PROJECT FINAL REPORT

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Project acronym: **FMD-DISCONVAC**

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4.1 Final publishable summary report

This section must be of suitable quality to enable direct publication by the Commission and should preferably not exceed 40 pages. This report should address a wide audience, including the general public.

4.1.1. Executive summary

The FMD-DisConVac project addressed specific gaps in our knowledge on all aspects of FMD control to enable implementation of enhanced animal-sparing vaccine-based control strategies. The project focused on:

(i) the refinement and replacement of in vivo FMD vaccine quality tests in light of the 3R concept. This was achieved by (a) determining correlation models between existing/new (IFN-γ ELISA; opsonising antibody levels) *in vitro* laboratory tests and *in vivo* protection, (b) developing monoclonal antibody based *in vitro* immunoassays to monitor vaccine purity, (c) improving methods to quantify the antigen payload content in the final product. Furthermore, serological methods were evaluated to estimate the protection level in vaccinated populations.

(ii) the assessment of heterologous protection by mono- and multivalent FMD vaccines against strains of the same serotype. Valuable results were obtained by performing a series of cross-challenge tests and comparing results with homologous challenge studies. Correlation models between cross-protection and existing/new (IFN-γ ELISA; avidity and IgG subtype ELISA) *in vitro* serological methods were determined. Guidelines were established for the reliable selection of reagents to include in *in vitro* matching studies and r-value determination between vaccine strains and FMDV field isolates was improved by harmonising test methodologies and organising ring-trials and international workshops. A method for predicting changes in r-values from sequence changes was proposed.

(iii) the development of new generation FMD vaccines and antivirals. The production and efficacy of a canine adenovirus- and an encephalomyocarditis virus vectored FMDV vaccine were evaluated. A replication defective human adenovirus- (with or without interferon alpha) and a Sendai virus vectored FMDV vaccine were developed for the reinforcement of the mucosal immune response to prevent infection at the oro-pharynx and are very promising. Mucoadhesive nanoparticles for mucosal delivery were formulated and recombinant baculoviruses, with or without additional CpG motifs, were assessed as adjuvants for FMDV vaccines. Three different antiviral compound classes were active against the different serotypes of FMDV. Proof-of-concept of antiviral activity *in vivo* was demonstrated in guinea pigs and in severe combined immunodeficient mice.

(iv) the improvement in FMD diagnostics. Ready-to-use ELISA kits were developed for the detection of antibodies against structural or non-structural FMDV proteins and for the detection and serotyping of FMDV antigens. These kits were used in poorly equipped laboratories to improve diagnostic capacity and FMD control in endemic countries. Extensive validation of mucosal IgA tests and NSP tests based on novel proteins and peptides was performed to help detecting infected animals, especially carriers, within a vaccinated population. Prototypes of Luminex multiplex immunoassays were developed.

(v) the enhancement of knowledge on FMD transmission. The between species transmission from infected cattle to sheep and from infected sheep to cattle was quantified. The contribution to transmission of the virus contaminating the environment was high. FMDV was transmitted from infected Asian buffalo to susceptible in-contact naïve buffalo and cattle by direct contact and FMD vaccination prevented this kind of transmission. The

involvement of wild boar or gazelle in FMD outbreaks was highly variable. Post outbreak NSP surveillance was a highly valuable tool to determine the virus spread in the field.

(vi) the development or adaptation of computerised FMD spread models to optimise vaccination schemes. Alternative vaccination strategies were studied by adapting three different models. A ranking of the simulation models according to their likely sensitivity to weak or missing information was submitted.

4.1.2. Summary description of project context and objectives

The Foot-and-Mouth Disease virus (FMDV) is a classic example of a highly contagious RNA virus that affects multiple species of cloven-hoofed animals. The virus is an Aphthovirus within the family of Picornaviridae. To date, seven different serotypes have been identified (O, A, C, Asia 1, SAT1, SAT2 and SAT3). The disease is characterised by the formation of vesicles and erosions in the mouth, nose, teats and feet.

Foot-and-mouth disease (FMD) is one of the world's most important infectious diseases of livestock. FMD has been identified as one of the priority animal diseases both within the European Commission's Animal Health Strategy "Prevention is better than Cure" (2007-2013) (http://ec.europa.eu/food/animal/diseases/strategy/ index_en.htm) and the European Technology Platform for Global Animal Health (ETPGAH) (http://www.ifahsec.org/europe/EUPlatform/Platform.htm). FMD outbreaks are very disruptive to normal life and economic activity. The incentive for FMD control, however, varies as the impact of FMD is largely dependent upon the international trade status of the respective country/zone. Economic considerations and access to lucrative trade markets are indeed by far the major incentives for controlling FMD.

The main concern of FMD-free regions such as the EU is to prevent virus introduction. Despite all efforts to control the borders, outbreaks will occur, as exemplified by the 2001 FMD outbreak in the United Kingdom (UK) and the 1997 outbreak in Taiwan.

The current version of the World Organisation for Animal Health (OIE) Terrestrial Animal Health Code makes provision of zoo-sanitary measures, movement restrictions, "stamping out" and/or vaccination to control outbreaks and regain FMD-freedom. Until recently, most FMD-free countries/zones not practising routine vaccination favoured a "stamping out" policy accompanied by zoo-sanitary measures and movement restrictions (e.g. Council Directive 90/423/EEC), mainly because of the short 3-month waiting period to recover FMD-free status as defined by the OIE. As a result of the mass slaughter of animals during the UK 2001 FMD epidemic, however, "stamping out" has been the topic of heated debate. Consequently, and accelerated by the availability of reliable "marker" vaccines and DIVA tests (Bergmann et al., 2000; Brocchi et al., 2006), both OIE and European guidelines were amended to facilitate the use of emergency vaccination (Council Directive 2003/85/EC and Community's Animal Health Strategy) and to reduce the waiting period from 12 to 6 months when applying such vaccination (OIE, 2007b). In FMD-endemic and FMD-free-with-vaccination countries/zones, outbreaks are primarily controlled by vaccination (Lubroth et al., 2007).

Although the Community's Animal Health Strategy (2007-2013) recognises that significant scientific and logistic advances in animal disease prevention, diagnosis, surveillance and control have been made in recent years, including progress in the field of FMD [e.g. through the Sixth Framework Programme projects FMD-ImproCon (SSPE-CT-2003-503603)], some drawbacks persist and need to be tackled to improve food safety aspects, as well as economic and animal welfare considerations. Therefore, this consortium seeked to build upon its

expertise gained in the FMD-ImproCon project (SSPE-CT-2003-503603) for addressing the remaining gaps, as identified by ETPGAH.

Therefore, the project aimed to (i) improve the quality of existing FMD vaccines and diagnostics, (ii) refine and replace in vivo vaccine quality tests, (iii) develop new generation FMD vaccines and diagnostics by cutting edge technology, and (iv) to increase/enhance our knowledge on FMDV spread and transmission following the use of high-potency monovalent or multivalent vaccines in free and endemic settings. Six priority work packages (work package [WP] 2-6) were identified towards which scientific and technological efforts were directed.

Work Package 2 (WP2), the Reduction and refinement of in vivo vaccine quality tests by in vitro methods, aimed to address and complement the first item listed in the Work programme topic KBBE-2008-1-3-02, i.e. (i) determine and validate correlation models between in vitro laboratory tests (serology, interferon-γ release by blood leukocytes and other cellular correlates to immunity) and in vivo protection based on experimental and field data, thereby harmonising tests to assess vaccine efficacy (refinement of the in vivo test), (ii) develop in vitro immunoassays to monitor vaccine purity by the reduction of FMDV non-structural proteins (NSP) content during the vaccine purification and in the final vaccine product (replacement of the in vivo test), and (iii) develop alternative methods to quantify the antigen payload content in the final product. As such, producer-independent vaccine quality test results will be available, while the confidence in the overall quality of the vaccine batch will be guaranteed in a verifiable form to end-users and other stakeholders (e.g. trading partners), strengthening the position of the EU on the global market.

