Rapid Field Diagnostics and Screening in Veterinary Medicine

Funded by EU FP7 - Grant agreement no 289364
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**Background**

Fast intervention is of utmost importance to limit the impact of infectious disease introduction into livestock. When farmers used to take care of the animals, each one was able to detect when a single animal appears to be affected by any kind of abnormality.

Intensified animal production systems with limited contact to individual animals require fast and reliable early warning systems for health abnormalities. The RAPIDIA projects seeks to deliver those approaches.

**RAPIDIA contribution**

We are working on an early warning system for animal diseases based on a computer-aided, non-invasive real-time monitoring system-on line (RTMS-ON). The aim is to detect and thus limit infections at the earliest possible time point through parameters such as body temperature, food intake and water consumption.
Real-time monitoring system-online (RTMS-ON)

Technical Basis

This technique is intended to detect the early stages of infection in sentinel animals by measuring physical or physiological changes through *in-vivo* sensors. The information is then remotely transmitted in real-time and an alert is issued when a certain threshold is reached.

Main Advantages

- Less invasive than continuous sampling
- Real-time transmission of data
- High sensitivity to early detect changes in patterns, like increased temperature, reduced water consumption or decrease in motion, potentially saving costs associated with surveillance of subclinical low prevalent or absent diseases

Drawbacks/Limitations

- Cost limits the use in chosen sentinels
- Requires prioritizing diseases and animals based on risk factors and knowledge about disease epidemiology
- Signals any potential infection independently of the etiology

ASSAY 1: Temperature and motion monitoring prototype device or Radio-Frequency Identification (RFID)

Background

Sentinel animals are used in surveillance as a cost-effective tool to enhance early detection of health threats, such as notifiable diseases. Once chosen by a risk-based approached, sentinel animals are periodically sampled. This method can miss infection if it occurs between two sampling periods. It is also an expensive method that requires continuous resources to trigger a potential alarm. The onset of infection is characterized by the presence of fever and pain that can lead to less movement and eventual
monitoring

prostration. The monitoring of animal and environmental indicators in animal health and production provides an excellent opportunity to improve the capacity of response to emerging threats.

**Procedure**

RFID systems have two main components: a transponder (transmitter-responder) and a transceptor (transmitter-receptor). Hand-made transponders are inserted in the body of the animal and contain a microchip with a unique identifier, and two sensors to measure motion and body temperature. Transceptors emit a continuous signal which results in data transmission once the transponder detects it. Transmission is amplified through a radio-frequency device that can be placed as an eartag or a collar. Transceptors contain a unit to process the data, an antenna and autonomous battery life. In pigs, the RFID must be less than 4 cm away from the inserted transponder, because body fat can hamper data transmission. Data stored in transceptors can be downloaded through Wi-Fi or cable internet connection to a core server, to any computer, smartphone or mobile device.
Background

In order to make a good diagnostic, sampling is more than essential, always taking into consideration the welfare of the animals.

RAPIDIA contribution

Easy to collect and non-invasive sampling procedures are a main goal within RAPIDIA project. In addition, all methods allowing the testing of a higher number of individuals like pooling or aerosol sampling will be included. Since molecular methods are more and more important also for field diagnostics, the extraction and handling of nucleic acids is part of the evaluation. Simple methods to prepare tissue suspensions will be evaluated by using inexpensive and disposable methods that can be used for high-impact exotic diseases without worrying about decontamination of equipment between premises with suspect cases of disease. Besides, the use of oral fluid will be studied, providing a rapid and inexpensive sampling strategy, which will reduce the sample collection time and decrease the stress on the animals.
Long-term sample-storage at room-temperature and rapid direct PCR for detection of viral DNA

Technical Basis

A simple and rapid three-step-procedure for detection of ASFV DNA fragments was developed. The procedure includes: a) long-term storage of diagnostic specimens on special swabs (GenoTube Livestock, Prionics, Schlieren, Switzerland) at room-temperature; b) direct PCR from swabs and tissue samples without prior DNA extraction; c) amplicon detection using nucleic acid lateral flow devices (NALF, Amodia) to avoid gel-electrophoresis.

a) GenoTube Livestock

The GenoTube Livestock is a swab originally designed for collecting genomic DNA from the nostril of cattle, horses and other animals but it can also be used for collection of diagnostic specimens. The principle of the long-term storage of nucleic acids on the GenoTubes is based on a desiccant that ensures complete and rapid drying of the swab after sample collection. Within the RAPIDIA-Field project the ability to store ASF viral DNA at room-temperature on the GenoTubes for a longer period was tested.

b) Direct PCR

For molecular diagnosis of ASFV usually PCR methods are applied which require time consuming nucleic acid extraction methods upfront the assay can be conducted. Those extraction methods furthermore need special equipment such as centrifuges and special consumables. Direct PCR systems without prior DNA extraction directly from pieces of e.g. tissues or swabs reveal a good opportunity to avoid DNA isolation. For detection of ASFV stored on GenoTubes as well as from infected tissues two direct PCR kits from ThermoScientific (XXX) were tested and proved to be very efficient.

c) Amplicon detection using NALF

In order to visualize PCR amplicons, typically gel-electrophoresis is used which requires a considerable amount of time and special equipment. Nucleic acid lateral flow (NALF) devices offer an easy and rapid alternative to gel-electrophoresis. By including specifically labeled primers and probes, the amplicons can be detected using those NALF devices within 10-15 minutes without the need of special equipment.

