New and Emerging Technologies: Improved Laboratory and On-Site Detection of OIE List A Viruses in Animals and Animal Products.

Summary of the EU research project LAB-ON-SITE (SSPE-CT-2004-513645) in the framework of the FP6 programme

On-site diagnosis with a portable PCR machine

Ten infectious, transboundary animal diseases (TADs), listed as notifiable to the OIE (World Organization for Animal Health) - foot-and-mouth disease (FMD), swine vesicular disease (SVD), vesicular stomatitis (VS), classical swine fever (CSF), African swine fever (ASF), bluetongue (BT), African horse sickness (AHS), Newcastle disease (ND), highly pathogenic avian influenza (HPAI) and swine influenza (SI) - were the subjects of this international research project of the EU. These diseases were chosen because of their ability to emerge and re-emerge, their highly infective potential, and above all, because they are TADs, which means that only internationally joint efforts from affected and threatened countries can lead to a better control of these diseases. Thus, this project is a good example of cooperation in a large European consortium formed by eleven diagnostic and research laboratories (nine partners, two subcontractors and a Third Party) including an SME, working together with a main objective: the improvement of the diagnosis of the above-mentioned diseases using new and emerging technologies. The applied diagnostic techniques were divided into two categories: (i) new ways of viral nucleic acid detection, including padlock probes, real-time PCR with PriProET, TaqMan and Minor Groove Binding (MGB) probes, isothermal amplification like the Cleavase/Invader assay or the loop-mediated isothermal amplification technology and the development of rapid kits for 'mobile' PCR; (ii) novel antigen-antibody detection systems, like simplified and more sensitive ELISA tests and Dip-sticks and Lateral Flow Devices for the on-site detection of the viruses in the field during the outbreaks.

Padlock probes are oligonucleotides of about 70-140 nucleotides with target complementary sequences separated by a linker. They provide highly specific recognition sites. This advantage allowed the design of target sequences in conserved or semi-conserved regions within FMD virus (FMDV), SVDV and VSV genome. A rapid method was developed using padlock probes and microarray-based read-out to detect simultaneously and differentiate FMDV, SVDV and VSV in a single reaction, as well as to provide serotype information in case of VSV infection. The assay developed within this project consists of three reaction steps during which only padlock probes that had been circularized were amplified and thus gave rise to a detectable microarray signal.

Primer-probe energy transfer (PriProET). Real-time PCR assays based on PriProET technology were developed and evaluated for ability to detect FMDV, VSV, SVDV, CSFV, ASFV, SIV, and BTV. This novel technology adds up more specificity to real-time PCR assays. A key point of the assay is tolerance toward mutations in the probe region, allowing the wide-range and robust detection of a high variety of the targeted viruses, irrespective to the variations. Melting curve analysis directly after PCR, with determination of probe melting point, confirmed specific hybridization of the virus strains. Single nucleotide mutations characteristic of some virus strains were detected by shifted melting points. All predicted mutations were confirmed by nucleotide sequencing. With the PriProET system, there is a chance to identify phylogenetically divergent virus strains, which may appear negative in other probe-based real-time PCR assays. Thus, this method provides a robust and reliable method for the detection of e.g. different SVDV strains. The amplification plot for closely related viruses remained negative, proving the specificity of this assay.

TaqMan real-time PCR. Although TaqMan probes are widely implemented currently in diagnostic laboratories, no real-time assays were available for some of the diseases of interest. Therefore, an rRT-PCR assay was developed for the detection of all the AHSV serotypes. The set of primers was designed to target a conserved region within the NS1 segment. The TaqMan probe used in this assay was labelled with FAM on the 5' end. The use of a TaqMan probe has proved to increase the specificity of AHSV detection. All the reference strains of AHSV and field strains tested in this study were detected with this rRT-PCR technique. Equine viruses producing similar symptoms to AHS and related viruses like BTV were included in the specificity assay, producing negative results

in all cases.