Work Package 3 (WP3), the assessment and improvement of heterologous protection by FMD vaccines is directly correlated to the second item listed in Work programme topic KBBE-2008-1-3-02 and intended to predict how well a vaccine (highly potent monovalent and bivalent) will protect against a challenge virus (or newly emerging field isolate) of another strain within the same serotype. In order to do this, a series of cross-challenge tests had to be performed since only limited in vivo data was on hand. The objective was to compare the results with those of homologous challenge studies. The work focussed on serotypes O and A as they represented the most likely threats to Europe, but also because serotypes O and A are significantly different from each other in terms of antigenic variability and induction of immunity. Moreover, it was the objective to study the correlations between the observed cross-protection and the one predicted by serology and by amino acid homology of the respective virus capsids. Hence, the depth of our knowledge and expertise regarding vaccine spectrum coverage will be increased. Furthermore, it was the objective to improve r-value determination between vaccine strains and FMDV field isolates by harmonising test methodologies and drafting guidelines for the reliable selection of reagents (i.e. bovine post vaccinal sera) to include in in vitro vaccine matching studies thereby avoiding future in vivo cross-protection studies.

Work Package 4 (WP4), the development of vaccines and alternatives (antivirals) with rapid onset of immunity and based on safer production methods, was related to the third item listed in Work programme topic KBBE-2008-1-3-02 and intended to increase our knowledge by investigating approaches for reinforcing the mucosal immune response in order to prevent FMDV infection at the primary portal for virus entry (i.e. the oro-pharynx). Animals vaccinated with current vaccines are generally protected against disease; however, no sterile immunity is achieved resulting in possible further virus shed and the development of the "carrier" state. Better vaccines could be developed if ways can be found to elicit immune responses at this site, rather than solely relying on parentally administered antigens that mainly induce systemic immunity. It was the objective to evaluate methods to elicit and

measure mucosal immunity against FMDV in cattle, which represent the major target species for vaccination. Ways to stimulate innate (rapid) and adaptive (lasting) mucosal immune responses will be investigated, using novel delivery systems, adjuvants and viral vectors. Furthermore, it was the objective to develop other new generation vaccines and to test them for their efficacy, avoiding the need for virus culture, thereby making the production of FMD vaccines environmentally safer.

An alternative approach by which the post vaccination "immunity gap" could be decreased is through the development of potent and selective anti-FMDV antiviral compounds that rapidly and completely prevent FMDV replication. Based on previous experience, it was the objective to screen and optimise possible compounds and to study the potential for inhibiting virus replication and disease transmission.

Work Package 5 (WP5), the improvement in FMD diagnostics, is responding to the fourth item listed in Work programme topic KBBE-2008-1-3-02 and focussed towards (a) increasing the availability of FMD diagnostics and (b) improving standardisation and harmonisation of FMD diagnostic results. Both objectives were to be obtained through the development of a panel of stabilised, validated, reliable diagnostic kits for FMD serology and antigen typing, ready for commercial exploitation. Secondly, there is a pressing and continuous need to develop new and, possibly, better diagnostic tools. For instance, confirmatory tests and/or test systems for NSP-serology are urgently required, and it was the objective to develop these tests in the framework of the project [confirmatory NSP test and IgA in saliva ELISA], as well as assays in which serum reaction profile are obtained simultaneously against a number of antigens (i.e. multiplexing), including some of clinically indistinguishable diseases. Although, the consortium recognised that several promising assays have been developed in recent years, their validation was generally still in the early stage. To increase our expertise, it was the objective to organise a workshop in which a selection of the best, worldwide available, assays were to be comparatively evaluated. Based on such sound scientific evidence, dossiers submitted to the OIE/EU to demonstrate/substantiate FMD-freedom in which the results of these tests are utilised, can be better interpreted.

Work Package 6 (WP6), Improving knowledge on FMDV transmission between species and in recently vaccinated animals, corresponds to the fifth item listed in Work programme topic KBBE-2008-1-3-02 and intended to obtain, previously unavailable, quantified knowledge on FMDV transmission within and between different FMDV susceptible species in the period shortly after applying emergency vaccination, and to study transmission dynamics in real-time outbreak situations to set-up early warning systems for FMDV penetration. It was the objective to set up carefully designed FMD transmission experiments to study the effect of vaccination in preventing FMD transmission through contact exposure to the virus. Furthermore, the Asian buffalo (Bubalus bubalis) is a livestock species of growing importance in rural areas (e.g. milk production in India), but has poorly been studied. It was the objective that during the course of the project, a newly developed infection model was utilised to study the ability of the Asian buffalo to transmit FMDV infection and to investigate the efficacy of vaccination to prevent this. It was the objective to study the role of wildlife (gazelles and wild boar) in FMDV maintenance and transmission as well as to quantify the presence of FMDV in viral secretions and excretions in different species (e.g. wildlife, sheep, etc). The obtained output can be used as parameters to adapt and improve computerised FMD spread models to optimise FMD vaccination programmes in free and endemic settings alike (WP7).

Work Package 7 (WP7), Development or adaptation of computerised FMD spread models to optimise vaccination schemes, addresses the sixth item listed in Work programme topic KBBE-2008-1-3-02 and intended to study the applicability and feasibility of modifying

existing simulation models for FMD spread to suit the exploration of vaccination strategies in the EU and other Western countries where FMD is considered an exotic threat. An existing Danish-funded project for 2008-2011 studied alternative vaccination strategies for Denmark involving three different models: (a) the InterSpread Plus model from Massey University, New Zealand, (b) The Davis model from University of California and (c) the North American Animal Disease Spread Model (NAADSM) developed by the Centres for Epidemiology and Animal Health (CEAH). The objective was to extend the study of alternative vaccination strategies by using the three referenced models, complemented with others models within the Consortium, and applying/modifying them to countries within the consortium having recently experienced repeated re-introductions or re-appearances of FMD outbreaks of FMD. Computer models, developed to enable the design of vaccination strategies for these high-risk regions, would be relevant step-stones to model vaccination strategies for truly endemic regions of the world, to achieve the ultimate goal of global control and eradication of FMD, as described by the "Global Roadmap for Improving the Tools to Control FMD in Endemic Settings in 2006" (http://www.endemicfmdroadmap.net).

A description of the main S&T results/foregrounds

Work Package 2: Reduction and refinement of *in vivo* vaccine quality tests by *in vitro* methods

Task 1. Refinement of in vivo vaccine potency tests by in vitro alternatives

Partner 14 (MERIAL) formulated inactivated vaccine to be used in experimental studies intended to refine the in vivo vaccine potency model and build a correlation between antibodies and protection against live virus challenge. Vaccine was formulated using as active ingredient a purified, double oil emulsion, inactivated antigen against FMDV strain O1 Manisa, produced by Merial FMD Vaccine Production Unit (Pibright, UK) according to Good Manufacturing Practice (GMP) and following the requirements laid down in the Ph.Eur. Monograph 04/2005:0063 (Eu.Phar. 2006). Five different vaccine batches had been prepared using this antigen batch, for a project called Altandi 3 started before the initiation of FMD-DISCONVAC. Each vaccine batch was divided into three identical bottles and subsequently used in 3 individual, identical Ph.Eur. FMD vaccine potency tests (a.k.a. PD50) for a total of fifteen tests. Three extra vaccine batches were produced to allow for the repetition of 6 of the PD50 studies. Partner 14 received from Partner 1 experimental sera from the 21 potency tests. 714 sera were tested by the virus neutralization assay, coming from 357 animals vaccinated with vaccine at variable payloads. All cattle were negative for FMD antibodies prior to vaccination, or prior to challenge concerning the control animals. All serum samples were titrated according to the reference virus neutralisation test (VNT) protocol described by OIE. Results were shared between the partners. Based on regression and correlation analysis, the relationship between the different serological techniques, vaccine potency and Ag payload were examined. In a similar manner, the correlation between the serological techniques were established between partners (P1 CODA-VAR, P9 ANSES and P14 MERIAL).

