Final report club 5 joint research
Maximum 4 pages;

Development and validation of alternative diagnostic tools for ASF

Participating partners:
ANSES: Marie-Frédérique Le Potier, **marie-frederique.lepotier@anses.fr**
CVI: Willie Loeffen, **willie.loeffen@wur.nl**, Helena Ferreira, **helena.ferreira@wur.nl**
DTU: Åse Uttenthal, **asut@vet.dtu.dk** (until 31.07.2013), Anette Bøtner, **aneb@vet.dtu.dk**, Bertel Strandbygaard, **bstr@vet.dtu.dk**
SVA: Lena Renström, **lena.renstrom@sva.se**

project leader

Name: Willie Loeffen

Email address and telephone number: **willie.loeffen@wur.nl** (+31(0)320 238696)

Proposal Summary (no more than 100 words)
The development and validation of additional virus detection and serology tests could improve and support African swine fever (ASF) diagnostics.
Currently, ASF virus detection tests use primary cells. Virus detection using established cell lines instead of primary cells would allow for large scale titrations, could lead to the development of more simple and harmonized test procedures, improved reproducibility and reliability.
Developing an IPMA using cell lines could lead to a simple, standardized, highly sensitive and specific serological confirmatory test for ASF. Given the large economic impact of ASF control measures, several highly reliable tests for ASF are needed.

Date started:	October 2012
Length of project	15 months
End of project:	December 2013

Project Team

Names	Institute	Role in project
Willie Loeffen	CVI	Main Coordinator
Anette Bøtner	DTU	M3 coordinator
Marie-Frédérique Le Potier	ANSES	M5/6 Coordinator
Lena Renström	SVA	M1/2 Coordinator
Helena Ferreira	CVI	M4 Coordinator

Research Questions

Explore different cell lines' suitability for ASF testing. Analyse the robustness of the methods by exchanging materials, ideas and results.
Implement protocols for ASF testing, both virological and serological, depending on individual partner institutes' needs.
Examine and compare developed tests to currently used tests for ASF.
Explore the use of different Monoclonal antibodies (MAbs) for the detection of all (known) ASFV strains. Study the possibility of using one MAb or a pool of Mabs for all ASFV strains.

Key Objectives:

Improve and standardize virus isolation/virus titration and serological confirmation tests using cell lines through cooperation and sharing of expertise between the Club 5 partner institutes.

Brief workplan, including key milestones

1.Exchange of experience with cell culture based techniques for virus isolation (VI) and immuno peroxidase monolayer (IPMA) (or other antibody detection technique).
2. Improve protocols in individual labs and where possible harmonize protocols, depending on individual lab preferences and needs (this may include exchange of personnel for short visits).
3.Testing different ASFV strains with different MAbs, with the aim of finding a MAb or pool of MAbs that can be used for the detection of all ASFV strains and compare to the use of polyclonal antibodies (PAbs).
4.Compare the sensitivity and specificity of VI on cell lines to VI in primary cells tested by haemadsorption or specific staining.
5.Create a list of assessment criteria to compare serological tests and comparing the IPMA to commercially available ELISA's.
6.Create a reference panel of sera that subsequently will be used for a ring trial of serological tests at a conclusion of this project.

Milestone No	Milestone title	Month
1	Exchange of experience and existing protocols for confirmatory tests	October-November 2012
2	Improve and test existing or new protocols in individual labs	December 2012-July 2013
3	Testing different MAbs, compare them to PAbs	January-March 2013
4	Comparison of virus isolation using cell lines and using primary cells tested by hemadsorption (HAD). This could take place during a workshop in one of the labs	May-June 2013
5	List of assessment criteria for comparison of serological tests and comparison of IPMA (or other alternative test) to ELISA	July-September 2013
6	Ring trial for serological tests in all labs	September-October 2013

Planned output*.(deliverables, to include possible publications and IP issues)*

Four intermediate reports and one scientific article.