Minor Groove Binding – MGB- real-time PCR. The use of fluorophore-containing MGB provides three novel features – the MGB (binds in the minor groove and raises the Tm allowing the use of shorter probes), an eclipse quencher (non-fluorescent quencher with a broad absorbance spectrum) and Super G, A and T modified nucleotides (improved base pair binding). In this project, MGB assays have been developed for ASFV (9GL gene), FMDV (3D gene), SVDV (2B gene), VSV Indiana (N gene), VSV New Jersey (N gene) and AHSV (VP7 segment).

Light Upon eXtension real-time PCR. The novel technology Light Upon eXtension (LUX; Invitrogen) has been applied for the detection of AIV and NDV, FMDV, SVDV (5'NTR), VSV Indiana (L gene), VSV New Jersey (L gene) and VSV Indiana (G gene).
Basically, the LUX primer set contains one oligonucleotide labelled at the 3' end by a fluorophore molecule while the other one is unlabelled. The labelled primer is designed to form a hairpin structure that quenches the fluorescence of the unbound primer. Upon the primer's annealing to its target the hairpin opens, the fluorophore is dequenched and the fluorescent signal increases and is detected in the real-time PCR instrument. The LUX technology enables melting curve analysis that provides a convenient way of confirming assay specificity.

Loop-Mediated Isothermal Amplification (LAMP). This novel PCR-based technology has proved to be an excellent method to amplify specific DNA or RNA regions without sophisticated equipment, thus, providing a feasible way of transfer this technology to third countries. A one-step RT-LAMP assay for the detection of SVDV has been developed. The assay detects the virus rapidly, within 30–60 min, and the result can be visualized either by gel electrophoresis or by the naked eye through the addition of SybrGreen. A collection of 28 SVDV isolates were tested positive, while heterologous viruses such as FMDV and VSV remained negative. The performance of the RT-LAMP was compared directly with real-time PCR using RNA from clinical samples including nasal swabs, serum and faeces. For nasal swabs and serum, the sensitivity of the RT-LAMP was shown to be at least equivalent to real-time PCR. Interestingly, for faecal samples the RT-LAMP assay was shown to be even more sensitive than real-time PCR, possibly because it is less sensitive to inhibitory substances. This RT-LAMP assay provides a number of benefits for the diagnosis of SVD, because the assay is sensitive, rapid, and the isothermal amplification strategy used is not reliant upon expensive equipment. It is particularly suited for 'front line' diagnosis of SVD in modestly equipped laboratories, in field stations or in mobile diagnostic units.

Invader squared technology is an isothermal (63ºC) nucleic acid detection method that was evaluated within the LAB-ON-SITE project as an alternative to PCR-based technologies. The method relies on the Enzyme Cleavase® XI to recognize a structure based on two target specific DNA molecules, which can only form on target specific nucleic acid, and the subsequent cleavage of one of these molecules. The resulting cleaved product is then free to bind to a reporter molecule inducing the release of a fluorescent substance (2nd Cleavase reaction), which will emit a fluorescent signal that can be monitored using either real-time PCR platforms or simple fluorescent plate readers. Both methods can be used quantitatively. In this project, invader squared assays were developed and evaluated for the detection of ASFV and CSFV. The ASFV invader assay was evaluated on samples extracted from pigs experimentally infected with ASFV strains E75, Malawi Lil 20/1 and Malta/78. The invader squared assay is not as sensitive (~2500 copies) as real-time PCR but it does offer other advantages. It is less prone to cross-contamination, the assay can be provided in a dried-down format and it can be run and read with simpler and less expensive equipment compared with PCR. This could make it a viable alternative for laboratories lacking real-time PCR instrumentation and for on-site diagnosis.

'Portable' real-time PCR. Rapid and accurate diagnosis is central to the effective control of exotic livestock diseases. The time taken to transport suspect clinical material to a central laboratory can be lengthy and this delay might preclude laboratory confirmation in the event that rapid decisions are required. To reduce the time taken to generate a diagnostic result, RT-PCR assays can be performed using 'mobile' or 'field-portable' equipment. Within the frame of the LAB-ON-SITE project, a kit for the "portable" real-time PCR has been developed in order to detect FMDV. Results from this study were encouraging, indicating that the limit of detection of the real-time assay was comparable with that of a laboratory-based diagnostic assay. Building upon these results, future work is directed towards the development of a simple-to-use and integrated robust template extraction protocols suitable for use by non-specialists in the field.