Main Advantages

- **Genotubes**: easy and minimal-invasive sampling method enabling long-term storage of nucleic acids at room-temperature
- **Direct PCR**: rapid, sensitive and easy-to-use; no DNA extraction needed
- **Amplicon detection using NALF**: rapid, sensitive amplicon detection without gel-electrophoresis
Drawbacks/Limitations

Direct PCR: The direct PCR systems from ThermoScientific do not include a reverse-transcriptase option making detection of RNA viruses difficult. Furthermore, these assays are end-point PCR methods and thus represent an open system for visualization of the amplicons. Opening the tubes after the PCR and visualization of amplicons using the NALF technology pose the risk of amplicon-carryover. To avoid producing potentially false-positive results correct handling and caution should be applied when handling amplicons.

ASSAY 1: Long-term storage of DNA on Genotubes at room-temperature and direct PCR for a rapid and easy detection of African swine fever virus (ASFV)

Background

In remote areas cooling of specimens after sampling or even long-term storage of viral DNA or RNA is challenging. Thus, sampling techniques are needed that enable long-term storage of nucleic acids at room-temperature. Within the RAPIDIA-Field project the ability to store ASF viral DNA at room-temperature on the GenoTubes for a longer period was tested. African swine fever virus is a highly contagious economically important DNA virus causing high mortality rates. Thus an early and rapid detection of ASFV infected animals plays an important role for livestock holders. To speed up diagnostic assays and keep them as simple as possible many sample handling steps should be avoided. Molecular diagnosis of ASFV is typically done by PCRs prior to that, DNA has to be extracted. Here we tested different direct PCR kits manufactured by Thermo Scientific with tissue and swab samples that were collected during an ASFV animal trial. To avoid a time and resource consuming amplicon detection by gel-electrophoresis, a specially labelled primer and probe set for a subsequent quick amplicon detection using nucleic acid lateral flow devices (NALF, Amodia) was included in the direct PCR.

Procedure

Genotubes:

In the framework of an ASFV animal trial, Genotubes (Prionics) were taken as oropharyngeal swabs from 15 ASFV challenged pigs. The Genotubes were stored at room temperature (RT) and DNA was isolated using the DNeasy Mini Kit (Qiagen) directly after sampling and after approximately 2 and 11 months of storage at RT. Real-time PCR was subsequently performed according to the protocol published by Tignon et al. (2011). Cq values obtained from DNA isolated from the Genotubes after approximately 2 and 11 months of storage at room temperature were higher than those directly after swabs were taken, but viral DNA was still detectable proving the ability to store ASFV DNA at RT for longer periods.

DirectPCR:
Pieces of the 15 Genotubes were used in direct PCR. Due to extremely low cycling times (denaturing for 3 sec, annealing for 5 sec and elongation) the PCRs were run within a very short time of approx. 30-40 minutes, depending on the thermocycler.

Amplicon detection using NALF:

The principle of the NALF technology is based on labelling the target sequence during amplification with a 5’ – biotin labelled primer and hybridization of a sequence specific labelled probe (5’ - FITC). One label (Bio) binds to gold labelled particles on the sample application pad on the LFD the other label binds to capture molecules immobilized on the test line of the LFD.

The figure below shows direct PCR results obtained from the Genotubes after 11 months of storage at RT. To compare the results cq values obtained by real-time PCR are shown. The amplicons obtained were visualized by NALFs and results compared to gel-electrophoresis. As shown in the figure even amplicons that were not detected by real-time PCR (sample 2, 9 and 15) revealed positive results. When comparing gel-electrophoresis to NALF detection, even samples that were not (sample 7) or hardly detectable (e.g. sample 2) in the gel were visualized much more intense by the NALF devices.

Type of samples

Swabs, tissue, blood

Application

For an early, rapid and simple detection of ASFV genome fragments as well as for a minimal-invasive sample collection method enabling sample storage at room-temperature, this three-step procedure including the Genotubes, direct PCR techniques and amplicon detection by using nucleic acid lateral flow devices proofed to be a very reliable detection method of ASFV genome without prior DNA extraction. Thus, the procedure described is a valuable tool for detection of ASFV in remote areas. Furthermore, other direct PCRs were tested by using blood and tissue samples from infected pigs.
Those direct PCRs proved to work as rapid and efficiently as the direct PCR applied with the Genotubes providing further opportunities of a rapid detection of ASFV from different sample types.
Background

In case of an infectious disease, it is obvious that a fast intervention could avoid or minimize the spread, not only in a single farm, but also, to the environment.

In some cases this kind of interventions, could mean important losses for the farmers. So any technical responsible would like to have tools that could confirm his suspicions and justify to the owners the measures to by apply.

RAPIDIA contribution

This has been one of the major tasks of the project, and many efforts on this sense have been done, working in many different approaches:

- Lateral flow devices
- Low density microarrays
- Immunotubes
- One step ELISA
- MIA
- ENIGMA system
Technique

Immunochromatographic test or lateral flow assay (LFA)

Technical Basis

This simple technique is intended to detect the presence (or absence) of a target analyte in a sample (matrix). The visualization of the antigen-antibody reaction is based on migration of the reagents by capillarity through a porous membrane. These tests are suitable for either antigen or antibody detection.

The technique is based on the use of different colored latex microspheres which are covalently linked to a control protein, and to the corresponding antigen/antibody. On the membrane, different lines are printed: the T line formed by the anti-analyte Ab (for Ag detection) or antigen (in the case of detection of antibodies), and the C line that is formed by a specific Ab for the control protein. The following figures show a schematic diagram of a simplex LFA (A) and a picture of a simplex and duplex lateral flow devices (B).

A)                                                                                                                              B)

In the case of a duplex LFA two different colored beads for binding the two antigens or antibodies are used. On the membrane two test lines are printed.

Procedure: add the sample to the test device and subsequently the running buffer. Wait for 10 minutes for interpretation of the result. After this period, the results are invalid.

In the case of a positive sample a colored line (T line) will appear under the detection zone of the strip as the result of the formation of complexes between the detection reagent and the capture reagent. In the case of a negative sample the appearance of a colored line in the control area (C line) confirms the proper performance of the test.