Partner 1 (CODA-VAR) analysed the sera from a series of in vivo vaccine potency tests (financed under the Altandi project) for the development of alternative serology based vaccine potency tests. Therefore an array of serological assays was employed by Partners 1, 9 and 14 to determine the anti-foot-and-mouth disease antibody titers (ab titer) in the post vaccinal sera of the individual animals. The correlation between the individual ab titers and the in vivo observed protection status of the animals was investigated and confirmed by logistic regression. This supported the development of serology based statistical models to estimate and predict the quality of a vaccine in a similar manner as the current European pharmacopeia standard protective dose 50% in vivo test. The alternative serology based vaccine potency method proved successful in a thorough external validation performed by partner 1. A proficiency test after the implementation of alternative serology based vaccine potency methods by the different participating laboratories (partners 1, 4 and 5) proved more difficult and demonstrated the need for harmonisation and validation of such methods for each individual laboratory.

Partner 12 (UGLA) worked with Partners 2 (PIR), 5 (FLI) and 8 (FUNPRECIT) to develop a better understanding of current in vivo tests to judge whether they could be replaced with serology. It was determined that because the efficacy of manufactured vaccines is actually quite high relative to the efficacy requirements, the false positive rate tends to be high and the false negative rate low. It was also determined that it is possible to devise live animal challenge tests that are more efficient in their use of animals at determining vaccine quality than the existing tests. Finally, it is also clear that the traditional Spearman-Kärber statistic is

better at determining PD_{50} than the currently recommended logistic regression. Using this information it is now possible to determine the quality of live animal challenge tests, and so set the standard for serological tests to replace them. Then a Bayesian modelling approach was used based on the individual wells in the serial dilution assays to predict protection from the associated serology. This enabled on average to better predict protection using this model than using the challenge tests itself, though work is still required to determine when sufficient challenge experiments have been carried out in training the model. This model was also extended to allow it to be used with the interferon-gamma assay described in the paragraph below, and data are now being gathered to allow this to be compared and combined with ELISA and VNT to improve predictions.

Partner 2 (PIR) further explored the relationships between vaccine induced protection against FMD and the antigen-specific priming of interferon gamma (IFN-γ) secreting T lymphocytes, primarily CD4 cells. This is measured by incubating anti-coagulated peripheral blood samples with FMDV vaccine antigens and then measuring the IFN-γ that is secreted into plasma with an ELISA. Problems of test reproducibility were initially encountered at partner labs to which the technology was transferred. These were overcome by optimisation of the assay, including standardisation of the antigen purity and content and alteration of the buffer in which it is suspended. In total, the IFN-γ test has been evaluated in a further 3 potency tests involving 2 additional partners (Partner 3, Partner 5). This has confirmed the association between the strength of IFN-γ test results for blood samples collected from vaccinated cattle on the day of challenge, to the subsequent protection afforded against FMD. A combination of serology and IFN-γ test results improves the prediction of vaccine efficacy. This work has been published in PLoS One by Oh et al. (2012).

Partner 8 (FUNPRECIT) has set up an IFN-γ ELISA based on a commercial kit, including the specific assay acceptance parameters and protocol standardisation. Analysis of heterologous IFN-γ responses in vaccinated cattle indicated that (i) cross-reactivity is frequent among strains even from different serotypes and (ii) the ability for inducing IFN-γ recall responses in FMD-vaccinated cattle was strain-related and dependant on the viral integrity, both in vivo and in vitro. Consequently, this parameter should be taking into account when performing and interpreting IFN-γ assays.

Partner 10 (IVI) has established several methods to measure opsonizing antibodies in porcine and bovine serum as well of murine monoclonal antibodies, which represent a novel highly sensitive way to assess vaccine-induced immune responses. These were used to evaluate the relationship to FMDV cross-reactivity, neutralization and protective immune responses. For the porcine system plasmacytoid dendritic cells (pDC) were employed. After optimization of cell purification and culture conditions the basic characteristics of this system were determined. Immune serum enhanced IFN-alpha responses of pDC at concentrations below its neutralization titre, and was found to be broadly cross-reactive. It was concluded that this method enables a sensitive identification of opsonizing porcine antibodies. The results were published in Veterinary Research and PLoSOne by Lannes et al. (2013). For cattle sera a primary cell culture system was established based on bovine monocyte-derived dendritic cells. This is based on enhanced infection and cytopathogenic effect of FMDV when complexed with antibodies. The results obtained are comparable to those of the porcine system. In parallel, to enable a high throughput measurement of opsonizing antibodies, a murine RAW264.7 macrophages cell line expressing bovine FcγRII (CD32) was established to measure the opsonizing activity of bovine sera obtained after vaccination. The RAW264.7 were resistant to most FMDV isolates unless the virus was opsonized. Opsonizing activity was observed against homologous serotype strains with a broader level than neutralizing activity. Cross-reactivity was also seen against some heterologous serotype strains. A panel of

cattle sera from vaccination/challenge experiments was tested to determine whether these novel tests have the ability to measure correlates of protection. In fact, in a heterologous vaccination/challenge setting a better correlation to the vaccine dose was observed with opsonising antibody levels compared to neutralizing titres. The number of tested sera is still far from being sufficient to conclude on the relationship to protection. This work will thus be continued beyond the end of this project. Using RAW264.7 cells we also tested a large panel of murine FMDV-specific monoclonal antibodies obtained from Partner 4 (IZSLER). This work permitted us to define the relationship between neutralization, opsonisation and epitope conservation. It was concluded that for opsonisation, much lower antibody avidities are required as compared to opsonisation. There are two manuscripts in preparation describing the work with bovine sera and murine monoclonal antibodies, respectively.

Task 2. Replacement of in vivo vaccine purity tests by in vitro alternatives

Partner 4 (IZSLER) investigated the feasibility of a simple blocking ELISA to detect and measure NSPs in FMDV antigen preparations. The test developed uses selected anti-3D and anti-3A MAbs for the measurement of 3D and 3ABC with its cleavage products 3A and 3AB and results suggest that a simple ELISA for detection of NSPs in native FMDV antigen preparations is feasible. Calibration of the assays with standard known concentration of NSPs will enable a quantitative determination of the analytes.

Partner 8 (FUNPRECIT) developed a monoclonal antibody (MAb)-based Western Blot assay to detect the presence of non-structural protein (NSP) 3ABC, and other proteins deriving from this precursor, in commercial vaccines and viral suspensions obtained during the vaccine production process. Lower detection limit was 4.5 ng of a recombinant 3ABC protein. This assay may provide qualitative information on the composition of contaminants NSP to complement quantitative assessment of NSP contents made by commercially available tests (PrioCHECK® FMD IPC 3ABC; Capozzo et al., Vaccine 2010 28: 6647–6652). The in-house test is not commercially available but can be made available after agreement with the developing laboratory.