Deliverable No	Deliverable title	Month
1	Full report regarding the protocols used in each institute. Each lab provides a summary of their methods.	May 2013
2	Report on the comparison of virus isolation using cell lines with virus isolation using primary cell tested by hemadsorption or staining.	July 2013
3	Report on the comparison of the serological tests developed in this project with available ELISA (including assessment criteria list)	August 2013
4	Ring trial report/ Results of test validation – and abstract for NASFL meeting 2014	October 2013
5	Publication – Scientific article on final results of this project	November 2013

Results (maximum 1,5 page)

At the start of the project, existing or preliminary protocols for virus isolation were send by DTU and CVI to all the partners. Virus isolation protocols were making use of different primary cells (VN2 cells (secondary Pig Kidney cells), PAM cells (Porcine Alveolar Macrophages) and PBL cells (Peripheral Blood Lymphocytes)) or a cell culture (MARC-145 cells). All protocols relied on immune-staining to confirm the presence of ASFV. Further protocols for a serological confirmation test (IPMA) were distributed by DTU and CVI. ANSES also had a protocol available, which was based on immunofluorescence, contrary to immonoperoxidase.
SVA did not have protocols at the moment and focussed on implementing and evaluating the DTU protocols for the rest of the project
All participants obtained the ASFV BA71VR strain and VERO-cells from the EURL in Madrid, which uses these as reference material for diagnostic testing. Where necessary, these materials could serve as controls.

DTU tested a panel of MAbs and PAbs, available from CISA-INIA and/or Ingenasa (see table below) against several ASFV strains. PAbs were reacting with all viruses tested. MAbs do not always react with all viruses, and in case of working with MAbs, a pool of different MAbs is advised. Additional work has been carried out with PAbs obtained from individual institutes in animal experiments. Most of these convalescent sera can be used in high dilutions in serological confirmation tests and virus isolation for the specific detection of ASFV. Stored in larger amounts (>100 ml) these sera are a practically unlimited source for diagnostic testing and can be easily shared among institutes if the need arises.

Antibody	Type	Virus				
		E70	E75	Malawi	Malta78	Georgia2007
Valdemos S+45	PAb	pos	pos	pos	pos	
Valdemos S+72	PAb	pos	pos	pos	pos	
Valdemos S+32	PAb	pos	pos	pos	pos	pos
1BC11	MAb	pos	-	pos	-	pos
17KC6	MAb	-	pos	-	-	pos
2CG12	MAb	pos	pos	-	pos	-
19DG8	MAb	-	-	-	-	pos
17KC1	MAb	pos	pos	pos	pos	pos
18FA4	MAb	pos	pos	pos	pos	pos
Pool 2CG12, 17KC1, 18FA4, 1BC11	MAb	pos	pos	pos	pos	pos

All institutes have been working on cell cultures as a possible matrix for virus isolation. Cell lines involved were mainly VERO cells, which are also being used by the EURL, and MARC-145 cells. At CVI a more extensive comparison of cell cultures has been carried out (also involving PK15, SK6 and a wild boar lung cell line), with three virus strains: Malta78, Brazil78 and Netherlands86. MARC-145 showed a combination of high sensitivity and easy use and were selected for both IPMA as a serological confirmation test and for use as a matrix in virus detection (staining cells with PAbs). While virus genome and proteins were being produced in these cell cultures, no viable virus was leaving the cells. Similar results were obtained by ANSES, using additional virus strains (Madrid, Uganda, Georgia2007). They compared the number of infected cells in cell cultures (VERO, MA104, MARC-145) to that on PAMs. Except for the Uganda strain, fewer cells became infected in cell cultures, compared to PAMs. This also suggests that virus may infect single cells, but is not always able to fully replicate and spread to other cells. Differences between individual strains do exist, though, and adaptation of individual viruses to cell culture is likely needed to optimize virus replication. Cell cultures are therefore usually not suitable for virus replication, especially for wild type viruses, but are suitable for virus detection and large scale virus titrations, which may for instance be needed in animal experiments to obtain valuable data that would not be easy to obtain using primary cells.
Further comparisons carried out at the CVI showed that the sensitivity of virus detection and