Main Advantages

- Simplicity: interpretation of the result is made by eye
- Rapid: one step assay
- User friendly: no equipment is required, neither trained personnel
- Portability: pen side test to be used at field level
- Cost-effective
Drawbacks/Limitations

Reduced sensitivity in some cases when compared with other techniques. Generally there is a tradeoff between time and sensitivity. These devices are very valuable as an initial field test in critical situations when positive results require immediate action.

ASSAY 1: Duplex ASFV/CSFV-CROM

Background

African swine fever virus (ASFV) is classified in the Asfarviridae family, genus Asfivirus. Currently, it is one of the most important diseases in domestic pigs. The VP72 protein is the main component of the viral capsid and one of the most immunogenic proteins in natural infection. The presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection, and persist for long periods, they are a good marker for the diagnosis of ASF.

Classical swine fever virus (CSFV) is a Pestivirus of the family Flaviviridae. Domestic pigs and wild boar are the only natural reservoir of CSFV. The E2 protein is a structural component of the virion and is considered essential for CSFV replication. Besides E2 is the most immunogenic protein of the virus, inducing neutralizing antibodies and protection against lethal CSFV.

The present Duplex ASFV/CSFV-CROM is based on the VP72 protein of ASFV and the E2 protein of CSFV allowing the detection of antibodies against both viruses in a given sample.

Procedure

Add 10 µl of the sample on the round window of the device and 4 drops of running buffer. Proceed as mentioned in the general protocol described above (Page 1).

In this particular case, three different microspheres are used: blue microspheres which are covalently linked to E2 protein of CSFV, red microspheres which are covalently linked to VP72 protein of ASFV, and green microspheres which are used as test control. On the membrane, two test lines are printed: T1 formed by the E2 protein of CSFV and T2 formed by the VP72 protein of ASFV. Furthermore, the test contains a control line formed by a specific MAb for the control protein. The results must be interpreted as follows:
Positive sample for ASFV: red and green lines will appear.
Positive sample for CSFV: blue and green lines will appear.
Negative sample: only a green line will show up.
In any case the control line must appear, otherwise the test has to be considered invalid.

<table>
<thead>
<tr>
<th>Type of samples analyzed</th>
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<tbody>
<tr>
<td>Serum, plasma and whole blood.</td>
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<table>
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<tr>
<th>Application</th>
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<tr>
<td>Differentiation between and ASF and CSF is not possible by clinical or post-mortem examination; therefore it is essential to send samples for laboratory examination. To this end, a rapid, one-step immunochromatographic strip capable of specifically detecting anti-ASF and anti-CSF antibodies in serum specimens represents a good front-line diagnostic tool for disease control.</td>
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ASSAY 2: Duplex AHSV/EIAV-CROM

<table>
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<th>Background</th>
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<tr>
<td>African horse sickness virus (AHSV) belongs to the genus Orbivirus, family Reoviridae. Nine antigenically distinct serotypes of AHSV have been identified by virus neutralization. AHS is a lethal viral disease of equids. The disease is endemic to Sub-Saharan Africa but sporadic outbreaks have had devastating effects in Northern Africa, Europe, and Middle East Asia. AHSV is composed of seven structural proteins that include VP7, the highly conserved major core protein and the target for antibody detection.</td>
</tr>
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</table>

Equine infectious anemia virus (EIAV) belongs to the genus Lentivirus, family Retroviridae. Despite the low rate of incidence of EIA in Europe and USA, most countries require a negative test result before allowing an imported horse into the country. The rp26 is one of the main viral internal structural proteins, well conserved among different viral strains and the most immunogenic protein of the virus. Thus the target antigen used in serological tests.
The duplex AHSV/EIAV-CROM developed within the RAPIDIA project is based on the VP7 and rp26 proteins from AHSV and EIAV, respectively. The initial results are promising: the test shows good specificity since each conjugate detects specifically the appropriate antibodies and no cross reactivity has been observed between both viruses.

**Procedure**

Add 10 µl of the sample on the round window of the device and 4 drops of running buffer. Proceed as mentioned in the general protocol described above (Page 1). In this case, the rp26 protein of EIAV is bounded to blue latex beads and the VP7 protein of AHSV is conjugated to red microspheres. Green latex beads are coated with the control protein. The results must be interpreted as follows:

**Positive sample for AHSV:** red and green lines will appear.

**Positive sample for EIAV:** blue and green lines will appear.

**Negative sample:** only a green line will show up.

In any case the control line must appear, otherwise the test has to be considered invalid.

**Type of samples analyzed**

Serum and plasma

**Application**

The economic impact of an outbreak of AHS or EIA was investigated by the Epidemiology and Economics Research Unit. The report identified an important horse industry worth, since the leisure riding sector (non-racing). Proper control of animal movements is important but the development and availability of a rapid and effective test for the diagnosis and differentiation of AHSV and EIAV represents also a promising tool for control the diseases.
TECHNIQUE

LFD-Comb

Technical Basis

The LFD-Comb is intended to detect the presence (or absence) of target analyte(s) of interest in serum, plasma, meat juice or oral fluid samples. The LFD-Comb combines ELISA based lateral flow device (LFD) technique and microtiter platforms. Each 8 nitrocellulose membrane strips printed with designed antigen (or antibody) in test line(s) and control line is casted into a comb, which fits exactly to a column (or a strip) of a microtiter plate. The detector conjugated with latex microsphere particles or Gold colloid is placed in the microtiter well and consequently mixed with the sample. The presence (or absence) of analyte(s) in a sample can be visualized in the test line(s). The control line serves as validity test and has to be visible in all the tests.