Novel ELISA systems. Several ELISA tests have been developed and/or improved within the LAB-ON-SITE project all based on the use of monoclonal antibodies that provide better diagnostic performances and further advantages in terms of standardisation, reproducibility and availability. These include:

1. A platform of sandwich ELISA tests for FMDV antigen detection, comprehensive of tests for the specific typing of the seven FMDV serotypes, as well as for pan-FMDV detection (type independent tests);
2. Two sandwich ELISA tests for typing of AIV H5 and H7, and another one for AIV pan-group A detection;
3. Validated solid phase competitive ELISAs (SPCE) for the detection of antibodies specific for the three most widely spread FMDV types, namely type O (O Manisa), A (A22 Iraq and A Iran 96) and Asia 1, in addition to other prototype SPCEs for SAT1 and SAT2 specific antibodies;
4. Two solid phase competitive ELISAs for the measurement of antibodies specific to H5 or H7 serotypes of AIVs.

The diagnostic performance of the developed ELISA assays was evaluated on experimental and field samples and estimates of sensitivity and specificity were produced. Furthermore, the examination of samples routinely

submitted to laboratory diagnosis led to expand data available and to confirm their power for a simple and rapid diagnosis, intended either for identification of isolates and serosurveillance.

Dip-sticks and Lateral Flow Devices. FMDV, SVDV, VSV, HPAIV, and ASFV dip-sticks have been developed and most of them were evaluated with good sensitivity and specificity. NDV and AHSV dip-sticks are going to be evaluated in the future. FMDV pan reacting dip-stick and the sample preparation kit, both have been launched by SVANOVA Biotech as Svanodip® FMDV-Ag penside test and the Svanodip® FMDV-Ag extraction kit that now are available on the market for diagnostic purposes. The test has been fully evaluated according to the OIE guidelines at the Institute for Animal health (Pirbright) and the data have been published in scientific journal. The SVANODIP® FMDV-Ag is a simple direct test for the detection of all seven serotypes of the FMDV antigen in swab and tissue samples. It is a rapid test, which may be carried out on the field, next to the animal. It may be used for early detection of infection, as first line diagnostics in order to control the spreading of infection. The test procedure is rapid and simple, providing a result within 10 minutes. The availability of this kind of tests provides a good tool for veterinarians to diagnose on-site FMD, as it was proved during the FMD outbreak that took place in the United Kingdom during 2007.

Comparison of the performance of nucleic acid extraction robots. A multicenter comparative trial was performed to evaluate automated nucleic acid extraction methods for virus detection. Identical sets of coded samples were prepared using serial dilutions of bovine viral diarrhoea virus (BVDV) from serum and cell culture propagated material. Five European veterinary laboratories (P1 SVA, Sweden; P3 DTU, Denmark; P9 IAH, UK; VLA, Weybridge and VLA, Penrith, both UK) participated in an exercise to compare the performance of nucleic acid extraction robots (12 separate instruments, comprising 8 different models). There was a good concordance between the results obtained for the different automated extraction platforms. The majority of robotic platforms displayed similar extraction efficiencies. There was approximately 100-fold difference in extraction efficiency between worst and best performers. No cross-contamination observed. These results provide reassurance to laboratories to indicate that the best performing optimised nucleic acid extraction systems can have similar performance. Obtained data can be used by laboratories to help selection of suitable robotic equipment for diagnostic use and further supports the use of optimised automated protocols for nucleic acid extraction.

