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PROJECT FINAL REPORT

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4.1 Final publishable summary report

Infectious animal diseases can have devastating effects on sustainable food production and the livelihood of rural communities throughout the world. For this reason, rapid and reliable detection and control tools are crucial to limit the impact of disease outbreaks and their spread. This is particularly difficult to realize in remote rural areas and in regions with only basic laboratory infrastructure, e.g. in Africa or some parts of Asia.

The RAPIDIA-FIELD project set out to develop and validate test systems and methodologies that could help to overcome those limitations through diagnostic systems that are suitable for non-expert use. In particular, technologies were targeted that allow pathogen and antibody detection directly under field conditions (pen-side) or in very elementary laboratories with basic equipment and limited resources. These test systems were linked to an early warning set-up, simple sampling schemes, processing workflows, and confirmatory tests that could be employed in sophisticated laboratories. Important notifiable diseases such as African swine fever (ASF), bluetongue (BTV), epizootic hemorrhagic disease (EHD), classical swine fever (CSF), foot-and-mouth disease (FMD), avian influenza, and African horse sickness (AHS) were used together with endemic diseases like porcine respiratory and reproductive syndrome (PRRS) as extendable proof-of-concept models, and resulting assays were validated with the help of partners and the Field-Net-Group.

Within the project, the above mentioned tasks were subdivided into 10 work packages (WPs) whereby WP1 dealt with the project management, and WPs 9 and 10 targeted standardization and dissemination of results, respectively.

Within WP2, an early warning component, i.e. a computer-assisted non-invasive health monitoring (real-time monitoring system online, RTMS-ON) was set up. The system uses behavioral changes and body temperature as parameters for an alarm system and the approach was successfully implemented under experimental conditions and in sentinel farms.

In the framework of WP3, sample collection and processing workflows were assessed that could be deployed under basic conditions. Among these were combinations of swabs samples, direct PCR, and nucleic acid lateral flow (NALF) detection. Moreover, pooling options were explored, and a virtual sample repository for assay validations was created.

A major achievement of WP4 was the development and validation of multiplex lateral flow devices (LFD) for the detection of ASF and CSF antibodies. The test was intensively assessed through an inter-laboratory comparison test. Moreover, LFDs for AHS/EIA antibodies and BTV/EHDV antibodies were developed and subjected to initial validation.

Within WP5, different PCRs were adapted to portable systems, and different isothermal amplification methods were employed. Among these adaptations was the use of the Enigma FL instrument for the detection of FMDV. This system combines nucleic acid extraction and real-time PCR. The results generated under field conditions suggest that the machine can accurately detect FMDV in a range of clinical samples from acutely infected as well as convalescent animals.

WP6 targeted antibody detection in small field labs and brought about prototype Q-ELISAs for the detection of CPV/CDV, LFD-COMB systems for ASFV/CSFV antibodies, and semi-quantitative LFDs for ASFV and CSFV antibodies. Furthermore, low-density antigen microarrays were established for detection of multiple pathogens.

Within WP7, pathogen detection was targeted. Here, especially the implementation of the cartridge-based ENIGMA ML machine for the detection of CSFV/ASFV and FMDV were major achievements of this project. Moreover, RPA was explored as simple, isothermal amplification method.
Among the results of WP8 was the optimization of next-generation sequencing workflows that do now allow more rapid data acquisition and evaluation.
Overall, the RAPIDIA-FIELD has brought about both antibody and pathogen detection techniques that are suitable for non-expert and warrant commercial deployment.

A summary description of project context and objectives

Prevention and control of animal diseases is of paramount importance for a sustainable production of safe and high-quality meat products and veterinary public health. Infectious diseases such as classical swine fever, African swine fever, avian influenza, Newcastle disease, bovine viral diarrhoea and border disease have shown tremendous socio-economic impact during the last decades worldwide. These diseases affect both domestic and wild animals and some of them have even zoonotic potential.
Since several of these viruses cause very similar, or even identical clinical signs clinical diagnosis is often complicated. Therefore, diagnostic tests are needed for a reliable and fast pathogen confirmation and to shorten the time elapsing between disease introduction, final diagnosis and control. However, accurate, sensitive and rapid diagnosis still remains a challenge for veterinarians. Nowadays, advances in technology enable implementing modern technologies for a quick and simple pathogen detection in basically equipped laboratories or even at pen-side level, e.g. in remote areas where human and technical resources are limited. However, many advanced techniques such as highly sensitive real-time PCR methods, high-throughput Luminex, and next-generation sequencing (NGS) are usually only available at the national reference laboratories or diagnostic centres while there is a lack of methods and tools that local veterinarians could rely on to make initial rapid tests under field conditions or close to farms, either to confirm or to exclude the presence of specific pathogens. To limit the impact of outbreaks and to take rational steps towards eradication of the diseases, rapid and reliable diagnostic systems are crucial. They provide rapid detection and confirmation, enable monitoring, and are of utmost importance for the implementation of control measures.
In order to overcome these challenges, the overall goal of the RAPIDIA-FIELD project was to develop easy-to-use, cost effective and robust diagnostic tests suitable for the use as pen-side tests and in laboratories with limited capacities. However, irrespective of the laboratory or pen-side diagnostic tools, an early recognition of diseased animals remained the initial and most critical steps of disease detection. Therefore, an early-warning system was developed that makes use of non-invasive observations of indicative parameters such as food and water consumption, body temperature and animal movement. After indications of disease would be detected, the next step would be for veterinarians to collect and prepare samples for the use in diagnostic tests and supplementary research. Acquisition of samples, especially blood or tissue biopsy samples is often a time-consuming step that requires skilled personnel. Where highly sensitive detection methods, especially for genome detection, are in place, non-invasive sampling procedures such as using oral fluid or aerosols can provide a useful method

for herd-level surveillance. Within the project, such samples were subjected to evaluation both for antigen and antibody detection. Furthermore, pre-assay processing steps to facilitate further diagnostic assays were tested and evaluated. Several assays for both pathogen or antibody detection for the detection of important animal diseases were developed and some of them are close to being brought to the market. They present a new level of validated veterinary diagnostic tools for many important diseases as well as endemic infections that impair food production in many countries. In addition, the use of simultaneous tests for several pathogens provides a new quality of diagnostic results in the field as well as in basic laboratories. Thus, the outcome contributes to the sustainable and safe production of meat and meat products through provision of better tools for the fight against devastating diseases. In terms of diagnostic tools, the focus of the project laid on multiplex assays (e.g. simultaneous detection of disease clusters that cause similar clinical syndromes) that can be applied at the point of care (pen-side) or in basic field laboratories without expensive and maintenance-intensive equipment. These tests should be simple to perform, fast, robust, inexpensive and should provide unambiguous results. Furthermore confirmation of initial field results, to generate background information for adaptation of assays, and to provide the possibility to detect unexpected variants or new pathogens, state-of-the-art technologies such as next generation sequencing, microarrays or Luminex technologies were utilized.
In summary: the primary aim of RAPIDIA-FIELD was to develop and bring to the market products and services that will contribute to Animal Health control at the field level.

In order to achieve the aims described, the work has been organized in 10 work packages (WPs) whereby WP1 was fully dedicated to the project management, while the main objectives were included in the other 9 WPs as described below:

WP2: Development and validation of a computer-aided, non-invasive, real-time monitoring system on line (RTMS-ON) that allows to measure relevant environmental and animal health parameters such as body temperature, water consumption and movement as early warning systems for animal diseases.

WP3: Development of simple-to-use methods that can be used in the field or in simple field laboratories for the collection, pre-assay processing and storage of diagnostic samples and to establish a virtual database of samples that can be shared amongst the project consortium.

WP4: Development of user friendly, low cost effective, sensitive and specific assays able to detect antibodies against multiple pathogens directly in the field. Modern Lateral Flow Device (LFD) techniques were utilized, both for detection of single pathogens and multiplex detection (e.g. for simultaneous detection of antibodies against BTV and EHDV.

WP5: Development of rapid multiplex pathogen detection methods for field diagnostic settings such as low-cost field detection devices, with proper preservation of diagnostic sensitivity and specificity/discrimination. Methods such as portable PCR systems, Loop-Mediated Isothermal Amplification (LAMP) assays and adapted LFD technologies to detect avian influenza virus (AIV) and Newcastle disease virus (NDV) as detection methods of

pestivirus infection in farm animals and for the differentiation of two haemorrhagic swine diseases, CSFV and ASFV were developed.

WP6: Development of innovative multiplex serology for the detection and quantification of antibodies to multiple pathogens in small field laboratories. LFD devices for the detection of ASFV/CSFV, BTV and EHDV were developed. In addition, Quick ELISA formats for the detection of companion animal diseases were developed. Finally, low-density antigen microarrays for multiplex detection of antibodies of several bovine and porcine pathogens were developed and validated.

WP7: Adaptation and validation of multiplex assays for simple and reliable pathogen detection in small field laboratories. Both direct genome and antigen detection methods were employed. PCR methodologies for the detection of CSFV/ASFV, FMDV and capripox viruses were transferred to the easy-to-use cartridge based ENIGMA Mini Lab system. Low-density antibody microarrays for the detection of several bovine and porcine pathogens were developed and validated.

WP8: Development and implementation of techniques for confirmatory reasons and as a basis for adaptations especially of molecular tools. Efficient and economic methodologies to sequence viral genomes using next-generation sequencing but also Sanger sequencing were developed and optimized. Several Luminex assays for serological detection several pathogens were successfully developed.

WP9: Validation of the developed rapid assays within internal proficiency tests (PTs) and together with international partners, resulting in ready to use field and laboratory tests for selected diseases. Several assays were tested in PTs and from members of the Field-Net-Group.

WP10: Dissemination of the objectives of the project and their results.

Finally, the ultimate goal of the RAPIDIA-FIELD project was the commercialization of the most promising assays to ensure availability for the improvement of animal health.