Main Advantages

- Simplicity
- Rapid and high throughput
- Flexible (both visual and digital readout)
- Objective through digital result interpretation
- User friendly
- LFD-Comb can be applied for detection of single analyte (Simplex LFD-Comb) and double analytes of interest (Duplex LFD-Comb) as indicated in Fig. 1

Fig 1. Simplex LFD-Comb and Duplex LFD-Comb

Drawbacks/Limitations

Like any other LFD test, LFD-Comb may have reduced sensitivity in some case. In the case of using visual readout, false positive or false negative might appeared in the cut-off range due to the perception of observer.
ASSAY 1: Prionics®-Check PrioSTRIP CSFV/ASFV

Background

Prionics®-Check PrioSTRIP CSFV/ASFV is intended to detect the presence (or absence) of antibodies against classical swine fever virus (CSFV) and african swine fever virus (ASFV) in order to quickly differentiate the two diseases.

In the Prionics®-Check PrioSTRIP CSFV/ASFV, duplex LFD-Comb, a recombinant E2 protein derived from a structural component of CSFV, and an affinity purified VP72 protein from the main component of ASFV capsid were sprayed on the nitrocellulose membrane on the test lines. As detectors, E2 and VP72 proteins are covalently conjugated to blue latex microspheres in order to minimize the deviation of the digital readout. The control line is an antibody against either E2 or VP72. The two conjugates are mixed and lyophilized in the microtiter wells.

Procedure

1. Add 80 µl of assay buffer to reconstitute the conjugate in the wells of the microtiter plate.
2. Transfer 5-10 µl test samples to the wells of reconstituted conjugate. Mix sample and conjugate gently.
3. Place the test comb into the wells containing test samples.
4. Leave the CSFV/ASFV Comb in the wells for 10 minutes.
5. Interpret the results within 20 minutes.

The readout of Duplex-LFD Comb can be visually and digitally. The visual result interpretation is described in Fig. 3, while the digital result interpretation is presented in Fig. 4.

0- Strip with test lines (orange CSFV and red ASFV), and control line (green)
1- Control line visible, CSFV and ASFV negative
2- Control line visible, CSFV positive and ASFV negative
3- Control line visible, CSFV negative and ASFV positive
4- Control line visible, both CSFV and ASFV positive
5- Control line is not clearly visible, the test is invalid
Type of samples analyzed

Serum/Plasma

Application

It suits for small laboratories with high throughput of test samples. It may be applied to rapid differentiation between CSF and ASF in serum/plasma samples, particularly as screen test.
One-step ELISA for Antigen detection

Technical Basis

The One step ELISA for detection of an antigen in a biological sample is based on a double sandwich assay in which two different antibodies against the antigen are used, a capture antibody labelled with biotin and a detection peroxidase conjugated antibody. Streptavidin coated plates are used as the support to which the sample and a master mix containing both antibodies is added. In the reaction the biotinylated antibody will bind to the streptavidin immobilized on the surface of the plate, the antigen in the sample will then be captured by the biotinylated antibody and the captured antigen will be then detected by the peroxidase conjugated antibody.

The following figure shows a schematic diagram of one step ELISA.

Procedure: add the sample to streptavidin coated plate and the master mix. Incubate the plate overnight at 4°C.

Main Advantages

- Simplicity.
- Rapid.
- User friendly.
- Cost-effective.

Drawbacks/Limitations

Less sensitivity than PCR and RT-PCR.
ASSAY 1: INgezim One-step PRRSV/PCV2

Background

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are two of the most important viruses in the swine industry worldwide. PRRS is caused by a small, enveloped positive-stranded RNA virus, which belongs to the family Arteriviridae, genus Arterivirus. Two main genotypes with only 60–70% nucleotide homology, the so-called North American and European PRRSV genotypes, are clearly distinguished. Porcine circovirus type 2 (PCV2), a small, non-enveloped, single stranded circular DNA virus belonging to the genus Circoviridae, is causative agent of several diseases and syndromes, collectively referred to as porcine circovirus-associated disease (PCVAD). In the diagnosis of these two virus diseases PCR and RT-PCR is commonly used. Detection of antigen instead of DNA or RNA is a good alternative for diagnosis which presents additional advantages. The One-step ELISA technique has been developed for PRRSV and PCV2 detection. Antibodies against PRRSV nucleocapsid protein (N) that recognize either the European and American genotypes, and antibodies to PCV2 capsid protein encoded by ORF2, have been used. The N protein and capsid protein are the most abundant viral proteins in these viruses and the most immunodominant antigens in the pig immune response to PRRSV and PCV2.

Procedure

- Add 25 µl of the sample into streptavidin coated plate.
- Add 75 µl of PRRSV or PCV2 master mix and incubate ON at 4°C
- Wash the plate thoroughly.
- Add TMB substrate and incubate during 5 min at room temperature
- Stop the reaction with sulfuric acid.
- Read absorbance at 450nm.

Type of samples analyzed

Serum

Application

The diagnosis of PRRSV and PCV2 are commonly determined PCR and RT-PCR. All these methods require several steps like sample preparation, thermo cycling reaction, gel running and gel visualization. One step under RAPIDIA project provides a rapid alternative for PRRSV/PCV2 detection.
TECHNIQUE

Rapid assay for IgG and IgM detection in immunotubes.

Technical Basis

This method uses immunotubes as support and it has been designed to detect the presence of antibodies in a biological sample. The principle behind this technique is similar to a double antibody sandwich ELISA in which two different antibodies are used, a capture antibody and a detection antibody.

The immunotubes are coated with an anti-specie IgG or IgM antibody. After addition of the sample the IgM or IgG will be captured. The antigen of interest is then applied to the tube and further captured by the IgG or IgM. A peroxidase conjugated specific antibody against the antigen is added, and consequently bound to the antigen. Finally the substrate is added and the reaction is stopped with a solution containing SDS giving a bluish color. The interpretation of the result is determined by eye.

