Chapter 36
Recent Advances in Veterinary Diagnostic Virology: Report from a Collaborating Centre of the World Organization for Animal Health (OIE)

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Introduction

Infectious diseases have a very high impact on animal and human health and welfare today, despite of strong efforts and good results in diagnostics, vaccine developments and control measures, including the early warning systems. There are many reasons, which contribute to the spread of infectious diseases, such as the open borders of the European Union, allowing rather free movement of animals over a whole continent, the globalization, the released and accelerated international and national trade and animal transfer. Simultaneously, the emergence and re-emergence of new or already known pathogens is a serious issue in veterinary and in human medicine. This scenario is clearly illustrated by the regular occurrence of transboundary animal diseases (TADs), such as foot-and-mouth disease (FMD), classical swine fever (CSF), African swine fever (ASF), among others. The recent occurrence of African swine fever in the Caucasus region and the spread afterwards to large territories of Russia clearly illustrates that our health authorities require a

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very strong preparedness, including prompt and powerful diagnosis, for the successful fight against the novel scenarios.

Considering these requirements the European Union (EU) is strongly supporting the development of powerful novel diagnostic methods, which are assured by international comparison, standardization and validation. Thus, the EU has launched and supported a large range of various projects for these purposes recently.

At the *World Organization for Animal Health (OIE) Collaborating Centre for the Biotechnology-Based Diagnosis of Infectious Diseases in Veterinary Medicine*, at the National Veterinary Institute (SVA) and the Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden, we have participated/participate in a high number of these projects, such as LAB-ON-SITE, EPIZONE, FLUTRAIN, FLUTEST, CSFV_goDIVA, ASFRISK, ConFluTech, AsemDialog, among others. As an example, the LAB-ON-SITE project (coordinated by SB) strongly contributed to the development of veterinary diagnostic virology. Ten infectious TADs, listed as notifiable to the OIE—FMD, swine vesicular disease (SVD), vesicular stomatitis (VS), CSF, ASF, bluetongue (BT), African horse sickness (AHS), Newcastle disease (ND), highly pathogenic avian influenza (HPAI) and swine influenza (SI)—were the subjects of this international research project of the EU. A range of new diagnostic assays were developed to to improve the current detection of the targeted ten viruses, such as novel real-time PCR assays, isothermal amplification methods, padlock probes, and novel ELISA systems. In order to improve the front-line, on-site diagnosis in the field, simple methods and equipment were adapted, such as portable PCR machines, simple thermo-platforms for isothermal amplification, dipsticks and lateral flow devices. Many of the developed methods were compared in the large international consortium, validation was performed and standard operating procedures (SOPs) of the new methods were disseminated via international organizations, such as the OIE with its 178 member countries and the International Atomic Energy Agency (IAEA) with its large networks in Asia, Africa and South America. Honouring the strong output, this project was considered as one of the Success Stories in FP6 of the European Union (http://ec.europa.eu/research/agriculture/success_labonsite_en.htm).

Considering the above-listed scenarios and requirements, *the prompt detection and very rapid and exact identification of various pathogens* is a very important and essential task in veterinary virology. While classical diagnostic methods, such as virus isolation, remain technically unaltered or show rather little steps of changes and development, the molecular diagnostic methods have advanced dramatically in the last decades. These new techniques provide powerful novel tools for the rapid detection and identification of a wide range of causative agents, as well as for supporting disease control and surveillance. For example, real-time quantitative PCR assays are highly sensitive and specific methods, which are widely used for detection of various pathogens in diagnostic units, including the Reference Laboratories and the Collaborating Centres of the World Health Organization (WHO; http://www.who.int) and of the World Organization for Animal Health (OIE; www.oie.int).

At the *OIE Collaborating Centre for the Biotechnology-Based Diagnosis of Infectious Diseases in Veterinary Medicine*, Uppsala, Sweden, great efforts have been made for the development, standardization and dissemination of new diagnostic

assays within the frame of several large EU and other projects in the recent years. Some achievements have been summarized in previously published review articles [1, 2]. Herewith, an update and selected examples are given in order to illustrate the trends of recent advances in veterinary diagnostic virology at our OIE Collaborating Centre, in collaboration with a wide network of partner laboratories in Europe and on other continents.