Task 3. Developing alternative methods for 146S (140S) quantification in viral products and vaccines

In general, epitopes present in the viral capsid structure are also conserved in the 12S sub-particles, with very few exceptions. For this reason most monoclonal antibodies (MAbs) recognise and react with both capsids and sub-particles. However, the concept that some MAbs recognising both 12S and 146S can actually generate immunoassays that detect only viral capsids, when used in catching and detector antibody combinations in sandwich ELISA, has been proved. These ELISAs are designed with one catcher MAb coated onto plates and a second detector MAb conjugated to peroxidase.

Partner 4 identified a set of simple MAbs-based sandwich ELISAs that can discriminate full/empty capsids (146S/75S) from 12S sub-particles, after evaluation of several dozens of MAbs, representative for different epitopes in any possible combination as catching and detector antibody. This test system does not require preliminary chemical-physical separation procedures or sophisticated equipment. It can be applied to any type of aqueous solution and to crude, non-purified materials, thanks to the function exerted by the capture MAb to both immune-purify antigens from crude preparations and present it for the binding by the second detector MAb. Thanks to linearity of the dose-response curves, quantification of the samples

may be achieved by testing serial dilutions and by reference to standard materials with known antigen concentration. Immunoassays detecting specifically (or better) capsid particles were identified for: (i) FMDV serotype O (European vaccine strains OBFS, Lausanne, Brescia 1964, etc., while the strain O Manisa requires further verification using sucrose-purified preparations), (ii) FMDV serotype A (European vaccine strains A5, vaccine strain A22 Iraq and A24 Cruzeiro), (iii) FMDV serotype C (Italy 1965 vaccine strain), (iv) FMDV serotype Asia 1 (vaccine strain Asia 1 Shamir) and (v) FMDV serotype SAT1 (strain Bot1/61). These tests are not commercially available but can be made available after agreement with the developing laboratory.

Partner 8 (FUNPRECIT) has fully automated a strategy for quantification 140S whole virus particles by sucrose gradient purification and spectrophotometry. The fully automated Density Gradient Fractionation System was complemented with a "fit for purpose" quantification software and resulted more accurate than the manual methods, especially when working with low virus concentrations. A protocol for purification of FMDV 140S and 75S particles by liquid chromatography (LC) was developed and tested, combined with spectrophotometry and a MAb-based ELISA specific for each vaccinal strain, for analysing FMDV capsids' integrity and identity in a tetravalent FMD vaccine. Results indicate that quality of FMDV vaccines and other antigenic preparations could be studied by a combination of LC with spectrophotometry and ELISA. This LC protocol may be scaled up as well, thus allowing large scale purification of whole virus particles for vaccine formulation.

Task 4. Validation of the use of serology to estimate the protection level in vaccinated populations

A selection of 21 days post vaccination sera from different vaccine experiments financed under the Altandi projects were used for the first phase of an inter laboratory study to estimate the protection level in a population of vaccinated animals. Therefore partners 1, 4 and 5 analysed sera with their routine diagnostic assays and the obtained antibody titers were evaluated by statistical models developed by partner 1 which resulted in an estimate for the protection level of the population (EPL). The differences observed in the EPL and the corresponding certitude of the EPL may reflect an actual difference between the different vaccines that were used in this study since the trends in the EPL and certitude follow the trend in the ab titer generated by the vaccines. However, these animals are vaccinated under experimental conditions, thus the EPL may not be a representative estimate of animals vaccinated under field conditions. The fact that field samples may originate from vaccinated and exposed and/or infected animals may still compromised this approach. Nevertheless, the second phase of the study will focus on results from field serosurveys.

Partner 3 (CVI) intended to answer the question if the relation between antibody response and protection can be standardised. The work started with a collaborative study in which three laboratories examined their own sera from O Manisa potency test and standardised the result by correlating the observed titre to the titre of a standard serum supplied by Partner 3, or by using a commercial ELISA, or by using both techniques. Inclusion of a standard serum reduced, however not completely, the variability between laboratories. For the ELISA the same slope was observed when analysing ELISA titers, whereas analysis of percentage inhibition often resulted in different curve slopes. Also for the ELISA the inclusion of a standard serum reduced the differences between laboratories (similar to the results observed by partner 8 Argentina), but still differences between laboratories are seen. The slope of the relation between antibody titre and protection observed in the analysis of the sera of European potency tests is equal to the slope observed in the sera from Argentina PGP tests (partner 8).

So although the location of the relation between antibody response and protection is different, the slope is the same which indicates that for the different strains tested a similar mechanism is present for this correlate of protection.

Since vaccines from different producers tested in the different countries did not produce the same results, explanatory variables were sought that could explain these differences using sera from one producer that tested various strains with different compositions. The analysis showed that also antigen dose and dose of the vaccine applied influenced the position of the curve describing the relation between antibody response and protection, although no significant relation was found between antigen dose and antibody titre. However, there was a strong relation between vaccine dose and antibody titre, showing again that the adjuvant is very important for the induction of the immune response. Improvement of vaccines should therefore focus on improvement of adjuvants.

The result of the analysis of the ELISA titres in relation to the protection has been used in evaluation of several vaccines in Pakistan, Iran and Egypt. Comparison can be made between antibody responses from different vaccines, locally produced and imported, based on the fact that the relation between ELISA antibody titre has the same slope for all strains tested, and is similar to the slope observed in Argentina (P8).

Partner 12 (UGLA) found that the model described under Task 1 was developed to predict individual and population-level protection. Predictions of protection are very good, but there is a significant problem for unprotected animals. VNT and ELISA even together cannot successfully discriminate between protected and unprotected animals, as a result of some protected animals having low VNT and ELISA titres. Further work will be needed to determine whether the IFN-γ assay will augment these tests sufficiently to allow them to discriminate better or whether new assays are required.

It was the aim to establish guidelines to evaluate the effectiveness of systematic mass vaccination campaigns against FMD by Partners 1, 2, 3 and 8. This evaluation should be based on the estimation of the proportion of protected animals and protected farms in vaccinated populations, as a function of the serological levels of antibodies against FMDV as measured by a serological test on the condition that a correlation between the serological titre and the animal protection is determined. Partners 1, 2, 3 and 8 decided that the impact of guidelines developed by this consortium might be too little to achieve the goal foreseen. Therefore the partners involved decided to present the idea of developing PVM guidelines at the meeting of the OIE and FAO FMD Reference Laboratories in India 2009 and to join forces. Since then the P1,2,3,8 participated in several meetings with OIE and FAO and the last meeting so far took place at FAO Headquarters, Rome, Italy, June 2013. The PVM guide will contain elements explained in the deliverable and will be published in 2015. This guide will be send to all partners of FMD-Disconvac and to the EC.