Main S&T results

WP2. Real-time monitoring system online

The early phases of infectious diseases are usually accompanied by unspecific clinical signs such as fever, depression, and decreased food and water intake. Those parameters are thus suitable to detect infections of sentinel herds or flocks at the earliest possible time point and can be integrated in early warning systems. Within WP2 of the RAPIDIA-FIELD project, such a prototype early warning system was developed for pig herds based on a computer-aided, non-invasive real-time monitoring system-on line (RTMS-ON).

Task 2.1 dealt with the implementation of prototype systems that allowed the continuous monitoring of animal parameters (body temperature and motion) and ambient factors (humidity, water flow, and room temperature) through the use of different room and animal sensors. For individual animal parameters, microchips were implanted to record the body temperature, and an eartag device with an accelerometer was used to assess motion. Both systems were based on a wireless radio-frequency identification (RFID) system. Based on initial data, alarm thresholds were defined: a first alert will be given if motion values drop below a certain threshold. This alarm triggers the recording of temperature data. Should both parameters (motion and body temperature) exceed the set thresholds, a strong alarm is given that should then lead to veterinary inspection and sampling.

The prototype system was subsequently tested under experimental conditions at the facilities of P8 (VISAVET-UCM). Despite some technical issues, it could be shown that the RFID systems detected fever at least at the day of first PCR positive results. In combination with motion data, the system could give an alarm up to two days prior to any PCR positive results.

Based on the first experiences, the RFID devices were improved to giver higher resolution through frequent measurements. The improved prototype was used in a second experiment. While the resolution power of the motion data was even able to distinguish different modes of action (e.g. playing, eating, lying), technical problems were encountered regarding battery power and robustness of devices. However, it could be proven that herd movement analysis through video-camera monitoring is an efficient and rather cheap method to detect significant changes before and after infection. Analyses of movement peaks per minute allowed detection of disease as early as one day post infection, i.e. three days before fever or virus could be detected.

The prototype RTMS-ON was also transferred to the facilities of P4 (Prophyl) and a Hungarian sentinel farm close to the Serbian/Croatian border (**task 2.2**). The animal rooms at P4 provided the possibility to test the system under conventional but controlled conditions and under the impact of different infectious diseases.

First experiments were done based on room temperature, humidity, and water flow data alone, subsequent studies included the live animal sensors. In this case, the body temperature of the animals was measured with the transponder-reader system. The transponder was read with hand held scanners and rectal temperature was measured with digital rectal thermometers to provide control data. Animal motion was obtained via accelerometers incorporated in reader devices. This system was tested in animal studies with different experimental settings and pathogens including Actinobacillus pleuropneumoniae (APP), PCV-2, and PRRSV.

In the APP studies it was seen that especially the motion data are adequate to register severe disease in a group of animals. Clinical signs in PCV-2 and PRRSV were very mild and did not trigger the alarm of the RTMS-ON. However, in these studies, a good correlation was established between reader data and rectal body temperature.
After each trial, RTMS-ON components were refined to allow the most optimal data collection. This optimized system was subsequently used in a trial with PRRSV (East European subtype; **task 2.3**). A clear correlation was established between the recorded motion and temperature data and the clinical signs recorded upon direct inspection.
As a last trial, parts of the RTMS-ON were transferred to a sentinel farm with suitable fattening units. Under these circumstances, water consumption, CO_2 concentration, ambient temperature (inside and outside), and humidity were recorded. During the reporting period, the sentinel farm was free from the most common diseases of pigs. When first signs of respiratory or intestinal disorders appeared, immediate antibiotic therapy was started to prevent the development of clinical signs or death. Data of the pre-diseased, diseased and post-diseased periods were collected. No trends could be found in the case of humidity, room temperature and CO_2 concentration as these parameters were more influenced by the outside temperature and the ventilation/heating system than by the animals. The most reliable parameter was the water consumption, which showed slight changes during therapy or clinical signs. The system also alerted the farm manager if the ventilation was flawed, or water pipe breaks occurred, therefore it was very useful to control the environmental influence and to discard the cause of disease because of a management failure in the room conditions.

In conclusion, the proof-of-concept studies yielded very promising results but also showed room for improvement, especially with regard to temperature reading, device robustness and innocuousness as well as threshold definition for movement data.

WP3. Collection and Preparation of Samples

The main objective of WP3 was to supplement and support the efforts towards development and validation of pen-side tests and tests for low-resource laboratories through the establishment of sampling and processing workflows. It also coordinates a sample database of specimens that could be used within the consortium for assay validation (Task 3.1). This virtual database now contains 5487 samples representing 15 different livestock and companion animal diseases. In addition to existing samples, partners have also exploited material from new experimental studies for a range of different viral pathogens (NDV, PRRSV, ASFV, PCV-2, BTV and EHDV). This database will remain active for future studies after the project is completed in 2015.
When considering sampling protocols, the project has explored practical approaches that minimise obstacles that could limit the deployment of these new technologies into the field and low-resource laboratories, and have focussed on methods that are appropriate for the non-specialist user. Sample preparation for molecular tests is particularly challenging since these detection technologies can be particularly sensitive to inhibitory substances that are present in sample matrices. Starting with the initial stages of sample preparation, this project has

demonstrated that simple disposable sample homogenisation methods can generate tissue suspensions that are suitable for down-stream analyses by molecular methods such as PCR (in the field and in simple laboratories using the Enigma FL and ML platforms) and LAMP methods (Task 3.2.1). The project has also continued to validate the use of oral fluids collected via chewing of cotton ropes by pigs as a simple alternative to collecting serum samples for ASFV diagnosis (Task 3.2.2). ASFV-specific antibodies could be detected in these fluids (11-30 days post-infection): results that were comparable to parallel testing of serum samples. Furthermore, these fluids were also suitable for qPCR analyses. Aerosol specimens have also been investigated (Task 3.2.3) as these represent a non-invasive sample type that is particularly appropriate for certain airborne viral diseases such as FMDV. Air samples collected with one of two portable air samplers (Biobadge® 100 and Biocapture®650 Mesosystems) during experimental FMDV transmission experiments (in sheep and pigs) were analysed using automated real-time RT-qPCR and RT-LAMP (including direct RT-LAMP – see Task 3.2.6). Apart from a single air sample collected from a sheep experiment (at two dpi), there was complete concordance between the results generated by RT-LAMP-LFD and real-time RT-qPCR highlighting the suitability of this sample type for pre-clinical and clinical diagnosis of FMD. Sample pooling (Task 3.2.4) has been investigated for oral swabs and milk samples. Mobile PCR platforms (such as the Enigma FL) and equipment that can be used to perform PCR in low-resource laboratories (such as the Enigma ML) adopt nucleic acid extraction procedures that are integrated into these machines. The performance of these processes (including the vital lysis step(s)) have been optimised and evaluated during this project (Task 3.2.5), prior to down-stream detection of pathogen targets (results presented in WP5 and WP7). The project has also investigated direct-detection of viral nucleic acid using rapid protocols without the requirement for nucleic acid extraction procedures (Task 3.2.6). In this context, a three step procedure that can be applied in simply equipped field labs for the detection of ASFV was established using samples collected into Genotube swabs (see Task 3.3.1). These Genotube swabs taken during an ASFV animal trial were used in a direct PCR without prior DNA isolation (Phusion Human Specimen Direct PCR Kit, Thermo Scientific). Additionally, tissue material (spleen) obtained after necropsy of the above described animals was tested in another direct-PCR system (Phire Animal Tissue Direct PCR Kit, ThermoScientific). As an alternative to time and resource consuming gel-electrophoresis, subsequent quick amplicon detection using nucleic acid lateral flow devices (NALF, Amodia) was applied. Direct PCR using the Genotube swabs and the spleen samples revealed PCR products even from samples with low viral load (cq ~ 37). The visualisation of the PCR products using the NALF procedure turned out to be even more sensitive than gel electrophoresis. In conclusion this three-step procedure proved to be a very rapid and reliable detection method of ASFV genome without prior DNA extraction from Genotube swabs and spleen samples that might be a valuable tool for detection of ASFV in remote areas. At P6, direct detection of FMDV nucleic acid by real-time RT-PCR was also achieved using the elution wash (nuclease free water combined with RNAse inhibitor) recovered from Ag- lateral flow devices (LFD's). As an alternative to PCR, the project has also evaluated direct RT-LAMP assays for FMDV. Using a simple dilution treatment of epithelial samples, the limit of detection of this RT-LAMP assay was demonstrated to be equivalent to that of a laboratory based RT-qPCR assay, and to have a 10,000 fold higher analytical sensitivity than the

FMDV-specific antigen LFD currently used in the field. In order to help visualise these different steps involved in the assays developed by RAPIDIA-FIELD (such as sample collection, sample pre-processing, nucleic acid extraction, target amplification, detection and visualisation of products and reporting of results), 14 different storyboard cartoons have been prepared and used to identify what extra equipment might be required to perform the test, and to highlight whether there are any logistical gaps in the diagnostic pipelines (Task 3.2.7).

In remote areas sampling systems are needed that enable long-term nucleic acid storage at RT (Task 3.3.1). P2 in conjunction with Prionics (P3) tested during the RAPIDIA-field project the suitability of forensic swabs (Genotubes swabs provided by P3) to store nucleic acids for longer periods at RT. Oropharyngeal swabs collected during the framework of CSFV and ASFV animal trials were tested directly after sampling and after 2-3 months intervals by manual nucleic acid extraction and subsequent routine PCRs for the detection of the respective pathogen. Results obtained for CSFV after two months of storage at RT showed that CSF viral RNA was still detectable. Results obtained for ASFV showed, that even after approx. 11 months storage at RT ASF viral DNA was still detectable. This proof-of-concept study showed that viral nucleic acids can be stored for longer periods at RT which is of significance in remote areas where freezing of samples in order to preserve nucleic acids is problematic. In a further study, Genotube swabs were investigated for their applicability to sample viral nucleic acids for pathogen detection from fallen animals (e.g. wild boar) using blood samples and organ samples (Petrov et al., 2014). To mimic transport conditions the immersed Genotube swabs were stored at RT and then subjected to nucleic acid extraction and the respective (RT-) qPCR. Results obtained for both ASFV and CSFV detection showed, that blood swabs are suitable for a reliable ASF and CSF virus detection. Furthermore, swabbing of organs is possible where no blood is available. In a supplementary study it was investigated whether antibodies against ASFV could also be recovered from the Genotube swabs (Blome et al., 2014). A set of EDTA blood samples were tested with an available ID Screen® African swine fever Indirect antibody ELISA (ID.vet, Grabels, France) which provides a protocol for dried blood on filter papers. In this study fragments of Genotube swabs were used and compared to samples stored on filter paper and to results obtained from corresponding serum samples as reference status as tested by the p72 antibody ELISA (Ingezim PPA Compac, Ingenasa, P1). Results obtained clearly demonstrated that antibody detection is possible also from Genotube swabs. The tested samples were in complete agreement with the serological status. Taken together, swab samples could be recommended as a pragmatic approach to sample especially fallen wild boar for passive swine fever diagnosis.