The following figure shows a schematic diagram of the rapid assay.

IgG detection B) IgM detection Immunotubes

Procedure: Step 1: Add the sample to the immunotube previously coated with an anti IgM or anti IgG antibody. Incubate 15 min at RT. Step 2: Add antigen. Incubate 15 min at RT. Step 3: Add peroxide conjugated specific antibody. Incubate 15 min at RT. Step 4: Add TMB and stop the reaction after 5 min. Visual determination. Several washes are done in between each incubation step.

Main Advantages

- Simplicity
- Rapid
- User friendly
- Cost-effective

Drawbacks/Limitations

Requires higher volume of sample than ELISA test. The test is valuable as a field test or in basic laboratories, especially in critical situations when positive results require immediate action.
ASSAY 1: INGEZIM-CPV IgM/ IgG detection

**Background**

Canine parvovirus (CPV) causes a frequently fatal systemic disease in a broad range of carnivore species, including domestic dogs and dogs entering animal shelters. An acute primary CPV infection may induce a specific antibody response as well as CPV vaccination. An antibody titer rise in paired serum samples is of decisive diagnostic importance or to clarify the immune status against the disease. Often the samples need to be sent to diagnostic laboratories for their analysis which are time consuming and costly. It would be clinically useful if there were diagnostic assays that could detect antibodies against CPV quickly in the veterinary clinic without the need of equipment or laboratory instruments. Therefore, we have developed and validated a rapid and sensitive CPV antibody assay.

**Procedure**

- Immunotubes coated with anti-canine IgM or IgG antibodies.
- Add 5 µl of sample and 500 µl dilution buffer and Incubate 15min at RT
- Wash 4 times.
- Add 5 µl antigen and 500 µl dilution buffer and incubate 15 min at RT.
- Wash 4 times.
- Add 5 µl peroxidase conjugate antibody against the antigen and 500 µl dilution buffer and Incubate 15 min at RT.
- Wash 5 times.
- Add 500 µl TMB and incubate 5 min.
- Add 500 µl stop solution (0.002% SDS).
- Visual determination.

**Positive sample:** Strong blue

**Negative sample:** Transparent or light blue

**Type of samples analyzed**

Serum

**Application**

The CPV immunization status or current CPV infections of dogs could be determined using a duplex IgG and IgM Rapid assay in an immunotube format.
Low density microarrays

Technical Basis

Microarray technology allows highly parallel visualization of specific bindings in a rapid and low-sample volume format. Therefore it plays a key role in many applications as diagnosis of diseases by Antibody-Antigen specific interaction. The low density microarray constitutes an important tool for either circulating Antibody detection or Antigen/Pathogen detection. Molecules in a microarray are attached to SuperEpoxy glass slides (ArrayIt®). Primary amines on the molecules surface act as nucleophiles, attacking epoxy groups and coupling the molecule covalently to the surface. Our customized slides contain 24 arrays (1X24 format) (Fig. below). Each of them can be sealed in individual reaction wells when assembled a hybridization cassette commercially available at ArrayIt® Corporation (Figure below) or just by separating arrays with hydrophobic inks. Therefore, 24 experiments can be performed at a time. Nanoliter amounts of target molecules are printed as 150 microns spots onto glass slides with a MicroGrid II TAS arrayer (BioRobotics, Genomic Solutions, UK) at Centro de Astrobiología (INTA). The visualization of the Antibody-Antigen reaction is based either on fluorescence or colorimetry.

Main Advantages

- Multiplex
- Sensitivity
- Reliability
- Low sample volume required

Drawbacks/Limitations

Printing and fluorescent technology are expensive.
ASSAY 1: Antigen microarray

**Procedure**

1. Microarrays printing of 24 bovine and porcine Antigens are prepared at 0.8mg/ml in a buffered spotting solution consisting of a mixture of commercial protein printing buffer 1X (Whatman International, UK) and 0.02% Tween 20. Total volume of 15 µl of each antigen is enough to print hundreds of microarrays. Each microarray must contain negative controls (BSA) and reference spots for subsequent grid positioning during image processing and quantification. Two different microarrays are produced according to the animal species.

2. Blocking the microarray for 1 hour with BlockIt™ Blocking Buffer Plus (ArrayIt®). Alternative home-made blocking solution can be used.

3. Washing with PBST (Phosphate buffered saline, Tween 20 0.1%) and subsequent centrifugation

4. Set up in the multi-chamber device

5. Incubation with test samples (40 µL) during 1 hour at RT

6. Washing with PBST and subsequent quick centrifugation to dry the slide

7. Incubation with the corresponding specific labelled Anti-IgG (Alexa647 fluorochrome for fluorescence or HRP for colorimetry) during 1 hour at RT

8. Washing with PBST and subsequent centrifugation

9. Only for colorimetry, 40 µl of TMB enzyme substrate addition for 10’ at RT

10. Scanning of the slide either with a fluorescence reader (GenePix® Personal 4100A Microarray scanner from Molecular Devices) or a colorimetric scanner (ArrayIt® SpotWare™) and subsequent image analysis with commercial software (e.g. GenePix® Pro software)

**Type of samples**

**Serum**

**Application**

Detection of circulating antibodies to pathogens in two different groups of animals: porcine and bovine. **The Porcine Antigen Microarray (PAgM)** contains 13 different antigens for antibody detection against the following viruses and bacteria: Circovirus, PRRSV, TGEV, Parvovirus, Aujeszky, Mycobacterium, ASFV, SIV, Brucella, Erysipelothrix, Mycoplasma hyopneumoniae and CSFV. **The Bovine Antigen Microarray (BAgM)** is composed of 11 different antigens for antibody detection against BTV, SBV, FMDV, AHSV and EHSV.
ASSAY 2: Antibody microarray

Procedure

1. Microarrays printing of 21 bovine and porcine monoclonal Antibodies (related to antigens in Page 4) are prepared at 0.8mg/ml in a buffered spotting solution consisting of a mixture of commercial protein printing buffer 1X (Whatman International, UK) and 0.02% Tween 20. Total volume of 15 µl of each antibody is enough to print hundreds of microarrays. Each microarray must contain negative controls (BSA) and reference spots for subsequent grid positioning during image processing and quantification.