Work Package 3: Assessment and improvement of heterologous protection by FMD vaccines

Task 1. Assessment of heterologous protection by highly potent, monovalent FMD vaccines

Partners 2 (PIR) and 6 (IIL) concluded a large study to evaluate cross-protection within FMD serotype O. Cattle were vaccinated with O Manisa and challenged with either the homologous virus or with the heterologous O Campos virus. Protection was compared with VNT-based serological predictions. Animals were vaccinated with different payloads varying from 60-0.94 µg of O1 Manisa antigen. The vaccinated animals and unvaccinated controls were

challenged by intra-dermolingual inoculation with either O1 Manisa (Group 1, 3 & 5) or O1 Campos (Group 2, 4 & 6). All controls developed generalized FMD. All vaccinated cattle in groups 1 and 3 challenged with O1 Manisa virus were protected (100%) while only one out of five (20%) in group 5 were protected against generalized FMD. In the groups vaccinated with O1 Manisa and challenged with O1 Campos zero (0%), three (60%) and five (100%) animals in groups 2, 4 and 6 respectively were protected. Despite relatively good cross-neutralization of O1 Campos by O1 Manisa antisera (r1 = 0.6), O1 Manisa vaccinated cattle were only partially protected against challenge with O1 Campos. The PD50 values calculated for the vaccine were 26.61 for homologous and 5.01 for heterologous challenge, respectively. Higher antibody titers were needed to protect against O Campos than against O Manisa. This implies that either some other immune mechanisms besides neutralizing antibodies are involved in protection or that O Campos is more virulent than O Manisa. Heterologous protection between serotype O strains were studied further by vaccinating cattle with the Indian vaccine strain O IND R2/75, followed by a heterologous challenge with O APKr 94/05. Surprisingly, while VNT results predicted poor protection against heterologous challenge (r1 = 0.28), the challenge experiment showed better heterologous than homologous protection.

Partner 14 formulated inactivated vaccine to be used in experimental studies intended to study cross-protection by challenging vaccinated animals with heterologous FMDV strains. A double oil emulsion (DOE) vaccine with 9 µg/dose, representing current vaccine bank vaccines for emergency use, was therefore formulated. Partner 5 (FLI) carried out cattle challenge experiments according to the European Pharmacopoeia (5 cattle full dose, five cattle ¼ dose, five cattle 1/16 dose, two unvaccinated control animals, challenge on 21 days post vaccination [dpv]) in order to assess cross-protection against recent serotype A isolates from the Middle East belonging to the A Iran 05 topotype. The purpose of these experiments was the assessment of protection against these viruses provided by vaccines of current European vaccine bank (AIran05 and A22). The challenge viruses used and several (alternative) candidate challenge viruses were passaged in cattle and checked for the generation of typical podal lesions. 21 days post challenge (dpc) sera were collected to establish a correlation between protection and VNT titres. Animals vaccinated with an A Iran 05 vaccine were challenged with A/TUR/1/06, A IRN/29/09, A ISR/11/09 and A TUR 64/11. The VP1 sequence homology (determined by the WRL for FMD) with early isolates of the A Iran 05 group was 99.22 to 99.84% for A/TUR/1/06 (quasi-homologous), 95.77 % for A IRN/29/09, 95.77 % for A ISR/11/09 and 92.64 for A TUR 64/11. The r-values in respect to A Iran 05 vaccines were taken as 1.0 for A/TUR/1/06 and determined as 0.42 for A IRN/29/09, 0.22 for A ISR/11/09 and 0.1 for A TUR 64/11. The protection results were 18.2 PD50 for A/TUR/1/06, 8 PD50 for A IRN/29/09, 6 PD50 for A ISR/11/09 and zero PD50 for A TUR 64/11. The lack of protection against A TUR 64/11 (despite good vaccine specific antibody titers) fully agrees with the total lack of challenge-strain specific antibodies in 21 dpv sera. The results confirm that A Iran 05 vaccines have been a valuable component of European vaccine banks. Data from the WRL for FMD indicate that they still cover many field isolates, but the failure against A TUR 64/11 emphasizes the need for a new vaccine strain for serotype A isolates from the Middle East. As many vaccine banks, including the European vaccine bank, contain A22 IRQ 24/64 antigen which, if administered as a high potency vaccine, gives a broad coverage, it was also tested if such a vaccine would protect against A Iran 05 strains. Despite the low sequence homology of A IRN/29/09 with A22 IRQ 24/64 being 82.16% and the r-value 0.27, the PD50 value of the A22 IRQ 24/64 vaccine in respect to A IRN/29/09 was determined as 8. However, the challenge trial with A ISR/11/09 showed that the heterologous protection induced by an A22 vaccine against challenge strains of the A Iran05 topotype is limited. In this experiment, when the identity again was just 82.16% and the r-value was 0.09, the A22 vaccine could not induce a satisfactory protection.

In the framework of the FMD-DISCONVAC project and the FP6 FMD_ImproCon project (503603) sixteen homologous and heterologous FMDV type A challenge experiments suitable for further analysis have been performed at the FLI. The sera from these experiments are kept for further analysis and a database of these sera was established on the restricted part of the project website as an excel table. The data from the challenge experiments will be sent to partner 12 (UGLA) for further analysis beyond the scope of this project. Generally, challenge virus specific VNT titers correlated well with protection, but the failure of A22 vaccine to protect against ISR/11/09 was unexpected.

It can be concluded that some recent isolates from the Middle East are not covered by even high potency type A vaccines which conferred broad cross-protection for years or even decades.

Moreover, two high-throughput ELISA techniques, avidity and IgG subtype ELISA, were set up by Partner 8 (FUNPRECIT) and used to further characterize heterologous antibody responses in cattle. Both assays were applied to a set of 64 previously characterized sera from animals immunized with an inactivated A24/Cruzeiro strain monovalent FMDV vaccine and challenged with the heterologous A/Arg/01 strain in four independent challenge trials. Single dilution avidity ELISA assessment showed that animals that were protected against A/Arg/01 challenge had higher avidity antibodies to this heterologous strain than non-protected cattle. This was also observed even when serum neutralizing titres were medium to low. Also, FMDV-IgG1 serum titres in protected animals were significantly higher than those achieved by unprotected animals, while IgG2 titres were lower. The difference in the subtype induced was particularly relevant for those animals with low levels of A/Arg/01 specific antibodies. In fact, bovines with that surpassed the heterologous challenge and had low or undetectable anti-A/Arg/01 serum-neutralizing titres presented higher IgG1/IgG2 ratio than non-protected animals. This study constitutes the proof-of-principle of integrating avidity determination and subtyping of specific antibodies to the indirect assessment of FMDV heterologous protection in cattle.

Task 2. Assessment of heterologous protection by multivalent FMD vaccines against strains of the same serotype

Partner 8 (FUNPRECIT) worked on bivalent and monovalent type A vaccines for South-America and carried out experiments on the correlation between in vitro measures of immunity and cross-protection within a serotype. Antigenic composition of the vaccines was 10 µg of A 24 Cruzeiro per dose (2 ml) for vaccine 1, 10 µg of A2001 Arg per dose (2 ml) for vaccine 2, 5 µg of A 24 Cruzeiro + 5 µg of A2001 Arg per dose (2 ml) for vaccine 3 and 10 µg of A 24 Cruzeiro + 10 µg of A2001 Arg per dose (2 ml) for vaccine 4. Ten animals (Hereford steers) were immunized with each vaccine. Serum and whole blood samples were taken at 0 and 30 dpv. The reactivity of these sera was studied by liquid phase ELISA (lpELISA) and virus neutralization test (VNT) against both homologous and heterologous strains. The r1-values determined by VNT for bivalent formulations exceed those of their monovalent counterparts although they consistently remained below the minimum value established for antigenic relatedness for this assay (0.3). The mean r1-value (0.32) for heterologous strain A/Arg 2000, determined with A/Arg 2001 considered as homologous virus and sera from the 10+10 bivalent vaccine group, was only marginally above the lower limit indicating relatedness. However, this group presented unexpected low Ab titres against the homologous strains, a fact that may lead to errors in the r1 value estimation. The r value estimates obtained by lp-ELISA coincided with those of the VNT as again r1-values for bivalent formulations exceeded those recorded for their monovalent counterparts. However,