During this project, P6 has also investigated the potential of using FMDV lateral-flow devices (LFD's) for dry transportation of clinical material for subsequent nucleic acid amplification, sequencing and recovery of infectious virus by transfection using electroporation. Positive samples (epithelial suspensions and cell culture isolates) representing four FMDV serotypes were detected by the LFD: after which it was possible to recover viral RNA that could be detected using rRT-PCR. Using this nucleic acid, it was also possible to recover VP1 sequences and also successfully utilise protocols for amplification of complete FMD virus genomes. When eluted RNA was directly inoculated onto susceptible cell cultures there was no infectious virus recovered, however following electroporation into BHK-21 cells and

subsequent passage, infectious virus could be recovered. The LFDs could be stored for periods of one month at temperatures as high as at 37°C. Therefore, these preliminary results support the use of the LFD to be used for the dry transportation of FMD positive nucleic acids to FMD reference laboratories. An added benefit of using LFDs is that that these can be used in the field to select suitable specimens that are confirmed to be positive for FMDV, and in the event of negative results, the devices can be used to inform rapid re-sampling of animals within a herd. These results provide evidence to indicate that positive lateral flow devices may pose lower biorisk should they be used for transportation of samples between the field and reference laboratories. In view of these data, further work to consider and agree appropriate biosecurity and IATA transport guidelines is required so that these new methods can be transitioned into the field for the safe preservation and recovery of FMDV (discussions regarding the possibility of sending non-infectious materials comprising RNA are now being considered within the network of EU National Reference Laboratories).

WP4. Antibody detection in the field

The aim of this WP was to develop user-friendly and robust assays able to detect antibodies to multiple pathogens in the field. The focus was placed on lateral flow devices for the detection of specific antibodies against disease clusters such as orbivirus infections (BTV/EHDV; **task 4.1**) or swine fevers (ASFV/CSFV; **task 4.2**). Apart from the mentioned primary targets, a third duplex LFD was later considered within this WP: the duplex for AHSV and EIA antibody detection (**task 4.3**), due to the availability of the target antigens and to the potential utility of such rapid tests for equid international trade, in which a strict control of both diseases' transmission between enzootic and AHS/EIA-free regions is fundamental.
All these assays are of great interest for an early differential diagnosis, especially in situations where laboratory support and skilled personnel are limited, and they can also be a useful supporting tool for rapid implementation of control measures to prevent further spread of diseases.
In initial steps, individual formats of double recognition LFD approaches were used to optimize the test conditions and to clear up problems in production and purification workflows.
It turned out that not all antigens were compatible with LFD development, especially the EHDV VP7 recombinant problem. The latter showed severe structural hindrances and despite all efforts, test performance could not be increased. For this reason, the duplex BTV/EHDV approach was cancelled.

In contrast, the duplex ASFV/CSFV LFD developed in Task 4.2 showed promising results both in the initial evaluation and with experimental sera. The internal evaluation conducted by P1 using reference sera, samples from experimental infections and field samples showed that the duplex ASFV/CSFV LFA correlated well with the OIE's ASFV reference ELISA (INgezim PPA COMPAC) and with the OIE's CSFV gold standard virus neutralization (VNT). Also, no cross-reactions were observed between the ASFV and CSFV test lines, and with other pestivirus (BVDV and BDV) or serum samples from ASF/CSF-free areas. The systems was intensively validated through a Proficiency Test (PT) under WP9, and to that

end, Partner 1 produced and sent out a total of 1624 test strips/horizontal cassette devices (in 3 batches) to the project partners and some Field Net groups (FNG) and Community Reference Laboratories (CRLs).
Overall, the results obtained under task 4.2 showed that the duplex AFSV/CSFV LFA is a reliable method for rapid detection and differential diagnosis of anti-ASF and anti-CSF antibodies. Further optimizations are, however, required in the production and purification of the E2 in order to obtain sufficient protein concentration for standard commercial manufacturing and to improve the CSFV's test sensitivity.
The duplex AHSV/EIA LFA developed in task 4.3 used the VP7 and RP26 proteins for specific AHSV and EIA antibody detection, respectively. Partner 1 produced and sent out a total of 255 individual horizontal cassette devices to P8 and to an external collaboration in Argentina (Equine Virus Laboratory of CICVyA-INTA, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas - Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina). The duplex AHSV/EIA LFA was evaluated using reference sera, samples from experimental infections and field samples from AHSV-vaccinated/infected horses, non-infected horses, horses infected with other orbiviruses, and from Cameroon and Ivory Coast AHSV-endemic regions, Argentina EIA-endemic regions and AHSV/EIA-free areas of Canada, Argentina and Spain. Results from this evaluation together with the internal validation conducted by P1 showed that the duplex LFA's performance is in good agreement with the OIE's AHSV ELISA (Ingezim Compac Plus, INGENASA) and with the OIE's EIA agar gel immunodiffusion (AGID). Partner 1 did also observe that the duplex LFA correlated well with the ELISA Ingezim Anemia Equina (INGENASA), and it specifically recognized the 9 different serotypes of AHSV (reference sera AHSV1-AHSV9). Moreover, no cross-reactivity was detected when analysing positive serum samples from other orbiviruses (EHDV and BTV). Despite these results, further evaluations using more samples from field and from experimental infections are still required to definitely validate the duplex LFA and to establish its analytical and diagnostic sensitivity/specificity.
The evaluation conducted in Task 4.3 highlighted the utility of the duplex AHSV/EIA LFA for the rapid detection of both AHSV-specific and EIA-specific antibodies on-field. This new duplex LFA can potentially be used as a first local diagnostic approach, mainly in countries where laboratory infrastructure is under development or missing.

WP5. Pathogen detection in the field

The main aim of this WP was to develop rapid, sensitive and specific multiplex methods for basic field diagnostic settings. These methods include portable PCR systems (**task 5.1**), multiplex isothermal amplification (LAMP) assays (**tasks 5.2 and 5.3**), and the adaptation of a RT-PCR assay for FMDV to use on the portable Enigma Field Lab (FL) instrument (**task 5.4**).
Within task 5.1, a three-level semi-nested PCR assay was developed at SVA for molecular pathotyping of H5 and H7 Avian Influenza Viruses (AIVs), using stationary real-time PCR platforms. The assay is based on the Promega Plexor® System employing a novel base-pair chemistry. In this task, the possible adaptation and implementation of the novel pathotyping assay to portable PCR instruments and systems, namely, T-COR4 and SmartCycler II was

investigated. The original pathotyping assay could be adapted to the SmartCycler II, but not the T-COR4, due to mismatches between the plexor chemistry and the design of T-COR4 assays. In addition, newcastle disease virus (NDV) was selected as a model pathogen because a pilot experimental infection study had been planned and carried out at P4, providing the advantage of getting access to samples for field evaluation of both, sample preparation and the portable PCR assay.
A real-time NDV RT-PCR assay was developed and evaluated together with P4. Viral nucleic acids were manually extracted in the animal facilities under field-like conditions using basic equipment. The real-time RT-PCR detection was performed with the portable T-COR4. An agreement of 83% (39/47) was reached when RNA preparations were tested in the portable assay and the Rotor Gene Thermocycler under laboratory settings, respectively. As positive results came from blood samples of birds challenged/infected with wild type virus, but not from vaccinated birds, it is possible to type the viruses based on detection of viral RNA in blood. The developed system simplifies the diagnostic procedures for the simultaneous detection and pathotyping of highly virulent and avirulent viruses by using a highly sensitive portable real-time PCR assay, successfully working under field conditions

Concerning multiplex amplification methods (**task 5.2**), a RT-LAMP assay was developed for the detection of pestivirus infection in farm animals, e.g., cattle. Unfortunately the performance of the assay was not satisfactory. The high genetic diversity of BVDV and BDV viral variants has limited the application of the LAMP technology in detection of pestivirus infection in farm animals. As alternative technology a portable real-time RT-PCR assay for the rapid and simple detection of pestiviruses (BVDV/BDV) was developed and evaluated. The assay did successfully overcome the limitation of the LAMP assay and has been transferred for testing field samples in China.

An additional RT-LAMP assay was developed for the detection and differentiation of CSFV and ASFV. In this case, LAMP product detection was done using LFDs. It could be demonstrated that the analytical sensitivity of the RT-LAMP-LFD for CSFV was highly sensitive, and no cross-reactions were observed with other pestiviruses. Combination with the LFD readout combined the strength of LAMP for rapid amplification of target nucleic acids with the simple readout of results. However, with the published ASFV LAMP assay, the duplexing of the two assays reduced the performance, regardless of the LFD readout. It is probably recommended to run the singleplex assays in parallel to ensure best sensitivity and specificity.

Furthermore, great efforts were made to adapt a multiplex ASFV/CSFV real-time PCR assay to the portable instrument T-COR4 for simultaneous detection and differentiation of ASFV and CSFV. A set of clinical samples was tested by the portable PCR assay at HVRI, China where CSF is endemic and at the NADDEC in Uganda. In general, good agreement was seen between in-house real-time PCRs and the portable assay.