2. Blocking the microarray for 1 hour with BlockIt™ Blocking Buffer Plus (ArrayIt®). Alternative home-made blocking solution can be used.

3. Washing with PBST (Phosphate buffered saline, Tween 20 0.1%) and subsequent centrifugation

4. Set up in the multi-chamber device

5. Incubation with test samples (40 µL) during 1 hour at RT

6. Washing with PBST and subsequent quick centrifugation to dry the slide

7. Incubation with the corresponding specific labelled Anti-IgG (Alexa647 fluorochrome for fluorescence or HRP enzyme for colorimetry) during 1 hour at RT to allow a sandwich (Antibody-Antigen-Antibody) on the reaction site.

8. Washing with PBST and subsequent centrifugation

9. Only for colorimetry, 40 µlof TMB enzyme substrate addition for 10 min at RT

10. Scanning of the slide either with a fluorescence reader (GenePix® Personal 4100A Microarray scanner from Molecular Devices) or a colorimetric scanner (ArrayIt® SpotWare™) and subsequent image analysis with commercial software (e.g. GenePix® Pro software)

Type of samples

So far, it has been tested with recombinant proteins. Further studies will be done with clinical significant samples (tissues, biopsies, secretions, etc.).
Application

Detection of antigens and/or whole pathogens in veterinary samples.

The **Porcine Antibody Microarray** (PAbM) is composed of 11 monoclonal antibodies to detect the following porcine pathogens: Circovirus, PRRSV, TGEV, Parvovirus, Aujeszky, and ASFV. The **Bovine Antibody Microarray** (BAbM) contains 11 monoclonal antibodies to detect BTV, EHDV, and AHSV.
Magnetic immunoassay (MIA)

Technical Basis

The antigen detection is based on the standard antigen-antibody interaction, as in ELISA or lateral flow device (LFD). In MIA, the specific antigen-antibody interaction is detected by the antibody that interacts with the antigen-antibody complex. This antibody is labeled by material which is magnetic, instead of conventional labels like peroxidases in ELISA. Since this label technology is magnetic, the assay is called magnetic immunoassay (MIA). The immuno-complex between the antigen and the antibody is captured to a solid phase which is an affinity-chromatography-like-consumable. The solid phase is a meshwork of antibody-coated plastic fibre, and thus facilitates the fast interaction space between the antigen in the sample and the antibody coated on the plastic surface. The more immuno-complexes form, the more magnetic material is attached to that complexes. The measurement of the magnetic signal is performed by a robust, 100% electronic, portable, 12V-battery-operable device. The amplitude of the signal is highly linear to the amount of the magnetic material contained in the solid phase. Due to the linear relationship of the signal amplitude to the magnetic content, the reader is able to give quantitative measurement of the amount of antigen. This MIA and its reader allow sensitive, quick and portable solution to the front line diagnostic testing at the field and small lab.

Main Advantages

- Simple and portable electronic device for measurement
- Non-optical signal
- Quantitative
- Faster than ELISA
- No need of heavy lab equipment

Drawbacks/Limitations

- No enzymatic signal-amplification, less sensitive than ELISA
- Slower than LFD
- Well-trained operator needed
- More expensive than LFD
- More labor intensive than LFD
ASSAY 1: MIA for BVDV Antigen detection

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### Background

The bovine viral diarrhea virus (BVDV) can directly transmit from animal to animal by contact. BVDV suppresses a cow’s immune system, making the animal susceptible to be a host of other infections. That is why BVDV is one of the world’s most costly bovine diseases, with high losses for dairy and beef producers alike. Stopping BVDV requires detecting and removing the infection source: persistently infected (PI) cattle that continually expose other animals to disease. Because PI cattle often look healthy and does not generate BVDV-neutralizing antibodies, a sensitive, specific diagnostic test is essential. PI cattle are the primary reservoir for BVDV infection in cattle herds, and hence are the major focus of control programs. The strategy of achieving detection of BVDV is the use of a mixture of monoclonal antibodies targeting E\textsuperscript{ns} protein, which is an envelope glycoprotein and is secreted in large amount into the extracellular environment.

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### Procedure

**Reagents:**

1. Detector antibody
2. Magnetic conjugate
3. Wash solution
4. Negative and positive controls

**Tools and disposables:**

1. Multi-channel pipette (max. volume=300 µL) and sample reaction tip fitted with coated solid phase
2. Round base tube, 1 mL
3. 96-tube rack, compatible with the dimension of the multi-channel pipette

The procedure here is described for 6 samples and using 8-channel pipette, as an example. Place 48 round base tubes into the 96-tube rack and fill the tubes with the reagents as stated in figure 1.

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**Figure 1. The layout of sample tubes and reagent tubes in the 96-tube rack for 6-sample-test**

Pipette the sample and detector antibody mixture according to figure 2, column 1. Then work through the rest of the columns sequentially, i.e. from column 1 to column 2, from column 2 to column 3 and so on.
Figure 2. The pipetting scheme, stepwise from column 1 to column 6. The drawing shows the sample reaction tips (inserted to an 8-channel pipette) is placed into the round-base tubes.

After the final wash at column 6, remove the sample reaction tip and measure the magnetic signal by inserting the tip into a magnetic reader. The positive control should give value of above 200 unit, while the negative control should be below 40 unit. Samples having higher than 50 unit are considered positive for BVDV Ag.