Sample Collection, Transportation, Storage, Enrichment and Nucleic Acid Preparation

Sample Collection, Preparation and Transportation

Proper sample collection, storage, transportation and enrichment are crucial for the reliable diagnosis of infectious diseases. To avoid potential degradation of the targeted nucleic acids, the samples are normally transported at low temperature using dry ice, cooling batteries and/or are stored in −20/70 °C freezers before further processing. Heat- and/or chemical inactivation of samples is required to avoid the transmission and spread of infectious agents. In order to diagnose the various known and "unknown" diseases in a safe and reliable way, proper methodologies are needed, which simplify the handling, processing of the samples, and the transport to the diagnostic laboratories. Currently, several simple tools, such as different filter papers or cards are commercially available for such purposes. For example, FTA™ Cards, produced by Whatman plc, now a part of GE Healthcare, are suitable for the collection, transportation and storage of samples at room temperature. The company claimed that such FTA Cards contain chemicals that lyse cells, denature proteins and protect nucleic acids from nucleases, as well as from the damages caused by heat, oxidative and UV effects. At the Collaborating Centre, our colleagues have used these types of cards for transportation of samples from other countries and continents to Sweden. For example, ASF serum samples were collected in Uganda, placed on the filter papers, and transported to Sweden at room temperature. The same type of filter papers was used for transportation of Peste des Petits Ruminants (PPR) samples from Pakistan. Bat coronavirus samples were sent from Brazil to Sweden in an envelope, by post. Viral nucleic acids, eluted from the cards, are successfully amplified by various PCR assays or detected by other means of molecular diagnostic virology. We perform real-time PCR assays, using small pieces of cards containing samples as templates and various viruses are detected in a convincing way after this simple way of sample transportation.

Sample Enrichment

The diagnosis in veterinary virology is frequently complicated by the fact that the amount of targeted viruses in the certain types of clinical samples, in food and feed products or in water samples, as well as in other diagnostic specimens, is often very

low. The low target content may lead to false negative results in the various PCR assays and in other molecular diagnostic procedures. In order to avoid or to reduce this very important bottleneck effect, diagnostic laboratories develop and apply a range of sample enrichment methods, in order to "fish out" the targeted pathogens or their components, such as nucleic acids or proteins, from the analyzed specimens. For example, DNA enrichment is achieved by functionalized magnetic nanoparticles that are coupled with probes to which the target DNA could be hybridized [3].

Nucleic Acid Preparation Processes

The proper preparation of viral nucleic acids as targets of PCR and other molecular diagnostic assays is also a very important task in the diagnostic laboratories. Simplicity and high-throughput capacity are major concerns in case of outbreaks where a huge number of samples are processed within a relatively short period of time. It is almost impossible to complete such a task by manual extraction methods such as TRIzol reagent or spin columns. The manual extraction processes are labour- and time-consuming and expensive. In addition, personnel costs and working processes have to be considered. For those reasons, various kinds of automated equipment have been developed and commercialized for nucleic acid preparation and/or handling of samples. At our OIE Collaborating Centre the automated nucleic acid extraction steps are performed with robotic extraction machines.

International Comparison and Standardization

In an exercise to compare the performance of nucleic acid extraction robots (12 separate instruments, comprising eight different models) in five European veterinary laboratories, similar results were observed from best performing robots when dilutions of a cell culture supernatant were tested, whereas up to 1,000-fold difference was obtained from less optimized robots when dilutions of a serum sample were tested [4]. It was observed that the same instrument performed differently when tested with different types of samples. Therefore, a proficiency test or "ring trial" of different clinical samples would be of great help to validate in-house assays, identify critical steps and improve performance of downstream PCR tests.

Real-Time Quantitative PCR Assays

Since the publication of quantitative real-time PCR in 1996 [5], it has become a very reliable, powerful, high-throughput and robust molecular tool for early, rapid and sensitive detection of pathogens in both human and veterinary medicine. Compared to the conventional gel-based PCR, the real-time PCR has several advantages, including high-throughput capacity, less hands-on time, lower risk of contamination,

and the potential to be fully automated [6]. By using a gene-specific probe, the specificity and analytic sensitivity of the real-time PCR may be further improved. The research activities at our Collaborating Centre aimed to develop real-time PCR assays for diagnostics to differentiate infected from vaccinated animals (DIVA), for specific detection of emerging viruses, and for detection of zoonotic agents, in particular water- and food borne pathogens.

Herewith, several examples are presented regarding specific problems, illustrating the diagnostic application of various real-time quantitative PCR assays developed and used at our OIE Collaborating Centre, in collaboration with other European veterinary institutes in various research projects of the European Union. Further important achievements are also listed here, for example the development of real-time RT-PCR assay using a minor-groove binder probe for the pan-serotypic detection of *foot and mouth disease virus* (FMDV) [7] and the application of loop-mediated isothermal amplification (LAMP) for detection of wild type *classical swine fever virus* (CSFV) [8] and vaccine strain [9], and *swine vesicular disease virus* (SVDV) [10].

Real-Time PCR Assays for the Detection of Various Variants of Classical Swine Fever Virus, Including Vaccine Strains

CSFV is a pestivirus within the family of *Flaviviridae*. It is the causative agent of classical swine fever (CSF), a highly contagious disease affecting both wild boars and domestic pigs. In Europe, the virus is largely maintained in the wild boar populations that serve as a reservoir for reintroduction to domestic pigs. Recently, a chimeric vaccine candidate, CP7_E2alf has been developed and has the potential to be used as a safe and efficient marker vaccine in wild boars, which enables differentiation of infected from vaccinated animals [11, 12]. A vaccine-specific TaqMan real-time RT-PCR assay was developed and evaluated, and a second, wild type-specific assay was modified from an established one in such a way that both can be performed in two wells side-by-side in a microplate in a single run [13]. Both assays could be applied in CSFV vaccination and control programs in the wild boar population.