Within **task 5.3**, a multiplex RT-LAMP-LFD assay, which can rapidly discriminate between FMD and SVD at the pen-side was developed. The RT-LAMP-LFD assay demonstrated

analytical sensitivity concordant with rRT-PCR and can be conducted on real clinical samples (epithelial suspensions) without the need for RNA extraction.

In order to detect and differentiate orbiviruses (BTV, EHDV and AHSV), new LFD assays were developed within **task 5.4**: a duplex EHDV-BTV LFD, and an individual LFD for AHSV detection. The demand for BTV and EHDV orbiviruses' differentiation using a duplex LFD was evident since both viruses can infect domestic and wild ruminants (sheep, cattle, goats, buffaloes, camels and deer). AHSV usually infects equids (horses, mules, donkeys and zebra) therefore it can be detected independently. The analytical performance of the duplex and individual LFDs was evaluated using each pathogen-specific VP7 recombinant protein and pathogen-specific viral samples. The duplex and individual LFDs detected only the corresponding VP7 recombinant protein and viral samples, and no cross-reactivity was observed when analysing BTV, EHDV and AHSV pathogens. The individual AHSV LFA was also compared to the INGENASA's AHSV ELISA (INgezim PEA DAS), by conducting a semi-quantitative analysis of the LFD test strips in the Qiagen's ESEQuant Lateral Flow Reader. Both assays correlated well but the AHSV LFD presented lower sensitivity than the ELISA for the detection of AHSV inactivated serotypes 2, 4 and 9. Partners 7 and 8 evaluated the diagnostic performance of the duplex EHDV-BTV LFA and ASHV LFA, respectively. Both assays presented lower diagnostic sensitivity than expected, and further optimisations should be conducted to improve this diagnostic performance including the analysis of more blood, serum, spleen and/or lung samples from infected animals. Despite the lower sensitivity, the duplex EHDV-BTV LFD had high specificity for pathogen detection in different matrices (plasma, blood, serum, and cell culture extracts), which supports its utility for the rapid and direct detection and differentiation of EHDV and BTV.

Within **task 5.5**, P6 (PIR) in conjunction with P12 (Enigma diagnostics) has brought the OIE diagnostic real-time PCR assay for detection of FMDV into a lyophilised format which can be assayed using a portable rRT-PCR machine (Enigma FL) combining integrated RNA extraction.

Although lyophilisation of rRT-PCR reagents by Enigma did not adversely affect the performance of the PCR part of the assay, the complete assay, run on the Enigma FL, showed an analytical sensitivity that was one log_{10} less than rRT-PCR using wet reagents and RNA extraction using laboratory robots, which reflects the less efficient. RNA step performed by the Enigma FL compared with standard laboratory robots. However, the test is simple to use and the RNA extraction and rRT-PCR can be performed directly on clinical samples. Commercially available field-based epithelial suspension preparation kits (Svanova FMD Ag sample extraction kits) have been validated for use during this study in the preparation of samples within field settings. Serum and probing samples can be added neat to the lysis buffer prior to running on the Enigma FL. Results of the test can be visualised simply at end point with minimum user interference. Furthermore the assay is rapid and the sample processing to test completion takes only 60 minutes. The Enigma FL performance has now been validated in two endemic settings within East Africa; Kenya and Tanzania. Results suggest that the Enigma FL can accurately detect FMDV in a range of clinical samples from acutely infected as well as convalescent cattle.

WP6. Antibody detection in small field labs

The aim of WP6 was to develop innovative multiplex serological assays for the detection and quantification of antibodies to multiple pathogens in small field laboratories. It complements the work done under WP4. Within the project a prototype multiplex LFD-COMB for the detection of ASFV/CSFV antibodies was developed. Due to the clinical and pathologic similarity, the differentiation of CSF and ASF is difficult and the two diseases have to be distinguished by laboratory diagnosis, which is currently performed by ELISA to detect antibodies or by PCR to confirm the virus in blood, lymph nodes, and spleen or serum samples of infected pigs. Both methods have to be carried out in well-equipped laboratories, they are time consuming and need highly trained personnel. Here, a simple diagnostic method based on a lateral flow device (LFD) to rapidly differentiate the two diseases was developed for front line disease control of CSFV and ASFV. Specific antigens to CSFV (E2 protein) and to ASFV (VP72 protein) were used as capture antigens to detect antibodies against CSFV and ASFV in serum. The prototype Duplex LFD-Comb CSFV/ASFV kit contains only three components: the LFD-Combs, the dried conjugate provided in the wells of the microtiter plate and the assay buffer. The advantages of the Duplex LFD-Comb CSFV/ASFV are its simplicity, speed in obtaining results and its high throughput as well as user friendliness. The assay is conducted in a microtiter plate format which accelerates preparation of sample and assay buffer by using multichannel pipetting. One assay runs in only 10 minutes followed by result readout and interpretation. The prototype Duplex LFD-Comb CSFV/ASFV test kit was subjected to validation organized by Partner 9 of the RAPIDIA-Field consortium as well as for evaluation by various field net groups (FNG). In summary, more than 1100 LFD tests of the Duplex LFD-Comb CSFV/ASFV have been sent to 9 laboratories in the consortium, 6 European reference laboratories and 4 FNGs. The validation results showed that the Duplex LFD-Comb CSFV/ASFV has equal or higher diagnostic sensitivity than conventional ELISA tests. The Duplex LFD-Comb CSFV/ASFV may be applied to rapid differentiation of CSFV and ASFV infection in pigs, particularly as a front line screening test. The simple workflow of the Duplex LFD-Comb CSFV/ASFV test requires only very limited training of involved personnel and is therefore suitable for small field laboratories requiring a high throughput to test samples for the presence and discrimination of CSF and ASF.

Apart from the LFD approaches, Quick ELISA formats were explored. **Task 6.2** had the overall goal to establish a Quick ELISA (Q-ELISA) test for the rapid detection of antibodies to multiple pathogens. These assays should be carried out using either, breakable ELISA plates or tubes, and droppers. Moreover, no system reader should be needed, making the assays usable in small labs. Important pathogens for companion animals were used as models: CPV, FCV and FHV. Canine parvovirus (CPV) causes multi-systemic and potentially fatal viral diseases in dogs. CPV serological assay can provide useful information to veterinarians regarding the immune status of healthy dogs in respect to CPV. However, veterinarians depend on diagnostic laboratories to obtain the immune status of their patients which can be expensive and time consuming. A presumptive diagnosis of CPV enteritis can be made based on clinical signs or CPV antibody detection using a diagnostic assay. Within Task 6.2, the

development and validation of a Duplex Quick assay for detection of IgG and IgM to CPV, has been carried out. Both assays are based on the canine parvovirus VP2 recombinant protein expressed in the baculovirus system as the target antigen for antibody detection. The VP2 is the major capsid protein containing the antigenic determination sites. This antigen is used in diagnosis for detection of antibodies in serum. For the Q-assays produced within RAPIDIA-Field project, canine IgM or IgG were captured in immunotubes with anti-canine IgM or IgG and detected with parvovirus VP2 recombinant protein followed by an anti-VP2 monoclonal antibody. The assay was tested using a collection of sera from dogs from an animal shelter located in Madrid (Spain). The results were compared with those of two commercial ELISAs considered as reference techniques for this study. A high correlation was found between the two assays, presenting an accuracy of 98% and 100% for IgG and IgM, respectively. In conclusion, the developed assay appears to be useful to determine the unknown immune status of dogs to CPV, especially in kennels and shelters where the rate of infection by CPV is relatively high.
Feline herpes virus (FHV) along with feline calicivirus (FCV) are the main causative agents for cat flu. Differential diagnosis of these two pathogens is necessary for appropriate control and treatment of the disease. One Q-ELISA was developed to detect FCV IgG and another to detect FHV1 IgG. The Q-ELISAs were developed based on the VP62 protein of FCV and purified FHV1 virus that were used as antigens on ELISA plates. The bound IgG antibodies presented in feline serum samples were detected via peroxidase conjugated monoclonal antibodies against feline IgG. Feline serum samples obtained from an animal shelter were analyzed by the Q-ELISA and the commercial ELISA kits available (EVL). The overall actual agreement between Q-ELISA and the commercial ELISA kits were 100% for FHV IgG and 91% for FCV IgG.

Task 6.3 aimed at the development of a semi-quantitative lateral flow device (LFD) to determine the antibody levels against EHDV and BTV in livestock. However, due to technical problems of compatibility between the recombinant EHDV antigen's stability (see WP4) and its use in the LFD, the development of this duplex LFD was not possible. Hence, another duplex LFD developed within the task 4.2 of this project (ASFV/CSFV LFA for simultaneous detection of specific antibodies of African and classical swine fever) was considered for the semi-quantitative analysis. The initial study and optimisation of the Qiagen's ESEQuant Lateral Flow Reader conducted with the simple LFD for BTV antibody detection (Ingezim BTV CROM) revealed that the semi-quantitative approach is of most interest mainly when analysing samples that have test line's intensities close to the positive/negative cut-off. In these cases, very weak positive test lines may not be correctly evaluated by the human eye but can be detected by the LFD reader. The development of the semi-quantitative duplex ASFV/CSFV LFD was performed using a similar strategy, in which positive, weak positive and negative cut-offs were defined for each ASFV and CSFV test lines. Here, the correlation between the qualitative results of ASFV-/CSFV-monoclonal antibody titration and the test line's intensity (in mV) was fundamental to estimate the antibody levels against ASFV and CSFV present in reference and field serum samples. Altogether, this task demonstrated that the Qiagen's ESEQuant Lateral Flow Reader is a valuable help in the identification of samples with different antibody titres, advancing the LFD qualitative positive/negative result.

This reader equipment is also a good option for field application: it can be used directly on-field (without a computer), or, if necessary, it can be easily connected to a computer in a small laboratory or veterinary clinic. Moreover, the Lateral Flow Studio software has an easy-to-use interface, and it does not require a qualified user to manipulate it.