they consistently remained below the minimum value of 0.4 which had been established for antigenic relatedness for this assay. Also the heterotypic induction of FMDV-specific cellular responses was studied. Whole blood samples taken at 0 and 30 dpv were used to analyze the capacity of these animals to induce IFN-γ after FMDV homologous or heterologous in vitro stimulation. A set of different strains of inactivated and purified FDMV particles were produced from virus concentrates or infected culture. Whole blood cultures were stimulated with both homologous (A24 and A2001) and 3 heterologous viruses (A79, O1 Campos and C3 Indaial). The production of IFN-γ was measured by a commercial ELISA. Interestingly, animals vaccinated with the A24 formulation (vaccine 1) were significantly more reactive than those immunized with the A2001 vaccine (vaccine 2), to homologous and heterologous stimulating antigens. Animals vaccinated with bivalent vaccines (vaccine 3 and vaccine 4), elicited IFN-γ with values that were between those of vaccine 1 and vaccine 2, following a dose dependent manner. A79 and A24 antigens were the most effective stimulants for blood cells obtained from animals vaccinated with any formulation. Samples obtained from animals vaccinated with the A2001 (vaccine 2) or bivalent vaccines (vaccine 3 and vaccine 4) induced very low IFN-γ levels when stimulated with the A2001 antigen. These levels were comparable to those obtained after stimulation with PBS or with strains from other serotypes (O1 Campos and C3 Indaial). Only whole blood samples from animals vaccinated with the A24 formulation (vaccine 1) were able to produce IFN-γ after stimulation with A2001 antigen. Incubation with O1 Campos only produced IFN-γ levels similar to those of non-stimulated samples. The results indicate that heterologous stimulation among serotypes for cell mediated immunity, measured by IFN-γ production, is less restricted than for humoral responses.

A24 Cruzeiro strain was more efficient than other serotypes in inducing IFN-γ responses when used as vaccine antigen (priming responses) or as stimulating antigen (anamnestic responses), an observation that may be related to higher stability of the FMDV capsids for this strain compared to others. To further investigate this finding, the capsid (140S) stability between A24 and O1 Campos was compared. Purified inactivated FMDV preparations were heated to 37ºC for 24 hours and the percentage of whole 140S particles was measured by ELISA. After one day at 37ºC, over 60% of the initial A24 Cruzeiro 140S particles were recovered, while only 20% of the O1 Campos 140S particles remained intact. Both of the purified virus preparations could no longer stimulate IFN-γ production by PBMC from FMD vaccinated cattle when 140S particles were disrupted to 12S particles. The results indicate that viral integrity is important for inducing IFN-γ responses in vitro and that stable strains may be better IFN-γ inducers.

Furthermore, the avidity and subtype of specific antibodies were determined and the results were used to indirectly assess heterologous protection against FMDV in cattle. It had been shown previously that vaccinated animals with low levels of specific IgG that surpassed the Protection to Podal Generalization test (PPG) had higher IgG1 than IgG2 titres. The avidity of antibodies was studied as it usually is associated with their opsonising capacity. Two high-throughput assays, avidity and IgG subtype ELISA, were developed to complement existing serological tests utilized to characterize heterologous antibody responses in cattle and to indirectly assess heterologous protection against FMDV. Single dilution avidity ELISA assessment showed that animals vaccinated with an A24/Cruzeiro monovalent formulation and protected against challenge with FMDV A/Arg/01 strain, had higher avidity antibodies to this heterologous strain than non-protected cattle. FMDV-IgG1 serum titres of protected animals were significantly higher ($p<0.0001$) than those achieved by unprotected animals, while IgG2 titres were lower. Bovines that were protected against heterologous challenge despite low or undetectable anti-A/Arg/01 serum-neutralizing titers had higher IgG1/IgG2 ratios than non-protected animals.

Partners 2 (PIR) and 6 (IIL) carried out animal trials with vaccine strains A IND 40/00 (Indian Vaccine strain) and A APS 66/05 (alternative vaccine strain) and field strain A RAJ 21/96 (heterologous challenge) strain. Monovalent and bivalent vaccines incorporating the two vaccine strains were prepared with different payloads and naïve cattle were vaccinated with different blends (monovalent and bivalent) of these vaccines while unvaccinated naïve cattle were used as controls. Animals were infected by intradermolingual challenge. Cattle vaccinated with A IND 40/00 (8 µg/dose) were protected against challenge with A APS 66/05 (100%) and A RAJ 21/96 (83%). Cattle vaccinated with A APS 66/05 (6 µg/ dose) were fully protected against A IND 40/00 and A RAJ 21/96 challenge. Bivalent vaccines (payloads 3 and 6 µg/dose of each antigen) offered protection against both homologous and heterologous challenges. All unvaccinated control cattle developed generalized FMD. The good heterologous protection results are in contrast to the poor protection predicted on the basis of VNT titres (r1 = 0.18 for A IND and 0.03 A APS 66/05). Further serological studies by VNT were carried with A IND 7/82 and A IND 17/82 antisera. The titres predicted protection by these vaccine strains against heterologous challenge by the field isolate A RAJ 21/96 (r_1 = 0.50 & 0.47, respectively). A pool of the two antisera against A IND 7/82 and A IND 17/82 gave even better neutralisation of A RAJ 21/96 than either on its own (r_1 = >1.00). In order to confirm the result, a bivalent vaccine (A IND 7/82 + A IND 17/82) was used to prepare an antiserum which was tested against the isolate A RAJ 21/96 in the VNT. New antisera to each monovalent vaccine (A IND 7/82 and A IND 17/82) were also prepared and tested. The antiserum to bivalent A IND 7/82 + A IND 17/82 again cross reacted with A RAJ 21/96 more strongly (r_1 = >1.00) than either of the individual antisera (r_1 = 0.14 & 0.59 respectively). This provides evidence that a combination of two type A vaccines could give better protection than can be provided by one alone. The vaccine matching work shows that two type A vaccines that would individually invoke moderately cross-reactive antisera to a field virus of the same serotype can be combined to improve the breadth of antigenic cover. This can be achieved without an overall increase in vaccine dose and suggest that combining different vaccine strains of the same serotype may improve the level of protection afforded by vaccination.

Task 3. Improvement of r-value determination

In a specific study funded by Partner 14, 5 cattle were vaccinated twice at a 21 days interval with A Iran 05 vaccine. About 500 ml of blood were taken from each animal 14 days after booster vaccination, to be able to supply consortium partners with sera for further research on r-value determination and standardization exercises.

Partner 1 (CODA-VAR) performed serological assays for r-value determination on sera derived from an *in vivo* study conducted previously (Altandi 2 Project 2008). This *in vivo* vaccine cross-protection potency study had been conducted with the same FMDV A Iran 96 (FMDV A96) vaccine batch as in the work package 2 study. Cattle were challenged with FMDV A_{22} Iraq 24/64 (FMDV A22) at 21 dpv and bled at 8 dpc. The potency test results for this vaccine ranged between 0.5 and 3.5 PD_{50}. The overall PD_{50} calculated over all the animals of all trials was 1.0 PD_{50} with bootstrap 95% confidence intervals between 0.6 and 2.7 PD_{50}. It was concluded that the highly potent A96 vaccine (20.5 PD_{50}) did not provide enough cross-protection against infection with FMDV A22. The 21 dpv serum titres in VNT and LPBE were used to calculate r_1 values. It was concluded that (i) the r_1 values calculated on pooled samples had less variance, and (ii) larger group size has positive effects by lowering variance of the r_1 values. Furthermore the r_1 values calculated for pooled samples

were in general lower than r_1 values for individuals, rendering these mean r_1 values more reproducible and more consistent with the *in vivo* observations.