DIVA Approaches

CSF remains endemic in domestic pigs in some countries, for example, China, where vaccination with the traditional lapinized live virus is still in practice. DIVA diagnostics is a potentially powerful tool to discriminate whether pigs are naturally infected with wild type strains or vaccinated with the lapinized live virus. The Chinese Hog Cholera Lapinized virus (HCLV) has been through extensive serial passages in rabbits in China in 1950s and is completely attenuated, but retains its efficacy as vaccine. Due to its safety and efficacy, this vaccine was introduced into European countries and named as “Chinese” strain (C-strain). “Riems” C-strain is one of the commercial vaccines derived from the Chinese strain. Development of a vaccine-specific real-time RT-PCR would be of great value, which could be used in

parallel with a wild type-specific or generic real-time RT-PCR for detection and differentiation of wild type viruses from vaccine strains. Leifer et al. studied differentiation of Riems C-strain-vaccinated pigs from animals infected by CSFV field strains using real-time TaqMan RT-PCR [14]. Leifer et al. also reported escape of CSF C-strain vaccine virus from detection by C-strain specific real-time RT-PCR caused by a point mutation in the primer-binding site [15]. At the Collaborating Centre, we have developed a real-time PCR based on primer-probe energy transfer technology (PriProET) for the improved detection of CSFV [16]. The PriProET technology was developed initially as a novel quantitative real-time PCR assay for the simultaneous detection of all serotypes of FMDV [17], and subsequently was used for detection of other pathogens, including SVDV [18], and *bluetongue virus* (BTV) [19]. Following PCR amplification, the melting curve analysis allows confirmation of specific amplicons, and differentiation between wild type CSFV and the HCLV vaccine strain (Fig. 36.1). Further evaluation of the assay demonstrated that in an RNA mixture of both wild type CSFV and C-strain vaccine, the melting curves displayed only one curve either a wild type-like or a vaccine-like depending on the dominating RNA [20]. Therefore, the PriProET melting curve analysis could identify the presence of CSFV field strain in equivocal samples or in animals vaccinated with C-strain, but would not reliably detect infections with wild-type virus in a population vaccinated with the Riems strain. In a recent study, a generic real-time TaqMan RT-PCR was developed for the specific detection of three lapinized vaccine strains, namely, the Taiwanese Lapinized Philippines Coronel (LPC), the Chinese HCLV, and the European Riems C-strain [21]. The new assay was able to detect the Riemser C-strain vaccine viral RNA in experimental samples, indicating that the assay could be a useful tool to facilitate outbreak control if a strategy using these lapinized vaccines is deployed.

Real-Time PCR Assay for the Detection of the Recently Detected Atypical Bovine Pestiviruses

Over the past few years, new bovine pestiviruses have been detected in biological products, e.g., foetal bovine serum (FBS) batches, and in naturally infected cattle. Due to nucleotide sequence variations, the identification of these viruses might fail, since primers and probes had mismatches to their target, leading to a false negative result. For example, the well-known "pan-pestivirus" primer pair 324/326 failed to react with D32/00_'HoBi' virus that was found in a batch of contaminated foetal calf serum [22]. To overcome the limitations of current molecular methods, a new one-step real-time TaqMan RT-PCR assay was developed for the specific detection of atypical bovine pestiviruses in clinical samples and in biological products [23]. This new assay provides a useful tool for highly sensitive and specific detection of atypical bovine pestiviruses and can be applied in combination with other diagnostic methods to ensure that biological products, including FBS and vaccines, are free from pestivirus contamination. Recently, we have tested 33 batches of commercial

