow high-throughput diagnosis. Simultaneously, the crosscontamination and carryover risks, which were considered as Achilles' heels of PCR diagnosis for a decade, are minimized. If we recall how much the introduction of the automated ELISA diagnostic systems did improve the reliability and speed of diagnostic work in the last 20 years, then we can state that a similar process in the PCR-based diagnosis is extremely important and useful today.

In parallel with the PCR-based diagnosis, a high range of other diagnostic techniques have been introduced in the diagnostic laboratories, as is outlined in this review shortly. We would like to emphasize the use of solid- or liquid-microarray techniques, which enable the simultaneous detection of a very wide range of pathogens in the same platform. This allows a very complex diagnosis and provides powerful novel means for the elucidation of the infection biology of various diseases scenarios, such as respiratory or enteric disease complexes, where various viruses and bacteria establish coinfections, influencing and/or aggravating the effects of each other. The microarrays allow a very complex investigation of these scenarios. The further development of microarrays includes improvement of sensitivity (e.g., with combination of amplification processes). Specificity and detection range is further improved by combination of wide-range and highly specific target sequences. The infection-biology investigation is improved so that microarrays will detect not only several pathogens, but, simultaneously, they will measure the immune response capacities of the hosts (e.g., cytokine profiles), antibiotic-resistance values, pathogenicity markers, population kinetics and other important factors of disease prognosis, development and control. Microarrays, especially the liquid-phase approaches, will also allow easy automation and the use of high-throughput, robust diagnostic platforms.

The arsenal of biotechnology-based diagnostic assays will be rapidly expanded in the coming years by the routine diagnostic application of a variety of further methods, such as improved detection of antigens by proximity ligation and by other approaches. The proper identification of virus variants and subpopulations by full-genome sequencing will be affordable to many laboratories, since prices are dropping and facilities and services will be provided. The analysis of a large number of viral genome sequences by powerful programes of bioinformatics will provide novel tools for viral metagenomics, molecular epidemiology and disease control. The novel methods of indirect diagnosis, such as liquid-phase microarrays, completing or replacing ELISA, will also facilitate the processes of pathogen detection and rapid identification, as well as the understanding of complex biology of infectious diseases.

Since emerging and re-emerging pathogens, as well as 'new' viruses, are posing a serious threat to animal and human health, including problems of zoonoses, it is very important to develop and apply powerful approaches for new virus detection and identification. In the last few years, a range of such techniques have been developed. So far, these methods were able to detect mainly small DNA viruses, such as bocaviruses and transfusion-transmitted virus. However, there is a need to further develop the new virus-detection techniques, with special regard to the detection of various RNA viruses, which play an important role in TADs, human epidemics and new emerging diseases worldwide.

Importantly, diagnostic laboratories should be encouraged to work simultaneously in two directions to introduce the novel biotechnology-based diagnostic techniques, as outlined in this article, to maintain the knowledge of classical virology and bacteriology, with special regards to virus isolation in cell culture, and *in vitro* and *in vivo* characterization of viruses and bacteria. Although classical virology and bacteriology are not the subject of our present article, we feel compelled to mention this here because we see that this profile of diagnostic laboratories is decreasing dramatically. This is a very dangerous tendency, considering that we cannot control infectious diseases effectively if we lose the

capacity to isolate and cultivate virus strains, which are absolutely important to understand the pathogenesis of the agents to develop diagnostics and vaccines and for many other purposes of infection biology.

Considering these facts, we believe that a practical combination of classical and biotechnology-based microbiology is required to combat infectious diseases effectively today and in the future.

## Five-year view

The detection and characterization of specific nucleic acids of medicoveterinary pathogens have proven invaluable for diagnostic purposes. Apart from hybridization and sequencing techniques, PCR and numerous other methods have contributed significantly to this process. The integration of amplification and signal-detection systems, including online real-time devices, have increased speed and sensitivity and greatly facilitated the quantification of target nucleic acids. They have also allowed for sequence characterization using melting or hybridization curves. Rugged portable real-time instruments for field use and robotic devices for processing samples are already available commercially. Various stem-loop DNA probes have been designed to have greater specificity for target recognition during real-time PCR. DNA fingerprinting techniques or postamplification sequencing is used to type pathogenic strains. Characterization according to DNA sequence is becoming more readily available as automated sequencers become more widely used. Reverse hybridization and, to a greater degree, DNA microarrays (as an example of high-throughput systems), are being used for genotyping related organisms, and can allow for the detection of a large variety of different pathogens simultaneously. Nonradioactive labeling of DNA, especially using fluorophores and the principles of fluorescence resonance energy transfer, is now widely used, especially in real-time detection devices.