Partner 1 (CODA-CERVA) and 8 (FUNPRECIT) organized a liquid phase blocking ELISA (lp-ELISA) "r" value ring trial with additional participating partners 2 (PIR), 5 (FLI) in order to improve the comparability of r1 estimates for serotype A. The 30 dpv sera used were derived from a previous study on the cross-protective potency of a monovalent FMDV A24 Cruzeiro vaccine. The four FMDVs strains provided by partner 8 (FUNPRECIT) were A24 Cruzeiro (homologous), A/Argentina/01, A-81/Argentina/87 and A/ Argentina/00. For all 3 heterologous strains there were in vivo data available that indicated that there was no cross-protection generated by the A24 vaccine. It turned out that in-house ELISAs of partners not familiar with these South American strains had to be adapted in order to obtain sufficient OD values and that afterwards they tended to significantly overestimate cross-protection. Therefore, a follow-up ring test was organised by partners 1, 2, 5 and 8 to generate an insight into cross-reactivity and possible cross-protection as it is estimated by r_1 values based on Liquid Phase Blocking ELISA antibody titers. In the first stage of the r_1 value ring test, the LPBE produced coherent results between the different partners indicating that the strains were not related. This was confirmed by the Virus Neutralisation Test performed later by the partners. In the second stage of the ring test the LPBE formats were adapted in an effort to determine the distance between the unrelated strains and the vaccine strain which resulted in highly variable r_1 values ranging from correct to wrong predictions. Profound understanding of this variability may lead to the development of ELISA assays formats capable of determining the actual distance between vaccine strains and less or unrelated field strains in a faster and safer way than the VNT. The final conclusion of this ring test is that a knowledge gap exits at the crucial point where well known vaccine FMDV strains meet emerging field strains of FMDV. A thorough knowledge about the assays and the vaccine strains used in the r_1 value estimation remains paramount for laboratories performing these r_1 value tests.

Partners 1, 5 and 8 organized and hosted a workshop "FMD Cross-protection, Vaccine-Matching and Vaccine Banks: Challenges and Opportunities" at INTA (Partner 8) in June 2011, Buenos Aires, Argentina. Due to problems caused by a volcanic eruption at this time, the workshop had to be finished in October 2011 in Tervuren, Belgium. Important topics were the status and strategies of vaccine and antigen banks around the world, criteria for the selection of strains, completed and on-going cross-protection studies, a database for reference reagents, the serotype A LPBE-based r-value ring trial mentioned above and the role of different parts of the immune system (cellular vs humoral) for cross-protection.

Partner 5 (FLI) found that the VNT protocol for r-value determination taken over from the WRL needs considerable adaptation and fine-tuning of the cell culture system used at the FLI to produce meaningful results. New protocols for washing, staining and reading the test were established and r-values for a number of recent type A isolates were generated. A collection of 21 dpv sera from homologous and heterologous challenge trials was made available to consortium partners for further research and standardization efforts exercises.

Partner 2 (PIR) has conducted an inter-laboratory comparative exercise to look at the equivalence of vaccine matching results obtained in different institutes. The work has been conducted on serotype A, in conjunction with other leading FMD laboratories who are members of the OIE/FAO Network of International FMD Reference Laboratories. The comparisons included tests based on *in vitro* virus neutralisation (VNT) and on antibody binding (lp-ELISA). It was concluded that, using common reagents and a standardised approach, it is possible to get good qualitative agreement between laboratories. Partner 2 (PIR) also worked on sequence based antigenic characterization of serotype A FMD viruses

from the Middle East, in particular of the A-Iran-05 group.. Analysis of the capsid sequence (amino acid) data revealed high sequence variation among these viruses, VP4 being least variable (5%) followed by VP3 (23%), VP2 (26%) and VP1 (46%). However, the critical residues for each antigenic site were found to be fairly conserved. While vaccine strain A Tur 06 may, in general, provide protection, the A BAR 08 and A AFG 07 sub-lineage may exhibit antigenic drift, necessitating a close monitoring of such outbreak strains. While it appears that phylogenetic trees do not provide a true indication of the antigenic phenotype of the isolates, it was concluded that some amino acid residues in the outer capsid (VP1- 40, 45, 65, 83, 96, 99, 110, 41, 171, 204; VP2- 64, 65, 134, 191, 195; VP3- 8, 59, 65, 70, 132, 220) provide an indication about the antigenic nature of the type A viruses.

Partner 12 (UGLA) and partner 2 (PIR) worked on the development of better ways of controlling for variability in r1-values determination and predicting changes in r1-values from sequence changes. It was found that there are more effects than just a variability consistent for the day of the experiment (which is already controlled for in established r1-value determination protocols by dividing by the homologous titre taken on the same day). It was shown that, after accounting for inter-experiment variability, the challenge virus and vaccine strain had significant effects. Where individual animal sera were used, also they had a significant effect. This allowed developing a refinement to the current r1-value calculation. With an extension of this model, capsid amino acids critical to intra-serotypic protection, were identified. Five mutations were predicted to affect antigenicity for serotype O. In co-operation with partner 2, site-directed mutagenesis at the three residues that were not parts of known antigenic sites were carried out and showed that they are indeed of antigenic importance. This now facilitates to predict antigenic changes from the sequence. Using the five residues mentioned above, a preliminary predictive model of cross-reactivity was established, which predicts r1-values better than previous in silico approaches.

This work has now been published: Borley DW1, Mahapatra M, Paton DJ, Esnouf RM, Stuart DI, Fry EE. (2013). Evaluation and use of in-silico structure-based epitope prediction with foot-and-mouth disease virus. PLoS One. 2013 May 7;8(5):e61122. doi: 10.1371/journal.pone.0061122.

A PhD was awarded to Dr Borley for this work at Division of Structural Biology, University of Oxford, The Henry Wellcome Building for Genomic Medicine, Headington, Oxford, United Kingdom.

Work Package 4: Development of vaccines and alternatives (antivirals) with rapid onset of immunity and based on safer production methods

Task 1. Production and evaluation of canine adenovirus vectored FMDV vaccines

Partner 9 studied the production of canine adenovirus vectored FMDV vaccines. Both bovine and porcine cell cultures supported transient canine adenovirus (Cav2) replication with viral genome persistence in PK-15 cells. In bovine cells, Cav2 replicates its genome without virion production. Since pre-existing adenovirus immunity might impair *in vivo* Cav2 mediated gene transfer, sera from random French cohorts of pigs and cattle were tested for Cav2 cross-neutralizing antibodies. Results showed that most pigs and cattle have no pre-existing antibodies against Cav2. Cav-G, a construct expressing the rabies glycoprotein, elicited a protective level of rabies virus neutralizing antibodies in pigs after a single immunization with Cav-G recombinant vaccine.

Two non-replicative canine adenoviruses (Cav2) have been made, expressing the FMDV capsid polyprotein (P1) and the 3C protease for its cleavage (Cav-P1/3C), or the VP1 capsid subunit protein (Cav-VP1), using coding sequences of FMDV strain O/FRA/1/2001. Both vector constructs expressed FMDV antigens *in vitro* and Cav2-P1/3C can be considered a potential marker vaccine against FMD, as intramuscular inoculation in mice and guinea pigs induced a humoral response against FMDV capsid. Cav2-VP1 did not induce a detectable humoral immune response in either species.

In a second experiment 8 guinea pigs were immunized twice with Cav2-P1/3C and were challenged with the FMDV O1 Manisa strain 21 dp the second immunization. The results showed that only one guinea pig seroconverted against FMDV and was partially protected after immunization with Cav2-P1/3C, whereas, a humoral response had been detected in 3/3 animals in the previous experiment. The origin of this difference is unclear. Therefore it was decided to delay the experiments in pigs until this problem is solved. This research will be continued with own funds in collaboration between P9 and 1.