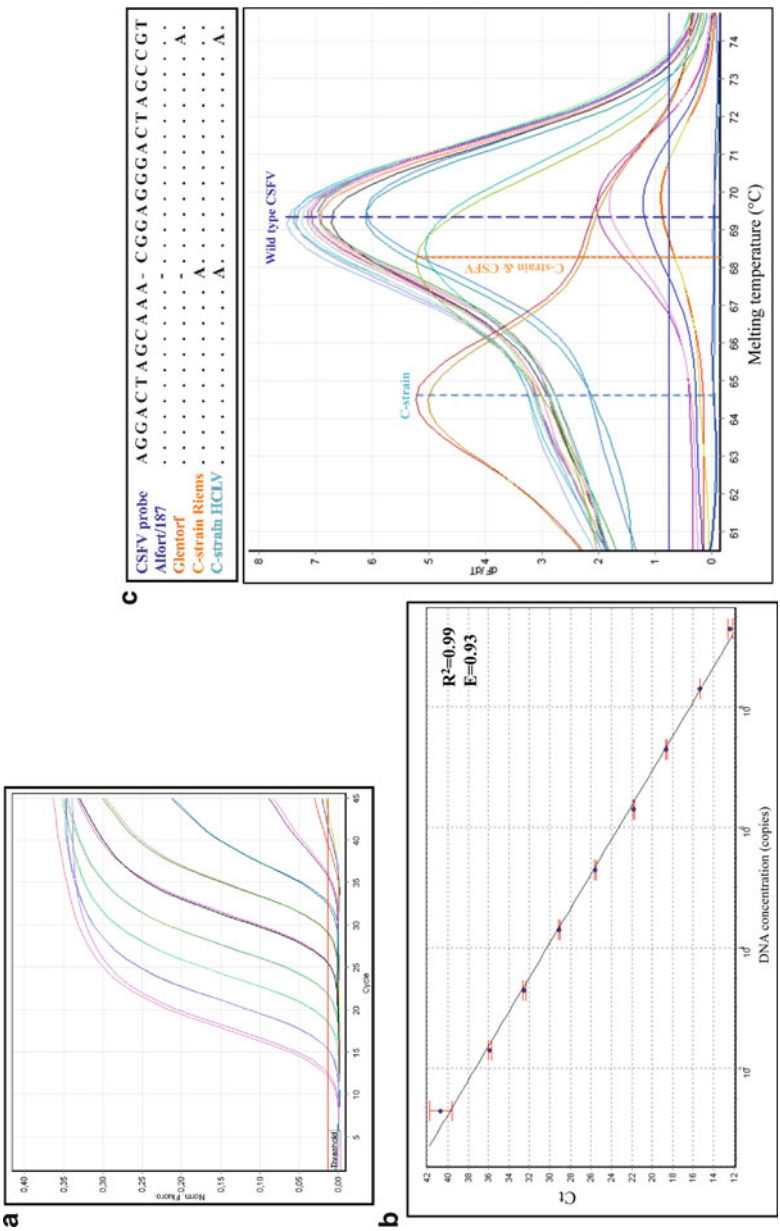


Fig. 36.1 A real-time RT-PCR assay based on primer-probe energy transfer technology for the improved detection classical swine fever virus. RNA standards containing the amplicon were ten-fold diluted in water and tested in duplicate. The results are demonstrated in amplification curve (**a**), linearity (**b**), of the PCR step for detection and melting curve analysis (**c**) to differentiate wild type viruses from the Chinese strain (C-strain) vaccine. Reprint from ref [16], with permission. Copyright © 2009, Elsevier

FBS by this approach and found all batches were positive for at least one species of bovine pestiviruses. The consequence of using contaminated FBS in diagnostic laboratories is detrimental. Isolation of bovine viral diarrhea virus (BVDV) in bovine turbinate cells is a routine method, which requires FBS during preparation of the cell cultures. A batch of contaminated FBS of South American origin had disturbed virus isolation. Tests showed presence of both the recognized species and the newly described atypical bovine pestivirus in the FBS batch. The contaminating atypical pestivirus was further characterized, as it is described by our group [24].

Broadly Targeting Real-Time RT-PCR Assays for the Generic Detection of Viruses

Apart from pathogens only affecting animals, a high number of pathogens can cross the species barrier(s) and cause diseases in humans, which is referred as zoonosis. Of more than 1,400 human pathogens, approximately 60 % are zoonotic, of which 25 % are estimated being able to be transmitted from human to human [25]. Therefore, it is of great importance to develop broadly targeting real-time RT-PCR for the generic detection of zoonotic viruses at our Collaborating Centre. Herewith, several examples are described.

Coronaviruses are the causative agents of a broad spectrum of animal and human diseases, including the Severe Acute Respiratory Syndrome (SARS) in humans. Considering this scenario, it is very important to develop very "general coronavirus" PCR assays, which are able to detect a wide range of viruses in various hosts simultaneously. In order to fulfil these requirements, we have developed a pan-coronavirus real-time RT-PCR, which is able to detect 36 strains of animal and human coronaviruses simultaneously, including HCoV-NL63 and SARS-CoV [26]. In this PCR panel the degenerated primers are targeting a conserved region of the ORF1b of the viral and have a low annealing temperature. We have compared this real-time RT-PCR system with two published TaqMan assays for detection of four animal coronaviruses in 75 clinical samples, and obtained the same results.

It has to be mentioned that eventhough this SYBR Green-based PCR assay is very promising in the general detection of coronaviruses, it also has weakness with regards to its specificity. Apart from the coronavirus positive samples, primer dimers and non-specific amplicons may also produce amplification curves and this can lead to false positive results. In order to avoid false diagnosis, melting curve analysis is needed in this assay. The melting curve analysis is a practical tool to verify the truly positive results. In summary, it was concluded that this SYBR Green-based real-time pan-coronavirus PCR assay, along with melting curve analysis, provides a powerful novel tool for the laboratory diagnosis of coronavirus infections and for detecting the emerging, yet to be uncharacterized new coronavirus variants. Considering the strong emergence rate of many RNA viruses, including the coronaviruses, the use of such pan-virus family PCR assays is very important. The new assay provides a novel tool for our diagnostic laboratories in veterinary and in human virology.