Other detection methods include the use of surface plasmon resonance and MALDI-TOF mass spectrometry. In addition to these technological advances, contributions by bioinformatics and the description of unique signatures of DNA sequences from pathogens will contribute to the development of further assays for monitoring presence of pathogens. An important goal will be the continuous development of robust devices capable of sensitively and specifically detecting a broad spectrum of pathogens that will be applicable for point-of-care use. Advances in biosensors, the development of integrated systems, such as lab-on-a-chip devices, and enhanced communications systems, are likely to play significant roles in allowing for rapid therapeutic and management strategies to deal with disease outbreaks.

It is evident that the molecular diagnosis of infectious transboundary and endemic diseases of animals and man will show a great deal of progress in the coming years. Intensive research will also be conducted in the field of food safety, considering the need for safe food products in our world of intensive globalization, where food products are transported at continental or at a global scale, causing problems for identification of the origin of these products and ensuring food safety requirements. The safe tracing, identification and testing of food and feed products is an increasing, very important requirement today, at a global scale. Considering this requirement, research will be conducted aimed at detecting and identifing the various infectious agents, chemicals, hormones and other factors of food safety, in very robust and complex diagnostic systems. Simultaneously, research will focus on answering questions regarding zoonotic infections, including the transmission of infections between various hosts in the animal and human populations.

The climatic variations and changes, observed worldwide, raise a number of questions for the emergence of new pathogens and the spread of diseases to new geographic areas, where they were not previously known. Since the geographic territories and the population biology conditions of insect vectors are strongly influenced by climatic changes, the issue of emerging vector-borne diseases, such as bluetongue, has become a very hot issue in recent years. It is evident that this tendency will be clearly seen, even in the coming period of time. The appearance of 'so far unknown' or 'completely new' pathogens will create new epidemiological situations in large regions of the world.

The one world, one health principle is extremely important, and it will be even more important in the future. Our world of highly intensified globalization, international trade and global tourism, struck by climatic changes, financial, energy and political turbulences, has become extremely sensitive and vulnerable concerning the subjects of this article: transboundary and endemic diseases, food safety and zoonoses.

If we try to outline a 5-year view, it is clear that there will be a vast need for the rapid, robust, high-throughput complex detection of known and unknown pathogens. The tendency of fervent development is clearly indicated in the listed examples of this article, which provides a short review of recent technologies that we believe will transform certain areas in molecular diagnostic virology. This field will continue to develop rapidly in the coming years, with many novel approaches for virus detection and characterization. Highly specific single-pathogen identification assays and very complex, wide-pathogen-range, high-throughput diagnostic panels will be simultaneously developed and applied for prompt detection and identification of pathogens.

The diagnoses will be extended to complex questions of 'infection biology', where not only 'known pathogens' will be detected in complex diagnostic panels, but also 'so far unknown agents', which may influence the health conditions of animals and humans by themselves, or in synergetic effects with other infectious agents, creating a very complex scenario of infection biology. Panels, such as wide microarrays, will be developed, which detect not only infectious agents, but investigate their populations and subpopulations, evolutionary aspects, in connection to the immune defence capacities of the hosts, such as cytokine expressions, or genetic, nutritional and other factors of complex disease development. Special attention will be paid to the development of complex diagnostic panels to detect emerging and re-emerging diseases, as well as maladies connected to climatic changes. Simultaneously, research will be conducted on the effects of agents, which are probably not direct pathogens by themselves but, in combination with other factors, are certainly capable of initiating or aggravating complex disease scenarios.

The wide range of methods of pathogen detection and identification outlined in this article serves as a stable basis for method development in the coming years. A wide range of additional methods of direct and indirect virus detection will be developed, based on further technological approaches, such as nanotechnology and other powerful technical principles. By using all these novel technologies to address the listed wide range of problems, there is a hope that the one world, one health principle will be followed and the quality of human and animal life will be improved.

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## Key issues

- Key issues in trends in viral diagnosis and epidemiology include:
  - The early and rapid diagnosis and control of disease pathogens – preferably before the onset of disease, or an outbreak.
  - Real-time confirmation of disease suspicion between field and laboratory.
  - Transfer of appropriate and sustainable technologies.
  - Real-time implementation of control measures based on the timely diagnosis and confirmation of diagnosis.
  - Harmonization and quality control of molecular diagnostic assays to ensure that tests used are fit for their intended purpose and that results produced are globally comparable.
  - Portable technologies, both simple and sophisticated, are poised to make an impact on the diagnosis of viral diseases as well as the way in which epidemiological data can be obtained.
  - New technologies, such as microfluidic-based PCR and rapid sequencing, will provide massive quantities of data. One challenge will be the managing and interpretation of this information.
  - The present advances in technologies are in amplification technologies, such as PCR, to achieve higher sensitivity and specificity.
  - Future advances will be in direct detection technologies – the detection of a virus on molecular level without the need to amplify its genetic material.

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