A very important part of this project consisted on the transfer of the technology developed. A number of workshops have been organized in East Europe, Middle East and the Far East in order to train laboratory personnel on the new technologies used for the diagnosis of animal diseases. Standard Operating Procedures (SOPs) have been collected in CDs and distributed to European, Middle East and Far East laboratories.
The information produced within the LAB-ON-SITE project has been disseminated in conferences, peer-reviewed publications and other journals meant for veterinarians and other sanitary personnel. Different partners organized workshops and seminars. In December 2008 IZSLER organized a 10-days workshop for demonstration and training on FMD diagnostic ELISAs. This was in the framework of an FAO project, named GTFS/INT/907/ITA (funded by Italian Ministry for foreign affairs), with the objective to provide training and to transfer diagnostic kits to five beneficiary countries (Afghanistan, Pakistan, Tajikistan, Uzbekistan, Turkmenistan). Three trainees from Afghanistan and three from Pakistan (plus two Chinese colleagues that were already in institute for a longer training on IZSLER activities) attended the workshop, during which they were trained on FMD ELISAs for antigen and antibodies detection. Then, our stabilised kits have been sent to four of the five countries where they are being used.
Our challenge now is to get feed-back from the laboratories where these techniques have been introduced and to further improve the diagnosis of the ten TADs by international standardisation and validation of the new laboratory-based and on-site diagnostic methods. Through the development of a wide range of new diagnostic methods and by providing international training, the LAB-ON-SITE project is contributing to reduction of economic and socio-economic losses caused by TADs worldwide and is improving the animal welfare, by following the "One World - One Health" principle.

Further details: www.labonsite.com
*This review is summarised by: Belén Rodriguez PhD, Facultad de Veterinaria, Madrid.

Sándor Belák[1] (co-ordinator), Mikhayil Hakhverdyan[1], Gordon Allan[2], Åse Uttenthal[3], J.M. Sánchez-Vizcaíno[4], István Kiss[5], Ann Nordengrahn[6], Emiliana Brocchi[7], Kristien Van Reeth[8], Donald King[9], Ulf Landegren[10], Jabbar S. Ahmed[11], Gerrit Viljoen[12] (partner leaders).

Acknowledgements: All participants of the LAB-ON-SITE project are greatly appreciated in the preparation of this article, obtained results and collaboration.

Participant institutes
1) National Veterinary Institute, Uppsala, Sweden
2) Department of Veterinary Science, Queen's University of Belfast, Belfast, UK
3) Department of Virology, National Veterinary Institute, Technical University of Denmark, Lindholm, Denmark
4) Department of Animal Health, Universidad Complutense de Madrid, Spain
5) Veterinary Institute of Debrecen, Hungary
6) SVANOVA Biotech AB, Uppsala, Sweden
7) Department of Research, Istituto Zooprofilattico Sperimentale della Lombardia e dell´Emilia Romagna, Brescia, Italy

8) Laboratory of Virology, Faculty of Veterinary Medicine, Merelbeke, Belgium
9) Institute for Animal Health, Department of Exotic Disease Control, Pirbright, Surrey, UK
10) OLINK AB, Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden (Subcontractor)
11) Research Center Borstel, Borstel, Germany (Subcontractor)
12) FAO/IAEA Joint Division, IAEA, Vienna, Austria (Third Party)

Technical information

Project acronym: LAB-ON-SITE

Project's official full title: New and Emerging Technologies: Improved Laboratory and On-Site Detection of OIE List A Viruses in Animals and Animal Products

Research priority: provide new, robust tests for the front-line diagnosis of the former OIE List A diseases by developing and applying more rapid and sensitive testing regimes based on recent advances in molecular biology for laboratory use.

European Commission scientific officer: Isabel Minguez-Tudela, DG Research (Isabel.Minguez-Tudela@ec.europa.eu)

COORDINATOR DETAILS:

Sándor Belák DVM, PhD, DSc, professor
Head of Joint R&D Division
Departments of Virology
The National Veterinary Institute
& The Swedish University of Agricultural Sciences
Ulls väg 2B, SE-751 89 Uppsala
Sweden
Tel: +46 18 67 41 35
Fax +44 870 13 63 214 (via UK)
E-mail: Sandor.Belak@sva.se
E-mail: Sandor.Belak@bvf.slu.se

TOP