Concerning other real-time PCR approaches, such as the TaqMan principle, we have found that it is challenging and difficult to develop a pan-coronavirus detecting system using a single probe. Even though the genomic stretches are relatively conserved, it is almost impossible to design a TaqMan probe for efficient general detection of coronaviruses. To overcome this limitation, three overlapping TaqMan probes were designed and used together with the above-mentioned primers for sensitive and specific detection of coronaviruses of all three groups [27]. We have found this three-probe approach reduced the degree of probe degeneration and maintained a high sensitivity and specificity. The assay detected coronaviruses in human nasopharyngeal aspirate samples and in duck faecal samples. Furthermore, this method was able to detect SARS-CoV in a reference material, and detect the synthetic sequences of bat coronaviruses (HKU3-CoV, HKU5-Cov and HKU9-CoV), indicating it is a useful tool for screening samples of animal origin for zoonotic or potentially zoonotic pathogens. The assay was used as a tool for epidemiological studies on coronaviruses in wild birds from the Bering Strait area [28].

Molecular Tests for the Improved Detection of Food- and Waterborne Zoonotic Pathogens

Food- and waterborne zoonotic pathogens frequently cause large outbreaks in regions where sanitation is poor. *Hepatitis E virus* (HEV) is an important food- and waterborne zoonotic pathogen, which is an emerging virus of global importance. This single-stranded, positive-sense RNA virus belongs to the genus *Hepevirus* in the family of *Hepeviridae* that includes at least four recognized genotypes. While genotypes 1 and 2 are associated with large outbreaks and epidemics in humans in developing countries with poor sanitary conditions, genotypes 3 and 4 HEV strains are able to infect both humans and various animal species, in particular, domestic pigs and wild boars, and are responsible for sporadic cases of acute hepatitis E not only in the developing, but also in the industrialized countries. It has been recognized that hepatitis E is a zoonotic disease. At our OIE Collaborating Centre, Gyarmati et al. developed two real-time PCR assays for universal detection of all four genotypes of HEV [29]. Both the TaqMan and PriProET assays were able to detect 20 viral genome equivalents per reaction. The results obtained from both real-time PCR assays were comparable to those from a nested gel-based PCR. However, the TaqMan assay had higher reaction efficiency and performed slightly better than the PriProET assay. Such tools are important not only for veterinary diagnostics but also for molecular epidemiology of HEV in humans, pigs and wild boars [30].

Proximity Ligation Assays

It is not widely known yet that real-time PCR assays can be used not only for the detection of viral nucleic acids, but *also for the detection of protein components or*

antigens of pathogens. This technique is referred to proximity ligation assay (PLA) [31]. In a proximity ligation assay, two oligonucleotides are coupled to antibodies. Binding of the antibodies to the same target will bring the two oligonucleotides sufficiently close to each other (or in proximity) such that the two ends could be ligated with the help of a third connector oligonucleotide. The ligation products are then used as target DNA for subsequent amplification and detection by real-time PCR. At the OIE Collaborating Centre in Uppsala, Schlingemann et al. used this technique to detect antigens of avian influenza viruses in biological samples [32]. We have found that the method is four orders of magnitude more sensitive than a sandwich ELISA, which utilized the same antibody. This study opens a new window for diagnostic virology, by using PLA for the highly effective detection of viral proteins in clinical specimens.

Suspension Microarray Technologies

Although real-time RT-PCR offers multiplex detection of pathogens, its capacity is limited to no more than approximately five targets in a single diagnostic block. By contrast, suspension microarray technology, such as Luminex xMAP technology (Luminex Corporation, Austin, TX) has the capacity of *detecting up to 100 targets in a single platform, or even more*. At our OIE Collaborating Centre a range of Luminex assays have been developed for the multiplex detection of pathogens important in human and veterinary medicine. A suspension microarray assay was constructed for detection and differentiation of pestiviruses including CSFV, BVDV-1, BVDV-2, *border disease virus* (BDV) and the atypical bovine pestivirus [33]. Following PCR amplification of target genetic region, one strand of the amplicons was removed by Lambda exonuclease to generate single-stranded PCR products for efficient hybridization to the microsphere-coupled oligo probes. After incubation with a reporter dye streptavidin-R-phycoerythrin, the median fluorescent intensity (MFI) of each reaction was measured in a Luminex instrument. The system was able to provide positive/negative results and to identify each species by comparing MFI values. Such an assay is a useful tool for the genetic detection of various pestiviruses in cattle.