titration on MARC-145 cells was similar to that on PBL. A comparison of ASFV detection by staining with PAbs versus HAD, using PBL, showed that both techniques had a similar sensitivity. Single infected cells could be detected microscopically with both methods. HAD has the advantage that plates can be read repeatedly on a daily basis. On the other hand, non-hemadsorbing viruses are known to exist, therefore preferring the use of PAbs. If necessary, both techniques can be combined in one plate. Both a virus isolation and IPMA based on MARC-145 cells has been set-up and initially validated at the CVI now.
DTU has continued working on VI on MARC-145 cells and PAMs. Isolation on PAMs is working satisfactory now, work on MARC-cells will be continued after the CoVetLab project. For an IPMA as a serological test, VERO cells turned out to be the preferred cell line in their hands and this test is now available for diagnostic testing.
SVA has primarily adopted the DTU and EURL protocols and worked on further optimization and subsequent implementation of an IPMA, based on VERO cells, which has been successful.
ANSES has chosen a slightly different path for confirmatory serological testing, by adapting an IPMA protocol to an immunofluorescence test. The main reasons for adopting the test were two-fold: 1) easier reading of intracellular immunofluorescence compared to colorisation, and 2) some health safety concerns for the substrate used in an IPMA. This protocol has been fully validated and implemented at ANSES.

All serological test protocols were evaluated in a small ring trial, using samples from the yearly EURL ring trial. Results of the ELISA and the confirmation test are shown in the table below. Shaded figures are positive. Overall the results compare very good between ELISA and confirmation test, and between individual institutes. DTU has one additional positive result, which matches with the slightly higher inhibition percentage in the ELISA. CVI seemingly has one false positive in the IPMA. Official results of the ring trial will be presented by the EURL in June.

Sample	ANSES		CVI		DTU		SVA	
	ELISA	IFMA	ELISA	IPMA	ELISA	IPMA	ELISA	IPMA
1	19.8	<20	15	160	18.1	<10	16	<80
2	18.9	<20	18	<40	19.8	<10	14	<80
3	21.2	<20	16	<40	19.2	<10	17	<80
4	100	240-480	104	320	100	≥1280	113	≥80
5	23.5	<20	20	<40	21.1	<10	23	<80
6	100	1280-2560	93	2560	100	≥1280	106	≥80
7	8.5	<20	16	<40	18.3	<10	17	<80
8	32.1	<20	24	<40	35.2	80	26	<80
9	98.8	2560-5120	104	2560	100	≥1280	104	≥80
10	100	≥10240	100	>=40960	100	≥1280	102	≥80
11	100	5120-10240	99	>=40960	98.9	≥1280	102	≥80

In addition to working on confirmatory tests, both CVI and ANSES evaluated a second ELISA, which became available during the CoVetLab project. This new ID-Vet ELISA was compared to the existing ELISA from Ingenasa, using in-house serum panels. Both institutes concluded that the overall performance of the ID-Vet ELISA was good, but the analytical sensitivity was somewhat less than of the Ingenasa ELISA. As a result there will be a delay of several days until pigs can be tested seropositive after an infection. For now the Ingenasa ELISA is still the preferred test.
A discussion on assessment criteria for serological tests was initiated by ANSES. Test characteristics of interest are primarily analytical and diagnostic sensitivity, analytical and diagnostic specificity, and repeatability of the test. Standard serum panels need to be put together, and criteria for these characteristics, for the specific panels used, need to be determined. For comparisons and standardisations between labs, exchange of reference samples to be included in these serum panels would be needed. This will require further work and could be followed up in due time.

Problems experienced

-
Value of cooperation for club 5 institutes
The value of cooperation in this project is visible on several levels. The exchange of protocols allowed for critically reviewing the own protocols for diagnostic confirmation tests and, in case of SVA, for the implementation of a protocol for an as yet unavailable test. Together, the partners were able to test more different methods, sera (e.g. MAbs vs PAbs) and viruses, than any individual institute could do. Exchange of these results allows for a further evaluation of the reliability of these tests in the broadest sense. As a result of both the cooperation within the CoVetLab project and the individual work of the partners, all have now confirmatory tests available.
Any other information
-

Evaluation	
Deliverables met?	Deliverables were not met as stated in the initial project plan. No individual reports on activities were written and a scientific publication was no longer seen as opportune. Instead this summarizing report was written. Main deliveries instead include for the individual institutes SOP's for the confirmation tests and preliminary or even full validation reports. Confirmatory diagnostic tests are now broadly available at a sufficient level in all participating institutes.
Start of project	Oktober 2012
Cooperation	Exchange of protocols and results, occasional meetings of partners present during other meetings (e.g. CSF_goDIVA or EURL-meeting), bilateral contacts of partners on specific issues.
Advice towards new calls / new projects	-