The objective of **task 6.4** was the development of low-density antigen microarrays for detecting antibodies against porcine and bovine pathogens (including other related livestock animals as horse and dear) in veterinary samples. Within the RAPIDIA-FIELD project, a bovine antigen microarray (BAgM) containing 11 different immobilized specific antigens and 6 inactivated viruses for detecting antibodies to bovine viruses and other orbiviruses (infecting dear and horse) was developed: VP7-BTV, NS2-BTV, VP2-BTV, BTV-1 inactivated and BTV-8 inactivated (for BTV); SBV N-protein (for SBV); FMDV-A, FMDV-Asia and FMDV-O (for FMDV); NS3-AHSV, VP7-AHSV and AHSV-4 inactivated (for AHSV); VP7-EHDV, EHDV-1 inactivated, EHDV-2 inactivated and EHDV-6 inactivated (for EHSV); and MPB83 (for Mycobacterium tuberculosis). Specific antigens were provided from project partners and the BAgM was validated with actual sera provided by RAPIDIA-FIELD partners.
Additionally, a porcine antigen microarray (PAgm) for the simultaneous multiplex detection of circulating antibodies against several pathogens was developed. The PAgM contains 13 different immobilized specific antigens: NPCV2 for circovirus; p10NEU for PRRSV; Coronavirus for TGEV; VP2-PPV for Parvovirus; ADV for Aujeszky; MPB83 for Mycobacterium spp; CP312 and VP72 for ASFV; H3N2V-SIV for SIV; LPS for Brucella spp.; SE for Erysipelothrix; MHYO for Mycoplasma hyopneumoniae; and E2 for CSFV. The PAgM was validated through a proficiency test with a collection of 21 randomized sera samples provided by Partner 9. In conclusion, two low-density antigen microarrays (BAgM and PAgM) were developed, that are ready use either in the lab or small field labs, for multiplex detection of circulating antibodies against different pathogens.

WP7. Pathogen detection in small field labs

The overall objective of this WP was the adaptation and validation of multiplex assays for simple and reliable pathogen detection in small field laboratories with basic equipment. Most detection systems are limited to being used in sophisticated laboratories by experienced scientific operatives and are thus lacking in many rural areas throughout the world; where both human and technical resources are limited. Nowadays, advances in technology allow implementing modern and reliable techniques for quick and simple pathogen detection either in basic laboratories or even at pen-side level. Within this project rapid and simple methods for direct genome as well as antigen detection for selected disease clusters were designed and validated.
One aim of this WP was to evaluate, adapt and validate direct genome detection methods including necessary extraction steps for further down-stream applications. Within this project, sample matrices and their preparation were comparatively assessed prior to assay optimization and transfer to the chosen technology platforms. In this context, simultaneous detection of CSFV and ASFV genome in serum and blood samples was tested successfully using an

existing nucleic acid extraction protocol. Another task within this work package was to combine routine single genome detection PCR protocols for species-specific disease clusters to multiplex PCR assays in order to transfer those assays to easy to use platforms (**task 7.1**, direct (multiplex) genome detection). One such diagnostic system is the cartridge-based ENIGMA® ML (MiniLab) consisting of an automated nucleic acid extraction with a subsequent real-time PCR and printout of the results. The system is easy-to-use and operators do not require special training. Furthermore the assays are freeze-dried, so there is no cooling of the cartridge required and implementation in arid or tropical areas is feasible. During this project, a triplex PCR for the simultaneous detection of ASFV, CSFV and an internal control (MS2) was successfully transferred to the Enigma ML and evaluated using EDTA-blood dilution series of different virus strains. Following this successful evaluation it was shown that the ASFV/CSFV multiplex could detect clinically relevant samples including cross-titrations of positive blood samples. Additionally, a PCR assay for the detection of FMDV and an internal control (MS2), which was already transferred to the Enigma Field Lab (FL) within WP5 was evaluated using cell-culture supernatant dilution series and bovine, porcine and ovine vesicle and saliva samples from different FMDV serotypes. This assay was further evaluated within an internal proficiency test and result from all of these evaluations showed that the ML could detect clinically relevant concentrations of FMDV and was furthermore shown to work in the field laboratory in Kenya with good correlation to comparison tests. Moreover, a PCR for the detection of Capripox viruses was successfully transferred to the Enigma ML. All these assays were only slightly less sensitive when compared to routine diagnostic (RT) qPCR methods but were able to detect even dilutions and samples with very low viral loads (cq-values ranging from 36- 39), and therefore the detection of acutely infected animals is very likely. Thus the developed Enigma® ML cartridge-based assays for the detection of ASFV, CSFV, FMDV and CPV are reliable and useful molecular based diagnostic tools for the early and rapid disease detection e.g. in remote areas.

Another task within this work package was the design of isothermal viral genome detection methods such as LAMP. The advantages of such assays are that they are rapid and can be run at a single temperature. Thus, no thermal cycling is needed and the assays can be run using a simple water-bath. Amplicon detection using lateral flow dipsticks is quick and offers an easy-to-use alternative to time and resource consuming gel-electrophoresis. During this project an RT-LAMP for the simultaneous detection of ASFV and CSFV combined with a lateral flow dipstick for visualisation of the amplification products was developed within WP5. In order to supplement the work on LAMP assays within WP5, alternative isothermal amplification methods were assessed. In this context, recombinase polymerase amplification (RPA) for the detection of an ASFV genome fragment combined with lateral flow dipsticks was developed. This assay proved to work very efficiently and the visualisation of the amplicons using the lateral flow dipsticks turned out to be more sensitive than gel-electrophoresis. Additionally a rapid and simple-to-use oligonucleotide microarray for the detection viral genome fragments from several bovine and porcine pathogens was developed and validated.

For direct antigen investigations a one-step ELISA for PRRSV and PCV2 detection was developed and tested. The assay was firstly optimized using tissue culture virus and recombinant proteins. Further analysis with real was carried out. Additionally two low density

microarrays were developed during the project: i) BAbM (Bovine Ab Microarray) for simultaneous detection of viral antigens or viral particles from BTV, EHDV, AHSV and ii) PAbM (Porcine Ab Microarray) for detecting antigens from circovirus, PRRSV, TGEV, Parvovirus, Suid herpesvirus 1 (SuHV-1), and ASFV. Both, the BAbM and PAbM have been validated with the antigens and viral culture supernatants by sandwich fluorescent immunoassay.

Finally, an oligonucleotide microarray has been designed and tested for the simultaneous and multiplex detection of nucleic acid sequences of: Aujeszky's disease virus (ADV), African swine fever virus (ASFV), Braquispira hyodisenteriae (BH), Braquispira pilosicosi (BP), Bluetongue virus (BTV), Brucella species, Porcine respiratory coronavirus (PRCV), Lawsonia intracelularis (EPP), Infectious Bovine Rhinotracheitis (IBR), Porcine circovirus (PCV), Bovine viral diarrhea virus (BVDV), Porcine parvovirus (PPV) and Porcine reproductive and respiratory syndrome virus (PRRSV).

Another objective of this WP was the evaluation of the AlphaLisa technology for direct antigen detection. AlphaLisa is a bead-based assay technology used to study biomolecular interactions in a microplate format. Assays were carried out in order to detect SBV or BTV viruses or viral proteins in biological samples. Assays performed for pathogen detection from biological field samples have not yielded satisfactory results. The beads coupled with antigens or monoclonal antibodies were efficient: these antigens and antibodies were used in the same time, with success, in a similar approach (Luminex technology (WP8)). However, the presence of residual hemoglobin in the tested biological samples (blood, tissue) interacts with the oxygen reaction needed for the revelation step.

WP8. Confirmation and reference techniques

The overall objective of this WP was the development and implementation of techniques for confirmatory reasons and as a basis for adaptations especially of molecular tools.

In detail, high technology strategies that can be applied to confirm the status of a sample at the genome detection level without prior knowledge using e.g. next-generation sequencing (NGS) and highly multiplexed genome detection methods e.g. microarrays were developed (**task 8.1**). In this project the emergence of a novel midge-borne orthobunyavirus (member of the Simbuserogroup: Schmallenberg virus, SBV) in Europe could be detected using NGS (Roche 454) and metagenomics analyses. The obtained sequence information helped to develop rapidly diagnostic tools and enabled culturing of SBV and other members of the Simbuserogroup for optimizing the full genome sequencing procedure. The generated sequences furthermore allowed in-detail phylogenetic classification of Schmallenberg virus within the Simbu-serogroup and enabled developing a universal pan Simbu-PCR. Additionally, based on the sequence information, first generation sequencing methods using conventional RT-PCR techniques of the S- and M segments from SBV samples were developed and allowed variant analysis of SBV field samples obtained from different host species from all over Germany. Thereby a mutation 'hot spot' within the M segment of Schmallenberg virus could be detected. Furthermore a virus discovery method based on