Type of sample analyzed

Serum and plasma.

Application

ELISA and RT-PCR tests for BVDV Ag require hours and specialized lab environment and equipment to obtain the results. MIA for BVDV is an alternative to LFD as a quick test, providing quantitative results instead of yes or no in LFD.
Recombinase Polymerase Amplification (RPA)

Technical Basis

The Recombinase Polymerase Amplification is a molecular based isothermal amplification technique that represents a rapid and simple alternative to the Polymerase Chain Reaction for the amplification of genome fragments in basically equipped field labs. In contrast to PCRs, which have to be conducted on thermocyclers to amplify genome fragments, the RPA reaction is working best at a single temperature, thus requiring only simple equipment such as a water bath. The optimal temperature of the RPA is between 37-42°C and results are obtained rapidly within 10 to 20 minutes. Similar to PCR, only two primers are needed, resulting in a very specific reaction and also multiplexing for the detection of more than one pathogen can be applied. The RPA reagents are provided in a lyophilized format by the manufacturer (TwistDx Limited, Cambridge, UK), thus no refrigeration is needed. The RPA process is based on a mixture of three enzymes including a recombinase that is able to recombine primers with homologous sequences of double-stranded DNA, a single-stranded DNA-binding protein (SSB) that binds to displaced stands of DNA and prevents the primers from being displaced and a strand-displacing polymerase, which synthesizes the DNA. These three enzymes can be combined with other enzymes such as reverse transcriptase for the amplification of RNA viral fragments or an exonuclease III that allows using exo-probes for a fluorescent based real-time detection. Additionally a specifically labeled probe can be included in the assay for subsequent amplicon detection using lateral flow devices.

Main Advantages

- Isothermal process: no thermocycler required
- Rapid and simple assay: results in 10 - 20 minutes
- Good sensitivity: single DNA molecule detection
- Portability: isothermal battery-powered instruments are available
- Stability: lyophilized reagents, no refrigerating is needed

Drawbacks/Limitations

TwistDx, the only supplier of RPA reagents, offers a basic kit which is an end-point detection system similar to conventional PCR. The RPA amplicons need to be purified before visualization by either lateral flow devices or gel-electrophoresis requiring time and further consumables. Additionally the tubes need to be opened after the RPA reaction, bearing a risk of amplicon carry-over and contamination. However, in this first proof-of-principle approach the RPA assay developed worked very efficiently and results serve as a basis to transfer the assay to a real-time RPA format which represents a closed system. These realtime RPA can also be performed in a portable system providing the opportunity to conduct the amplification as penside tests directly in the field.
ASSAY 1: Rapid RPA assay for the detection of African swine fever virus (ASFV)

Background

African swine fever virus (ASFV) is a double stranded DNA virus and belongs to the genus Asfivirus within the family Asfarviridae. ASFV is the only known arthropod-borne DNA virus that is transmitted either through their vectors (Ornithodorous ticks) or directly from animal to animal. ASFV is highly contagious and causes hemorrhagic fever with high mortality rates in domestic pigs causing great economic losses in pig industries as no vaccines are available to date. To prevent the spread of the disease in disease-free countries, regions or animal holdings, only protective and restrictive measures can be applied. Thus, it is of utmost importance to have simple-to-use and quick methods available for early pathogen detection to apply rapid countermeasures to avoid further spread of disease. In this approach a RPA assay for the rapid detection of ASFV was developed, that can be applied in basic field labs by using basic equipment e.g. a water bath.

Procedure

The RPA master-mix was prepared according to the manufacturer’s recommendations (TwistDx Limited, Cambridge, UK) and the RPA reaction was incubated at 37°C for 4 minutes. The tubes were inverted 10 times, briefly centrifuged and incubated again for another 16 minutes at 37°C. The RPA products can be either visualized by using gel-electrophoresis or by using nucleic acid lateral flow devices. For the latter, a probe specifically labelled must be included in the reaction. The figure below shows a positive sample (left side, two lines) and a negative sample (one line, control line).

Positive sample: two lines appear
Negative sample: only one line appears

In any case the control line must appear, otherwise the test has to be considered invalid and must be repeated.

Type of samples

DNA from serum and plasma, tissues or swabs

Application

Molecular detection of ASFV genome fragments is based on PCR techniques requiring thermocyclers and a considerable amount of time. Using RPA technology, no sophisticated PCR cyclers are needed as the reaction is carried out best at 37°C within a very short time (approx. 20 minutes). Thus RPA technology for detection of ASFV genome fragments provides a rapid and simple alternative to PCR applicable in basically equipped field labs or directly in the field when using in combination with a portable instrument.
field test/small lab tests

TECHNIQUE

Fully automated PCR designed for non-expert users in non-laboratory settings

Technical basis

Real-time PCR is the basis of many of the laboratory reference tests for infectious organisms, especially for viruses where it provides the most convenient and sensitive method of detection. The basis of RT-PCR is the selective amplification of nucleic acid sequences of the target organism and specific detection of these sequences as amplification occurs. This provides a semi-quantitative output. The reference RT-PCR tests generally involve several independent steps which are carried out with different items of laboratory equipment, often in separate laboratories, the steps being:

1) Lysis to release nucleic acid from cells or virions
2) Nucleic acid purification to extract as much of the target nucleic acid as possible whilst efficiently removing substances that may interfere with the PCR process
3) Addition of the extracted nucleic to the PCR reaction mixture
4) Performing RT-PCR by thermal cycling of the reaction mix whilst monitoring signals from sequence-specific probes which, most commonly, are fluorescent-labelled
5) Visual analysis of results which are in the form of amplification curves on a computer screen.