Another Luminex suspension microarray assay was developed at our laboratories for the serological diagnosis of bovine viral diarrhoea (BVD) [34]. In this blocking system, microspheres were coupled with a monoclonal antibody (MAb) and were incubated with viral antigen and serum samples. After addition of another MAb that is biotinylated, R-phycoerythrin-conjugated streptavidin was added and the MFI values were measured on a Luminex 200 analyzer. This blocking microsphere-based immunoassay had intra- and inter-assay variability of 4.9 % and <7 %, respectively, and variability of bead conjugations of <6.6 %. By testing a total of 509 serum samples, the assay had a sensitivity of 99.4 % and a specificity of 98.3 % relative to a commercial blocking ELISA. The new microsphere immunoassay provides an alternative to conventional ELISA systems and can be used for high-throughput screening in the BVD control and eradication program.

As BVDV is one of the pathogens causing bovine respiratory disease (BRD) complex, our ongoing work at the Collaborating Centre is to develop a multiplex Luminex assay for the high-throughput, simultaneous detection of antibodies against four major viruses that cause BRD, namely, BVDV, *bovine respiratory syncytial virus* (BRSV), *parainfluenza type-3 virus* (PIV3) and *bovine coronavirus* (BCoV). This novel multiplex assay not only allows a higher sample throughput but also reduces the time and labour required. The Luminex platform has a strong potential to replace ELISA as a powerful novel system for the complex diagnosis of pathogens.

Genome Sequencing and Viral Metagenomics, Detection of "Unknown" or Emerging New Viruses in Veterinary Medicine

A pathogen's genome can be determined by various methods, such as the traditional cycle sequencing and by the advanced "next-generation sequencing" technologies. While the cycle sequencing is still the method of choice in most laboratories, next-generation sequencing is becoming available in some laboratories. Both methods are a powerful tool in veterinary diagnostic virology. Furthermore, genome sequencing will contribute substantially to a better understanding of pathogens, which would be helpful in veterinary diagnostic virology. Particularly, viral metagenomics is a generic technology using large-scale sequencing to identify viral genome sequences without prior knowledge. Viral metagenomics has helped researchers with the *investigation of complex diseases, diseases of unknown aetiology, and identification of emerging novel viruses in samples.* In the followings, several examples are given on the use of viral metagenomics at our OIE Collaborating Centre to detect new and/or emerging and re-emerging viruses in animals.

Detection of Novel Viruses in the Postweaning Multisystemic Wasting Syndrome (PMWS) of Pigs

PMWS is a viral disease worldwide affecting postweaning pigs of about 2–4 months of age. *Porcine circovirus type 2* (PCV-2) has been found to play an important role in the complex infection biology of PMWS. PCV-2 is a small circular DNA virus belonging to the family *Circoviridae.* The virus is ubiquitously distributed in almost all pig populations, not only in PMWS pigs but also in clinically healthy ones. Therefore, it is hypothesized that other known or unknown factors may have a contribution to the development of PMWS. Aiming to find possible factors contributed to PMWS in piglets, our group tested lymph node samples that were collected from Swedish pigs with confirmed PMWS, by using GS-FLX 454 technology for large-scale sequencing of randomly amplified products [35]. Analysis of about 9,000 unique sequences obtained from the 454 run showed presence of PCV-2, *Torque Teno virus* genotypes 1 and 2 (TTV-1 and TTV-2), as well as a novel

parvovirus-like agent, termed as porcine boca-like virus in the lymph nodes from the diseased pigs. Based on this study, Blomström et al. screened 34 PMWS pigs and 24 animals without PMWS by PCR for the presence of PCV-2, TTV-1, TTV-2 and porcine boca-like virus [36]. PCV-2 was found in all PMWS pigs and in 80 % of pigs without PMWS. Presence of all three viruses TTV-1, TTV-2 and porcine boca-like virus was found in 71 % of the PMWS pigs and in 33 % pigs without PMWS. The exact role of these viruses, particularly the porcine boca-like virus in the development of PMWS requires further investigations.

Detection of Novel Astroviruses in a Neurological Disease of Minks, Termed Shaking Mink Syndrome

At our OIE Collaborating Centre viral metagenomic approaches, including the GS-FLX 454 technology was also used to investigate the cause of a neurological disease of minks termed *shaking mink syndrome*. The disease was first observed in farmed mink kits in Denmark in 2000 and subsequently in Sweden, Denmark and Finland in 2001, and in Denmark again in 2002 [37]. General virological investigations, including virus culture, negative-staining electron microscopy, immunoelectron microscopy, broad targeting PCR for herpesviruses, adenoviruses, pestiviruses and coronaviruses, and specific tests for six viral diseases, for three protozoa, for bacteria and intracerebral inoculation of neonatal mice were conducted but with negative results. It was postulated that it is likely that the disease was caused by a yet unidentified virus [37]. To investigate the cause, brain samples from experimental infection were prepared for nucleic acid extraction and random amplification and large-scale sequencing using GS-FLX 454 technology [38]. Analysis of the 454 sequencing data revealed eight sequence fragments similar to mink astrovirus. Based on the result, new primers were designed in order to determine the nucleotide sequences of the complete viral genome. The comparative analysis of complete genome sequences showed a similarity of 80.4 % to that of a mink astrovirus causing pre-weaning diarrhoea in mink. As the virus was not detected in healthy mink kits, we suppose an association between the astrovirus and the neurological disease of mink.