random amplification and next generation sequencing (DNase SISPA) was developed and enabled the sequencing of SBV from RT-qPCR positive field samples from Belgium.
Additionally deep sequencing using the Illumina MiSeq platform revealed unique isolates of recent highly virulent type 2 bovine viral diarrhoea virus (BVDV-2). The coexistence of three distinct BVDV genome variants could be confirmed whereby the major portion (ca. 95%) of the viral population harboured a duplication of a 222 nt segment within the p7/NS2 encoding region and the minority reflected the standard structure of a BVDV-2-genome. The results obtained suggest that the variant with duplication plays a major role for the highly virulent phenotype.
Taken together, these findings highlight the importance of metagenomics and full-genome deep sequencing in combination with in-detail data analysis for the investigation of viruses in fundamental research and diagnostics.
Within this task furthermore pre-treatment protocols for RNA virus metagenomics in serum and tissue samples were developed and applied to several diagnostic and research cases. Thereby coincidently the contamination of commercial RNA extraction reagents with oceanic viruses or viral nucleic acids could be documented, illustrating the importance of integrating metagenomics studies in a diagnostic framework.
To improve sequence knowledge with the aim to improve test design and evaluation, it is important to develop efficient and economic methodologies to sequence viral genomes (**task 8.2**). To this means, workflows are needed that optimize sample preparation and nucleic acid extraction as well as random amplification, subsequent sequencing, and data handling steps. Alternative workflows may be needed for samples with low genome loads. Furthermore shipment of highly contagious viruses e.g. ASFV from affected countries for collaborative in-detail studies, for example within the ASFORCE project, is almost impossible. In these cases, sequencing of overlapping PCR fragments is an option that can be followed and optimized.
Within this task a method was developed to generate full genomes from ASFV from Russia by sequencing PCR products directly or stored on nucleic cards (collaborative approach within ASFORCE and RAPIDIA-FIELD). After data analyses a tandem repeat insertion in the intergenic region between the I73R and the I329L protein was detected which revealed a higher resolution in ASFV molecular epidemiology. This proof-of-principle approach showed that it is possible to sequence ASFV full genomes from PCR products directly or stored on nucleic cards and its value for investigating genome fragments that contribute to obtaining a higher resolution in ASFV molecular epidemiology. While data analysis was mainly part of the ASFORCE collaborative project, optimization and design of methodological approaches was performed as part of RAPIDIA-FIELD. This is an example how synergistic effects can be generated and used among projects.
Additionally a method was developed that enabled full genome sequencing of CSFV type 2.3 from samples with low viral load (collaboration and continuation of approaches started within the CSFV_goDIVA project). The generation of full genomes is often hampered by the lack of samples with sufficiently high genome loads, but cell culture passaging may lead to biased variant amplification. To avoid these effects and to obtain original complete sequences from field samples, a primer-walking method covering the full open reading frame and parts of the non-translated regions was developed. This method was employed to investigate spleen samples collected during an outbreak in French free-ranging wild boar in the Vosges du Nord

Mountains between 2003 and 2007. The generated sequences allowed in-detail comparative sequence and evolutionary analyses and results obtained revealed a slow evolution of CSFV strains over the years whereby substitution rates varied among protein genes. Furthermore, a spatial and temporal pattern could be observed. These findings highlight the value of methods that enable the generation of full-genomes from samples with low viral loads.

DNA viruses such as ASFV or poxviruses have large genomes (approx. 180-220 kbp) with sometimes complex structural variations e.g. insertions, deletions and repeat regions. This often complicates the assembly of complete genomes from short second generation (e.g. Illumina MiSeq or 454/Roche sequencing) sequence reads. Within this WP, the value of third generation sequencing (Pacbio) for DNA virus whole genome sequencing was investigated. This technology produces very long sequence reads, however, currently still with a higher error rate than second generation sequencing. Pacbio sequencing was applied to several viruses, including total DNA extracts of suid herpesvirus 1 (SuHV1, Aujeszky's disease) isolates. The resulting long sequence read data were used to generate whole genome sequences and resulted in full genome length de-novo assembly contigs. For each sample, Illumina short read (higher accuracy) sequence data was also produced. In addition, a sheep- and goatpox whole genome amplification strategy based on overlapping PCRs was designed. This was necessary as insufficient DNA was present in poxvirus stocks to reach the range of highly purified DNA necessary for the Pacbio library preparation protocol. Pooled PCR products were used as input for Pacbio sequencing and resulted in whole genome poxvirus assemblies. For each sample, also Illumina short read (higher accuracy) sequence data was produced. Final data analysis in both cases is ongoing to investigate the relative value of Pacbio vs Illumina vs hybrid (using both datasets) assembly for the efficient generation of high accuracy whole genome assemblies.

Another task within this work package is to develop systems enabling highly multiplexed genome detection (**task 8.3**). Within this task methods for the generation of starting materials for subsequent multiplex or singleplex genome detection were optimized. The focus was laid on techniques to simplify and speed up nucleic acid extraction for downstream applications using rapid extraction protocols based on magnetic particle technology. To this means, two open extraction platforms KingFisher™ Duo (Thermo Fisher Scientific) and BioSprint® 15 (Qiagen) as well as the fully automated EZ1® advanced XL instrument (Qiagen) were used and validated using blood and serum samples from animals infected with the ruminant diseases SBV and BVDV. The results of this study showed that the speed-optimized novel extraction protocols allow rapid and simple nucleic acid extractions for a variety of target viruses without significant loss of sensitivity compared to standard procedures. For this reason they represent valuable contributions to overcome the limitations associated with the sample processing step in mobile diagnostic tests.

Despite of these sequencing methodologies and genome based detection methods, immunoassays for viral diagnosis with a Luminex platform for multiplexed antibody detection were developed within this project (**task 8.4**). Microsphere immunoassays (Luminex assays) for the detection of antibodies against flaviviruses, orbiviruses and orthobunyaviruses were successfully developed. An innovative high-performance multiplex Luminex assay for serodiagnosis of flavivirus-associated neurological diseases in horses has been developed which provides a powerful alternative to ELISAs and VNTs for the detection of antibodies

against West-Nile virus, Japanese encephalitis virus, and tick-borne encephalitis virus in veterinary diagnosis. A multiplex Luminex assays for serological detection of African horse sickness virus was also successfully developed. Furthermore, Luminex assays for the detection of antibodies against BTV and SBV were established and proved to enable antibody detection in several ruminant species tested. Taken these results together, the Luminex assays developed in this project demonstrate that this technology allows to detect specific Ab against arboviruses (flaviviruses, orbiviruses and orthobunyaviruses), in a multiplex assay and in a single tube using only small quantities of serum. The specificity and sensitivity of this technology is similar to conventional serological tools as ELISA or sero-neutralization assays.

WP9. Standardization

Newly developed diagnostic assays should fulfil the requirements laid down in the OIE "Manual of Standards for Diagnostic Tests and Vaccines" (OIE, 2008). To ensure compliance with these guidelines within the RAPIDIA-FIELD project, proficiency tests (PT) were carried out with four promising candidate tests that had passed the initial validation at the developing laboratory. The PTs were executed according to ISO 17043 guidelines under the auspices of P9 (CODA-CERVA) using well characterized samples provided by the partners of WP3 and the FNG. The aim of the PT's was to demonstrate the sensitivity and specificity as well as repeatability and robustness of the tests in other laboratories than the developing laboratory. Both RAPIDIA partners and FNG participants took part and the statistical analysis of results was performed according the implemented macro's by P9 according ISO 5725-2 and ISO 17043. A qualitative as well as a quantitative analysis was performed. The latter included estimates of the precision (repeatability and reproducibility estimates for each lab and for each sample) and if possible a graphical representation.

The following PTs were carried out:

1. **Evaluation of a commercial LFD for the detection of Bluetongue-specific antibodies**

In this PT, six laboratories took part of which five were national reference laboratories for BT. The PT comprised serum samples from infected, vaccinated, or naïve animals of different ruminant species (cattle, sheep, goat) and of various BTV-serotypes (samples from previous EU ring trials, in-house samples from the participants, and samples prepared by the PT provider.

The diagnostic sensitivity, evaluated with 37 positive sera, was 100 % (95% C.I. [90.5-100]) where as the diagnostic specificity, evaluated with 21 negative sera was 95.2 % (95% C.I. [76.2-99.9]). Qualitative repeatability or accordance and qualitative reproducibility or concordance were 100% for seropositive samples but were lower (45 - 89 %) for some of the seronegative samples due to false weak-positive reactions. Analytical sensitivity was evaluated by testing positive sera at increasing dilutions and determining the limit of detection: it was better for the BTV LFD (1/16-1/32) compared to some commercial ELISAs (1/8-1/16). Furthermore, seroconversion of an infected sheep was detected at 4 days post infection compared to 6 days post infection with commercial ELISAs. Analytical specificity was impaired by cross-reactions observed with some of the samples seropositive for epizootic

haemorrhagic disease virus (EHDV). Agreement (Cohens's kappa) between the LFD and a commercial BTV competitive ELISA was 0.79 (95% CI [0.62-0.95]).
In conclusion, the BTV LFD device is a rapid and sensitive first-line serological test that can be used in the field to screen clinical cases suspected of bluetongue, especially in areas endemic for the disease where there is a lack of diagnostic facilities. However confirmation by a more specific serological assay such as ELISA is needed in case of weak positive reactions or if EHDV infection cannot be ruled out.

2. **Evaluation of two LFD for the detection of classical swine fever virus (CSFV) and African swine fever virus (ASFV)-specific antibodies:** ASFV/CSFV Duplex LFD Comb of PRIONICS (Switzerland) and Ingezim ASFV-CSFV antibody of INGENASA (Spain).

The PT was carried out by nine partners from the RAPIDIA-Field group and 11 participants from the FNG. The samples comprised four categories with serum samples from infected, vaccinated, or naïve individuals of domestic pigs, wild boars and warthogs (samples from previous EU ring trials, field samples, in-house samples, and samples provided by the PT provider). Several genotypes of ASFV and CSFV were represented, and BVDV samples were included to assess cross-reactions.
For samples obtained from infected or vaccinated animals it was demonstrated that the Prionics ASFV/CSFV Duplex LFD Comb has a high sensitivity and a variable specificity. Based on the results of dilution samples for CSF and ASFV, the LFD assay was demonstrated to be as positive as the reference test (CSFV) or even more sensitive compared to the reference test (ASFV). Some false positive reactions on the ASFV line by the evaluation of CSFV samples were noted as well as positive cross-reaction results for other pesti viruses such as BVDV and BDV. For the Ingezim ASFV-CSFV (Ingenasa), it can be concluded that the LFD assay has a variable sensitivity and a high specificity. Variable results were demonstrated for the accordance and concordance and some false positive reactions due to cross contamination were noted. Few false positive reaction on the ASFV line by the evaluation of CSFV samples were noted.
For the samples of previous EU ring trials (from 2013), the LFD assay of Prionics has a relative sensitivity of 93% and a relative specificity of 71%. Accordance and concordance were 100% for 4 out 7 samples; sample 3, 4 and 5 with as duplicate sample 20, 19 and 18 had an accordance as well as concordance of 75 %. Some false positive as well as false negative reactions were noted. Some positive reactions on the ASFV line were noted for the samples 3, 14, 20, 21 and 24. The Ingezim ASFV-CSFV (Ingenasa) has a relative sensitivity of 97% and a relative specificity of 92%. Accordance and concordance were 100% for 6 samples, sample 3 with a duplicate sample 20 had an accordance of 75 % and a concordance of 42%. Some false positive as well as false negative reactions were noted. Field samples showed varying results.
In conclusion, the LFD-duplex assays for the detection of ASFV/CSFV specific antibodies are easy and fast to use in field and laboratory conditions. The use of proficiency trials allowed evaluating the repeatability and reproducibility of the measurements within and between laboratories. The Se of the LFD assays should be improved especially for ASFV. The relative Se is depending on the FNG partner. The readability of the results (lines) can be improved by

using an automatic system (reader) with an optimized cut off value in order to discriminate in positive and negative samples in a more objective way.