With instruments that act robotically on single-use cartridges, Enigma systems integrate sample handling, nucleic acid extraction, RT-PCR and results analysis. The cartridges contain all of the nucleic acid extraction and purification reagents and components as well as the RT-PCR reagents and the thermal cycler. Photographs of the ML instrument and cartridge (left) and FL instrument and cartridge (right) are shown below, together with diagrams of their cartridges to reveal more detail.

Procedure: For the ML, sample in a standard tube is slotted into the cartridge. Sample details are entered through the user interface and the barcode on the cartridge provides assay instructions to the instrument. The user places the cartridge on a tray presented by the instrument which then runs the test and gives the result on the screen and on a printout. For the FL, sample is added to the cartridge, the user selects the test, adds the cartridge, shuts the door and presses the start button.
Main Advantages

Eliminates: operator variability; cross-contamination between samples; need for a cold chain for reagents (ambient storage of cartridges for 12 months); specialist facility requirements; specialist skills required for the operator. Provides: fast results (55 minutes for FL; about 70 minutes for ML).

Drawbacks/Limitations

The ML requires mains power. The FL needs further development before commercialization so more cost-effectively produced ML components can be used. The FL is single cartridge only (ML is 1 to 6).

ASSAY 1: FMD

Background

Foot and Mouth Disease virus (FMDV) is a small, non-enveloped, positive-sense RNA virus within the Family Picornaviridae, Genus Aphthovirus. There are several distinct serotypes of the virus. It infects cloven-hoofed animals including sheep, cattle, pigs and goats. It is highly contagious and causes acute vesicular disease with fever, painful blistering, lameness and loss of appetite and may be fatal in young animals. The acute phase lasts about a week and may be followed by secondary infections. The suffering caused together with the loss of yield make the control of its spread important, especially in FMD-free regions.

Diagnosis of FMDV infection is made difficult by similarities in clinical signs with other diseases including Swine Vesicular Disease, Vesicular Stomatitis and Bovine Calcivirus.

Procedure

Tissue from lesions is prepared by manually grinding excised tissue in buffer with sharp sand and then pipetting 2ml of the supernatant into a sample tube containing granular lysis reagent. Alternatively, 2ml samples of serum or vesicular fluid may be added directly to the sample tube. The tube is sealed with a transport cap.

The outside of the tube can be sterilized with, for example, bleach whilst the contents of the tube are inactivated by the lysis reagent and this allows the sample to be transported off the infected premises for testing. The sample tube is taken to a vehicle outside the biosecurity zone or to suitable near-field premises where the ML instrument and a supply of ML cartridges are stationed.

The transport cap on the sample tube is replaced with a piercable foiled cap and the tube is then loaded into the sample tube holder in an ML cartridge which is then folded down into the cartridge body.
The user enters test details through the touch-screen on the instrument and/or via the in-built barcode reader to record user and sample information. The cartridge barcode is scanned by the in-built barcode reader in the front of the instrument.

On pressing the Start button, the instrument opens the door of the processing module. A tray is presented and the user is asked to insert the cartridge. After the cartridge is placed onto the tray, the user presses the close button and the automated analysis starts.

The time taken for the whole process for FMDV is about 75 minutes. It includes: releasing the lysed sample from the tube; adding a control; preparing the nucleic acid using a magnetic particle-based process with capture, wash and release steps; rehydrating a freeze dried RT-PCR mix with the nucleic acid preparation; reverse transcription to convert RNA to DNA; 45 cycles of DNA amplification; and automated analysis of the real-time PCR fluorescence data)

At the end of the process, the results are presented to the user on the touch-screen and on a paper printout. The result information given is kept simple: FMD Positive or FMD Negative or, if for some reason a result cannot be called, Test Failure is reported. Additional information records: Date and Time of Test; Operator ID; Expiry Date/Lot Number/ Identification Number for the cartridge; Serial Numbers/Software Version for the instrument. Electronic data download via LIMS or USB can also be made available.

The user is presented with a touch button to eject the cartridge which is removed for disposal through normal means, typically incineration, and the system is ready for another test.

The configuration of the ML instrument shown above is one Control Module (CM; to the left) and one Processing Module (PM; to the right). Up to six PMs can be attached to each CM, operating independently through the same touch-screen. This provides flexibility in terms of throughput. In terms of portability, the one-plus-one or one-plus-two configurations are most suitable.

<table>
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<th>System Configuration</th>
<th>Width (mm)</th>
<th>Height (mm)</th>
<th>Depth (mm)</th>
<th>Weight (kg)</th>
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<td>355</td>
<td>430</td>
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<td>355</td>
<td>430</td>
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Types of samples analyzed

Epithelium, vesicular fluid and serum.

Application

The system provides laboratory-standard nucleic acid based test results in field or field-lab settings. This provides timely, high confidence results to be obtained so that appropriate measures can be taken quickly. Delays from sample transport to centralized laboratories and reporting-back of the results can be avoided. The same system may be flexibly used for a range of infections through the use of disease-specific cartridges, programming information for the test being specified through the cartridge barcode.

The cartridges are single-use and a cold-chain is not required for their transport or storage.
confirmatory tests

Background

The obtained results of the field diagnostics should be confirmed applying all the sophisticated and accurate techniques by the reference labs. Especially in case of notifiable diseases.

RAPIDIA contribution

Development and implementation of techniques for confirmatory reasons and as a basis for adaptations especially of molecular tools.

Methods will include sequencing (next generation sequencing with 454/Roche technology) and highly multiplexed genome detection methods including microarray systems. These technologies will be of use in diagnostically difficult situations like (re-)emergence of unexpected pathogens or pathogen variants.

Multiplex immunoassay for viral diagnosis (e.g. FMDV, BTV, West Nile Virus) by using the Luminex liquid array technology will be developed/tested and used as confirmatory technology. This assay will allow e.g. 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.
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<tr>
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