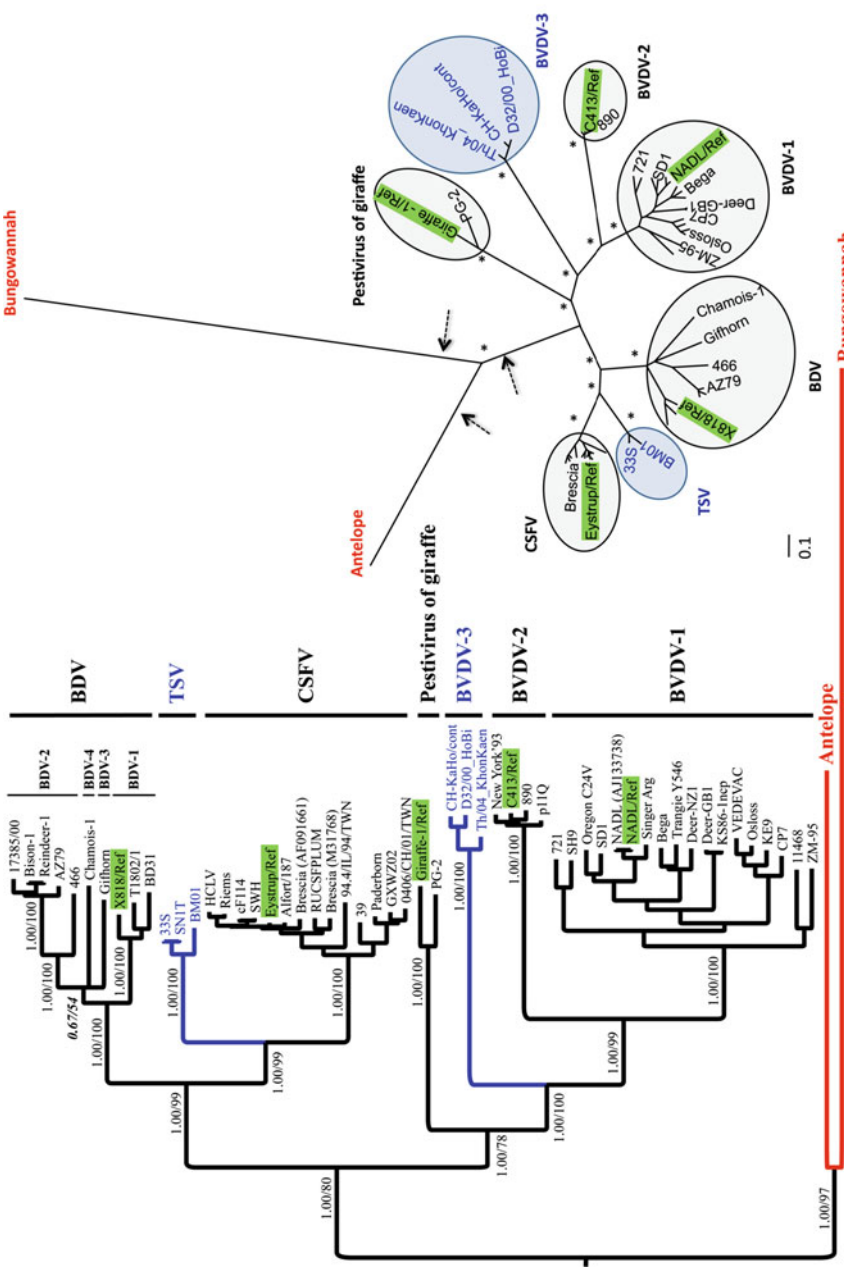
Genetic Characterization of Novel Bovine Pestiviruses in Biological Products, Such as Foetal Bovine Serum

Genome sequencing and subsequent phylogenetic analysis have been considered as important tools for the exact identification of the “unknown” or emerging new pathogens. From the initial discovery of the pestivirus D32/00_‘HoBi’ in a batch of contaminated FBS, it was unclear how this virus is related to the two recognized bovine pestivirus species: *bovine viral diarrhoea virus* 1 (BVDV-1) and *bovine viral diarrhoea virus* 2 (BVDV-2). The answer to this question has implication in veterinary diagnostic virology as well as in the BVD control and eradication program in various countries. Phylogenetic analysis of three genetic regions showed three different

relationships to both BVDV-1 and BVDV-2 [22]. To unequivocally solve the relationship, the pestivirus strain Th/04_KhonKaen was recovered from a serum sample of a naturally infected calf and the complete genome sequence was determined [39]. Phylogenetic analysis of complete genome sequences by Neighbor-joining, maximum likelihood, and the Bayesian approach, unanimously placed Th/04_KhonKaen in a single lineage, distinct from the established pestivirus species, and close to BVDV-1 and BVDV-2. A further evolutionary study proposed to term these newly described bovine pestiviruses as BVDV-3 (Fig. 36.2) [40].