3. **Evaluation of a portable machine for the detection of Foot and mouth Disease virus (FMDV):**

In this PT, four laboratories took part. The samples comprised different serotypes (O, A, Asia 1, SAT1, SAT2). Two aliquots/sample/lab (n=12) were shipped in a randomized way.
The accuracy was 99%. Only one false positive reaction in one lab with one aliquot was observed. The repeatability (within-lab) was 0 – 6.4% according to sample and lab, the reproducibility (between-lab) = 2 – 8.9% according to sample. In conclusion, the portable Mini Lab (ML) of ENIGMA is a sensitive device for the detection of FMDV antigen. It is easy to use and quick. There is no need for a very skilled operator as all steps are automated, including nucleic acid extraction. However detailed results of the test are not available for the user. There may be a need for confirmation of weak positive reactions.

4. **Evaluation of an Antigen Micro Array for the detection of antibodies to several swine diseases:**

Three laboratories took part in this PT. All of them received 21 reference samples that were positive for different viruses (Aujeszky virus (AUJ), ASFV, BVDV, CSFV, porcine circo virus 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV)). The resulting accuracies were: AUJ 100%; ASF: 33.3%; CSF 94.5%; PCV2: 100%; PRRS: 67%. The repeatability (concordance) was 84 – 100% according to tested disease. Some cross-reaction occurred with BVDV for CSF. The evaluation of accuracy was done only for 5 diseases. False positive reactions were detected for ASFV and false negative reactions for PRRS. The assay is also designed to detect antibodies to 7 other diseases: parvovirus, coronavirus, mycobacterium, swine influenza virus, brucella, erysipelothrix, mycoplasma hyopneumoniae. Further evaluation is needed.

Carrying out these PTs proved to be very helpful in assessing the designed tests and to help refining and optimizing assays.

WP10. Dissemination of results

A specific project website (www.rapidia.eu) was created to disseminate the main outputs from the project. It also offers a private access for Consortium partners to upload project progresses and advances. The RAPIDIA-FIELD website stores all of the presentations of the annual meetings where progress of work is shown. It also stores a database of sample banks that will be maintained after the end of the project. A list of publications and communications is also available. All partners contributed to transfer the technology developed under the RAPIDIA-Field project around the world through presentations as invited speakers at Universities, research centres and international organizations like the OIE General Session of the World Assembly of Delegates or at FAO conferences; at international seminars, scientific meetings and workshops; at EU conferences like EPIZONE or EAVALD (European Association of Veterinary Laboratory Diagnosis); at national and international congresses of veterinary,

virology, microbiology, biosafety and One health; and at disease-specific symposiums, particularly for FMD, ASF and zoonosis. Technology transfer was also performed through the publication of scientific articles in international peer-reviewed journals of high impact in the veterinary area. Finally, some partners also tried the new tests in countries of the Field-Net-Group.
In the two and a half years of the project, the Consortium has presented more than 60 communications in 23 different countries, including 11 non- European Union member countries and has produced more than 20 peer-reviewed publications in prestigious scientific journals and editorials. We are expecting the acceptance for publication of at least 5 more articles from different partners during the next months. A leaflet with a summary of all assays and detection methods developed within the project was disseminated at the first international workshop with Animal Health Organizations. Dr Almudena Sánchez-Matamoros, from UCM (Partner 8), developed part of her PhD under the RAPIDIA-FIELD project framework, in collaboration with ANSES (Partner 7). The results were shown in several international congresses and in two scientific articles.
Two workshops were organised at UCM, one in 2014 21-22 May, and the other one, following the extension of the project, in 2015. Both workshops were a success and the Consortium had the opportunity to directly show the results of their research to different stakeholders in animal health through a hands-on exhibition and through presentations. There was a fruitful discussion in both workshops among all stakeholders on the advantages and disadvantages of the new diagnostic tools presented.

The potential impact

Over the last decades, outbreaks of notifiable diseases such as classical and African swine fever, foot-and-mouth disease or avian influenza have shown tremendous socio-economic impact. In developing countries, high impact trans-boundary animal diseases pose a direct threat to food security, nutrition and income of rural communities that are dependent on livestock. These threats are very real and more than a merely trade related problem. Deadly and economically devastating livestock epidemics have existed throughout history but there is no doubt that more pathogens are emerging – and spreading. Thus, safe-guarding animal health is a keystone for sustainable food production and veterinary public health throughout the world. Prevention and control strategies have to be based on early-warning methodologies and powerful diagnostic tools that can be implemented in a wide range of settings, from sophisticated laboratory environments in industrialized countries to point of care applications in remote rural settings. The RAPIDIA-FIELD project has worked based on state-of-the-art materials and methodologies as well as creative applications of alternative techniques. Based on the consortium's experience in the field of veterinary diagnostics both in research and development as well as commercialization, easy-to-handle, cost effective and robust diagnostic tests have been made available and are suitable for the use as pen-side tests and in laboratories with limited capacities. They present a new level of validated veterinary diagnostic tools for many important notifiable diseases as well as endemic infections that impair food production in many countries. In addition, the use of parallel testing for several pathogens (**multiplexing**), has provided a new quality of diagnostic results in the field and in

the low level laboratory. Moreover, reference techniques that can be applied to confirm the status of a sample without prior knowledge and identification of new pathogens were under the focus of RAPIDIA-FIELD project. Overall, the outcomes of the project have contributed to the sustainable and safe production of meat and meat products through provision of better tools for the fight against devastating diseases. In consequence, the common market will be strengthened and the losses caused by animal disease outbreaks reduced.

The particular **expected impact of this project was:**

- Availability of a reliable and validated, **non-invasive early-warning-system** that allows monitoring of farmed animals for behavioral alterations and body temperature raises in order to detect introduction of animal diseases at the earliest possible time point.

Integral part of all prevention and control concepts is the timely detection and reliable diagnosis of pathologies to allow for an appropriate and timely intervention. Here, the first step lies inevitably with the owner or person responsible who has to perceive and report abnormalities. Implementation of early warning approaches can thus lower the impact of disease incursions through the possibility of apt interventions.
This aspect has been addressed by the RAPIDIA-FIELD project through the development and validation of a computer-aided online system for farmed pigs, the RTMS-ON (real-time monitoring system on line). This system takes advantage of the fact that most infectious diseases will be accompanied by alterations of body temperature, behavior, and water intake. These parameters were used as sensitive indicators for the detection of abnormalities and were measured non- or minimal invasive. The developed system has shown potential and will be available for implementation after final adjustments. Its use under industrialized settings is well-suited for the reduction of the high risk periods of transboundary diseases such as swine fevers or foot-and-mouth disease and to facilitate treatment of endemic infections such as PRRS or SIV.
The detection of abnormalities has to be linked with appropriate sampling and processing schemes for a reliable herd- or population-level surveillance and especially downstream laboratory diagnosis. In this respect, different settings require different approaches. In the above mentioned farm setting, non-invasive sampling for pathogen specific testing can be optimal. Candidate matrices are e.g. air samples or oral fluids from playing ropes or baits. In this context, the project partners benefited from the advances in biotechnology and detection techniques that have paved the way for the use of such non-invasive samples. It could be shown that oral fluids could be valuable samples for e.g. ASFV antibody detection. Generally, lowering the labor-intensity and time requirements for sampling is expected to facilitate the compliance of farmers and practitioners and thus increase sample submissions for pathogen exclusion/confirmation (= efficient early warning).
Similar methods can be applied to design pragmatic approaches for wild life or livestock in remote rural areas (e.g. swabs). Such systems have been validated within the project and most promising results have been obtained for the detection of ASFV and CSFV genomes and ASFV antibodies from dry blood swabs. Integration of these approaches into some national workflows has already taken place and extension to other settings and pathogens is envisaged.

- Commercial availability of new validated and standardized (**multiplex) methods** applicable **at the point of care** (pen-side) and **in basic laboratories**.

With regard to the laboratory diagnostic approaches, several aspects have to be taken into consideration when developing sampling and testing schemes. To date, most countries with industrialized animal production have routine laboratories with sophisticated equipment and well-trained personnel. In contrast, laboratory capacity is low in remote rural areas of e.g. Africa or South-East Asia, but also in rural areas within countries neighbouring the EU. Unfortunately, especially both of the latter have a high burden of transboundary diseases and the impact on their livelihood is severe if animal diseases strike. Under rural conditions, timely laboratory diagnosis is hampered by several factors including obstacles with sample taking, cooling and transport (long distances, no courier service, lack of money for transport costs, no appropriate packing materials) as well as laboratory capacity itself. For this reason, manageable tools are needed that work under limited conditions, either directly in the field (pen-side) or in very basic laboratories. The basic laboratories that are available have usually the advantage that they are close to the problem and are accustomed to local structures and traditions. However, limitations in water supply, temperature management, electricity, acquisition of consumables (kits, specialized laboratory plastics), machine equipment (ELISA reader, PCR cycler), and personnel for assay implementation and maintenance exist. Taking those aspects into consideration, lateral flow devices (LFDs) and cartridge-based PCR systems with nucleic acid extraction included are particularly well suited. Exactly those techniques were employed by the RAPIDIA-Field partners to bring about both pen-side tests and tests for basic laboratories that can be combined with simple processing workflows. Exemplary pathogens were chosen representing diseases or disease complexes with high impact. One complex was the simultaneous detection of swine fevers.