Summary

During the last decades substantial progress has been made in veterinary diagnostic virology at our laboratories at the National Veterinary Institute (SVA) and the Swedish University of Agricultural Sciences (SLU) in Uppsala, Sweden. As the *World Organization for Animal Health (OIE) Collaborating Centre for the Biotechnology-Based Diagnosis of Infectious Diseases in Veterinary Medicine*, we have developed and tested a wide range of novel molecular diagnostic methods. Many of these novel assays provided powerful novel tools for the improved detection of viruses in veterinary and human medical virology. A wide range of the novel molecular diagnostic methods has been internationally compared in ring tests and validated. In order to illustrate this trend of development, several examples are summarized in this chapter. For molecular methods, upstream *nucleic acid extraction* is crucial for the success of the downstream diagnostic tests. We came to the conclusion that the *real-time PCR* principle, using different chemistries, such as TaqMan and PriProET, provides very reliable, highly sensitive and specific novel diagnostic tools for the direct detection of a wide range of pathogens, as it is demonstrated here with several examples. Simultaneously, the real-time PCR technology allows the development of *novel DIVA tests*, which are highly required for the improved control of infectious diseases, using marker vaccines and accompanied diagnostic packages. In parallel, high-throughput *suspension microarray technologies* enable the simultaneous detection and identification of multiple pathogens in single test platforms. The liquid-phase microarray platforms, such as Luminex panels, are accelerating the detection of emerging animal viruses and zoonotic, in particular, the water- and foodborne pathogens. *Proximity ligation* assay has emerged as a novel method for the highly sensitive and specific detection of the viral proteins. *Viral metagenomics and large-scale genome sequencing* establish powerful tools for the detection of "unknown" viruses, as well for the identification of emerging and re-emerging pathogens. These novel approaches strongly support the investigation of disease complexes and/or emerging novel disease scenarios in veterinary diagnostic virology, with regard to diseases in domestic animals and in wildlife, with special regard to zoonotic infections, by following the principles of "One World One Health."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C413/Ref
890
BVDV-2
721
SD1
NADL/Ref
Bega
Deer-GB1
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Osloss77
ZM-95
BVDV-1
Chamois-1
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Bison-1
Reindeer-1
AZ79
466
Chamois-1
Gifhorn
X818/Ref
T1802/1
BD31
33S
SN1T
BM01
HCLV
Riems
cF114
SWH
Eystrup/Ref
Alfort/187
Brescia (AF091661)
RUCSFPLUM
Brescia (M31768)
94.4/IL/94/TWN
39
Paderborn
GXWZ02
0406/CH01/TWN
Giraffe-1/Ref
PG-2
CH-KaHo/cont
D32/00_HoBi
Th/04_KhonKaen
New York'93
C413/Ref
890
p11Q
721
SH9
Oregon C24V
SD1
NADL (AJ133738)
NADL/Ref
Singer Arg
Bega
Trangie Y546
Deer-NZ1
Deer-GB1
KS86-1ncp
VEDEVAC
Osloss
KE9
CP7
11468
ZM-95
Antelope
Bungowannah
0.3
1.00/100
1.00/100
0.67/54
1.00/100
1.00/100
1.00/100
1.00/99
1.00/100
1.00/99
1.00/100
1.00/100
1.00/100
1.00/78
1.00/100
1.00/100
1.00/99
1.00/100
1.00/80
1.00/97

Fig. 36.2 Phylogenetic analysis of pestiviruses by maximum likelihood and Bayesian approach. The molecular dataset contains 56 sampled pestiviruses and 2,089 characters, comprising the 5′UTR, N^{pro} and E2 gene regions. PHYML (v2.4.4) was used for phylogeny inference according to maximum likelihood criterion. MrBayes 3.1 was used for Bayesian analysis. This is a representative consensus tree: mid-point rooted (*left*) showing all sampled pestiviruses and their relationships, and unrooted (*right*). The reference sequences are highlighted in *green*. The new species proposed in this study are in *blue* (BVDV-3 and TSV) and in *red* (Antelope and Bungowannah). The numbers at a node are posterior probability (*left*) and percentage of 1,000 bootstrapping replicates (*right*). A "*" indicates strong statistical support for a node by a posterior probability value of 0.99–1.00 and by a bootstrap value of 78–100 %. The scale bar represents changes per site. The *arrows* show the probable placements of the root for the given unrooted network. Reprint from ref [40], with permission. Copyright © 2009, Elsevier

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