African swine fever (ASF) is one of the specific examples of a disease affecting livestock and causing important economic losses worldwide. There is no treatment or prophylaxis available, and control is based on rapid and early detection of the infection. **Classical swine fever (CSF)** is a highly contagious disease, causing major losses in swine populations almost worldwide. Although the virus has been eradicated in many countries, the disease has been recently reported in Hungary, Lithuania, Serbia, Israel, the Russian Federation, and Latvia. With the spread of ASFV from the Caucasus, the probability of areas encountering both viruses is increasing, and the involvement of wild boar increases the risk of introduction into the domestic pig population. Due to these reasons, and taking into consideration that both pathogens are listed as notifiable diseases to the World Organization for Animal Health (OIE), early differential diagnosis of these diseases is of great value for immediate implementation of control measures to prevent further spread of the diseases. However, differentiation between CSF and ASF is not possible by clinical or post-mortem examination, therefore it is essential to send samples for laboratory examination.

The ASF diagnostic techniques most frequently used nowadays are PCR for virus detection and ELISA or immunoblotting for serological analysis. In the case of CSF the diagnosis is based on identification of the agent by PCR and virus isolation or detection of antibodies by ELISA or neutralization assays. However these methods are time consuming and require well equipped laboratories and personnel, possibly delaying the disease diagnosis in remote areas. The duplex LFD ASFV/CSFV that was developed within the RAPIDIA-Field project by Partner 1 offers a rapid and simple to use diagnostic tool suitable for field application, allowing the early and specific diagnosis of ASF/CSF. Furthermore, the assay has been designed to test either serum or blood, thus making the sample processing quite easy and feasible even at field level, no equipment neither skilled personal is required. These features make these devices very suitable for small field labs or task forces, supporting in many cases local decisions, especially in countries where laboratory infrastructure is under development or even missing. In the same manner Partner 3, has developed a duplex LFD-Comb

ASFV/CSFV for detection of antibodies against ASFV and CSFV, but in an 8-strips comb format which ensures suitability for 96-well plates. The developed assays allows the analysis of 8 samples at one time and is suitable to be performed in basic laboratories with limited resources, since it does involve the use of minimal reagents. In the same disease cluster, partner 10 developed a LAMP assay for the detection of CSFV combined with a subsequent visualization of the amplification product using lateral flow technology. The RT-LAMP-LFD assay combines the efficient one-step isothermal amplification of CSF viral RNA and the simplicity of the LFD to read the results within two to five minutes. Seven genotypes of CSFV were successfully detected by the RT-LAMP-LFD assay, indicating that the method has a broad range of detection and can be applied in different geographical areas where CSFV strains belonging to these genotypes are present. The performance of this RT-LAMP-LFD assay was similar to that of the real-time RT-PCR. The analytical sensitivity was about 100 copies per reaction when testing two genotypes (1.1 and 2.3). No cross-reactivity to non-CSFV pestiviruses was observed. This RT-LAMP-LFD assay can be a useful novel tool for the rapid, simple and economic diagnosis of classical swine fever in the field. For the detection of CSFV and ASFV genomes, a cartridge-based system has been developed that includes the detection of an internal control for quality assurance.
Beside the swine fevers, **foot-and-mouth disease (FMD)** was targeted as important pathogen and as differential diagnosis. Foot-and-mouth disease outbreaks occurred worldwide, resulting in significant economic losses. Early detection of the FMDV is therefore critical in the control and eradication of the disease and in order to minimize losses in livestock. Diagnosis of FMDV infection is difficult by similarities in clinical signs with other diseases including swine vesicular disease, vesicular stomatitis and diseases caused by bovine caliciviruses. Current diagnosis of FMDV is often based on ELISA and virus isolation. These methods are either not very sensitive or are very time-consuming and require high biosafety standards. Given these limitations, rapid, sensitive and easy to handle assays in clinical samples are required. Two different approaches have been carried out within the RAPIDIA-FIELD project to provide alternative diagnosis methods for FMD:
ENIGMA MiniLab (ML) system: adaptation and validation of single and multiplex assays for simple and reliable pathogen detection in small field laboratories, were transferred to easy to use platforms like the Enigma ML system. This system is a fully automated, compact, ruggedized instrument designed for PCR testing in the field. It can be powered from a battery, the mains or from the auxiliary power supply in a vehicle. PCR tests are supplied in the form of sealed, pre-packaged disposable cartridges which can be stored without refrigeration for a year. No additional reagents or chemicals are required. The procedure of nucleic acid extraction and subsequent PCR for detection of viral genome is done in one run. Three different protocols have been adapted and transfer to the ENIGMA ML system, including FMDV:

a. Triplex RT-qPCR to detect CSFV/ASFV/MS2
b. Duplex RT-qPCR for the detection of FMDV/MS2.
c. Duplex qPCR for the detection of capripoxviruses and MS2.

ENIGMA Field (FL) system: adaptation of RT-PCR assay developed at Partner 6 facilities for FMDV to be used on the Enigma FL instrument. Partner 6 in conjunction with Partner 12, has adapted the OIE diagnostic real-time PCR assay for detection of FMDV into a lyophilised format which can be assayed using a portable rRT-PCR machine (Enigma FL) combining integrated RNA extraction. Besides, the Enigma ML system with FMDV specific cartridges was also evaluated in a proficiency test in several labs organized by Partner 9. The results suggest great potential for field-based molecular testing without need for expert training or extensive sample pre-treatment. The threshold settings in the software may require additional

optimization to improve the assay sensitivity. Field-testing was carried out by Partner 6 in endemic settings in both Kenya and Tanzania using the Enigma FL system operated from the back of a truck. Analysis of the results showed that the Enigma system detected FMDV in all suspect samples of epithelium and vesicular fluid. It also detected FMDV in serum when the infection was recent (days 1-3 p.i.), but not after several days (6-7 p.i.). The results suggest that the Enigma FL can accurately detect FMDV in a range of clinical samples from acutely infected as well as convalescent cattle.
Additionally to the diseases mentioned above, other pathogens affecting livestock were under the scope of the RAPIDIA-FIELD project. With that purpose, low-density antigen microarrays for detecting antibodies in veterinary samples for porcine and bovine pathogens (including other related livestock animals as horse and dear) were developed by Partner 5, and validated with real sera in collaboration with Partner 1. As result, two low-density antigen microarrays (BAgM and PAgM) are ready to be used in small field labs, for multiplex detection of circulating antibodies to different pathogens. The test was validated within a PT organized by Partner 9, where several RAPIDIA-FIELD partners participated.
In total, systems are now available for the detection of CSFV/ASFV antibodies, BTV antibodies, AHSV/EIA antibodies, and for the detection of CSFV/ASFV genomes, FMDV genomes, and capripox virus genomes. Most of these tests were not only designed but also successfully tested in inter-laboratory proficiency tests, providing confidence in the reliability of the developed assays but also pointing out existing pitfalls that can now be amended or circumvented.

- Optimization and validation of high-end confirmatory test systems based on e.g. Next Generation.

Results obtained in the field or in basic laboratories may require confirmation by specialized laboratories. Moreover, emerging diseases such as MERS or Schmallenberg virus infection may need special diagnostic approaches that allow diagnosis without prior knowledge of the pathogen. In this context, next-generation sequencing approaches were optimized within the RAPIDIA-FIELD project. The suitability and value of those approaches was confirmed by the investigation of the Schmallenberg epidemic, in-detail studies of new variants of BVDV type 2, and the epidemiological study of CSFV and ASFV isolates. The last two studies also showed that problems with sample shipment and nucleic acid content can be nowadays solved by alternative samples (nucleic acids on FTA cards or filter papers) or additional approaches (e.g. primer walking systems).
In the main, the broad applicability of metagenomics approaches has a high impact on the diagnosis of emerging and re-emerging pathogens as well as the adaptation of diagnostic tools after emergence of escape variants or other mutational events that render the routine methods unsuitable.
Apart from NGS-approaches, Luminex liquid array technology has been developed and used as confirmatory technology. This assay allows the simultaneous detection of antibodies or antigens, in a single reaction, to multiple viral antigens coupled to microspheres labelled with different proportions of fluorescent dyes. During the project period an innovative high-performance multiplex Luminex assay for serodiagnosis of flavivirus-associated neurological diseases in horses has been developed, which provides a powerful alternative to ELISAs and VNTs for the detection of antibodies against West-Nile virus, Japanese encephalitis virus, and tick-borne encephalitis virus in veterinary diagnosis. Good results have been obtained to detect antibodies against BTV VP7 for all 24 serotypes and VP2 for BTV8. Many sera of wild and domestic animals have been tested (in parallel with ELISA) and the results were promising. The Luminex approach has been used to detect antibodies against different

proteins of FMDV: VP1 of serotypes O, A22 and Asia1, but also 3D and 3ABC. Moreover, a Luminex multiplex approach has been assessed to detect at the same time in one-step, antibodies against BTV, Schmallenberg and Foot-and-mouth disease virus.
Finally Partner 8 in collaboration with partner 7 used the Luminex approach to develop a DIVA assay allowing the differentiation of AHSV infected and vaccinated animals. The antibodies against VP7 and NS3 protein of AHSV were detected simultaneously in infected animals, while vaccinated animals showed only antibodies against VP7.

Altogether, the RAPIDIA-FIELD project has achieved deployable proof-of-concept tools that can be extended to other pathogens and applications. The diagnostics have been combined to rational work-flows and prototype tests were assessed through proficiency tests.
Reliable, easy-to-handle pen-side tests are now directly available to support decision making ("data-based suspicion"), and basic laboratories can be equipped with systems that are suitable for non-expert users and do not need specific consumables from different sources.