Task 2. Production and evaluation of encephalomyocarditis virus vectored FMDV vaccines

Partner 9 studied the production of encephalomyocarditis vectored FMDV vaccines. A deletion in the 2A gene from a variant of EMCV, avirulent for mice, was introduced into an infectious cDNA clone (EMCV1.26) and a viable recombinant virus (EMCV1.26Δ2A) was produced and stably replicated after transfection of BHK-21 cells. EMCV1.26Δ2A had an altered phenotype in cell culture suggesting that the 2A protein is required for inhibition of apoptosis during EMCV infection. Mice infected with EMCV1.26Δ2A did not exhibit clinical signs, or central nervous system pathology, unlike EMCV1.26 infected mice. Moreover, the EMCV1.26Δ2A virus protected mice from challenge with a lethal dose of wild type virus (Carocci et al., 2012).

The FMDV P1 genes were incorporated into EMCV1.26Δ2A and FMDV antigen was detected in transfected cells but without live virus recovery. To see if the production of EMCVΔ2A-P1FMDV recombinant virus is limited by processing of the FMDV P1 by the 3C of EMCV, vectors expressing the P1 and 3C of FMDV and EMCV were generated and used to transfect BHK-21 cells and study the P1 cleavage. Results suggest that FMDV P1 is not cleaved by EMCV 3C and that cleavage site mutagenesis could be used to correct this defect and allow creation on FMDV P1 of sites recognised by the 3C of EMCV. If successful, the mutations will be introduced into the full recombinant cDNA to try to rescue the EMCVΔ2A-P1FMDV recombinant virus.

Task 3. Development of methods to reinforce and measure mucosal immunity against FMDV in cattle

A viral vector system has been evaluated for delivery of FMDV capsids at mucosal surfaces to elicit a local and protective immune response in order to block FMDV infection by the respiratory route. A replication defective human adenovirus was modified by insertion of serotype A FMDV genes to express FMDV capsid precursor proteins (P1-2A). Complementary insertion of the FMDV 3C protease gene led to cleavage of the capsid precursors and formation of empty viral capsids, confirmed by electron microscopy (EM).

Partner 2 showed that intramuscular (i.m.) or intranasal (i.n.) delivery of recombinant vector vaccine in mice induced systemic FMDV-specific humoral and cell mediated immunity. The

i.n. route gave rise to similar systemic responses, plus a FMDV-specific IgA response in the lungs. The recombinant replication defective human adenovirus-FMDV was used to immunize and boost cattle by i.m. or i.n. routes with subsequent FMDV aerosol challenge. Only the i.m. vaccinated group of calves was fully protected clinically and furthermore, did not develop antibodies against FMDV non-structural proteins, suggesting little or no virus replication. The same adenovirus vector but expressing interferon alpha (AdV-IFNα) instead of FMDV genes was tested for early protection against an aerosol FMDV challenge. Calves vaccinated i.n. with AdV-IFNα at a high dose on the day prior to challenge were not protected from clinical disease, although the lesions and viremia were delayed by 2-4 days compared to unvaccinated control calves. Publication planned: Adenovirus vector based FMDV empty capsid vaccine for increasing immune responses. Authors: Babu A., Zhang F., Gilbert S., Paton D., Taylor G., Parida S.

To evaluate systems to deliver mucosal vaccines, partner 7 gave conventional FMD vaccine antigens to calves with different adjuvants by the sublingual and i.n. spray routes. The best responses were obtained when high doses of antigen were delivered sub-lingually, using a needle-less injector. This gave rise a local IgA response in nasal fluid and a systemic neutralising antibody response in serum.

Nanoparticles formulated by chitosan-PLGA (poly(lactide-co-glycolide) loaded with DNA vaccines encoding FMDV capsids and interleukin 6 and by chitosan-trehalose carrying FMDV serotype A inactivated antigen were used by partner 7 as mucoadhesive i.n. delivery vehicles. A micro-ball mill produced dry powder particles of the correct size and stability for nasal delivery. The DNA vaccines were tested initially in guinea pigs and rats (Wang et al., 2011) and later both DNA vaccines and inactivated FMDV antigens were tested in cattle, with up to three immunizations prior to challenge by contact with FMDV infected donor cattle. The i.n. delivered nanoparticle vaccine prototypes did not provide complete clinical or virological protection, but reduced disease severity and virus excretion and delayed disease onset. Induction of secretory IgA, systemic humoral and cell-mediated immune responses for the vaccinated animals were studied in detail.

Task 4. Asssessment of recombinant baculovirus as adjuvants for FMDV vaccines

To develop emergency vaccines with a reduced time between vaccination and induction of protective immunity, partner 8 studied recombinant baculovirus (BV) as an adjuvant for FMDV vaccines and as an antiviral agent for FMDV infection. Experiments were mainly performed in a mouse model previously developed in the laboratory and a small-scale experiment was also conducted in cattle.

BALB/c mice were immunised with inactivated FMDV antigen (iFMDV), with or without BV and challenged with live FMDV at 2, 4, 7 or 14 days post-vaccination (dpv). This showed that co-inoculation of iFMDV and BV increased the innate and humoral immune responses as well as protection against viral challenge at 2, 4 and 7 dpv, but not at 14 dpv. Combining BV with single-emulsion oil iFMDV vaccines increased protection against viral challenge to 14 dpv (Quattrocchi et al. Vaccine, 2013). This indicates that the BV is able to induce a very rapid antiviral state that does not interfere with the induction of FMDV-specific antibodies by conventional FMD oil vaccines.

With this background, partner 8 conducted a pilot-scale experiment involving three cattle as a proof of concept on the capability of the BV AcNPVbovCpG (developed for the DISCONVAC project) to induce early protection to FMDV infection. Two animals received $5x10^9$ pfu of the AcNPVbovCpG intravenously, at 6 or 24 hours before FMDV challenge; the

third animal received only insect cell medium 24 hours before challenge. All the challenged cattle developed FMD without differences between experimental and control animals regarding time course or severity of the symptoms. Production of IFN-γ was not detected by ELISA or ELISpot assay in any of the animals, whilst quantification of type I IFN's and pro-inflammatory cytokine mRNAs is in process. A more extended study, including additional experimental groups with different immunization regimes, may be necessary to assess the actual capacity of BV to induce an effective anti-viral state in cattle. The study highlights the difficulties in transferring successful approaches tested in mice to the natural hosts.

Task 5. Development and optimisation of potent and selective inhibitors of FMDV replication

During the FMD-DISCONVAC project, partner 1 further optimized the *in vitro* antiviral activity of the CHI-67 and CHI-68 compounds by exploring their structure-activity relationships. More than 100 new analogue molecules were designed and synthesized in different rounds. A new lead compound was obtained with an EC50 value in the low μM range. The CHI-compounds were active against the different Eurasian serotypes of FMDV and inhibit the early intracellular phases of the replication cycle. The non-structural 2C protein is their molecular target. Following the *in silico* screening of 8.5 million small chemical compounds for chemical structure similarity, 240 selected molecules were tested *in vitro*. Subsequently, 75 new analogue molecules were designed and synthesized, resulting in a more potent lead compound with an EC50 value in the high nanomolar range. However, the most active CHI-compounds were found to be racemic mixes of active and inactive molecules that could not be segregated. Consequently, it was concluded that further pharmaceutical development of the CHI-compounds would not be cost-efficient when compared to emergency vaccination or pre-emptive culling. Additionally, 517 inhibitors of human entero- and/or rhinoviruses were tested, but none was active against FMDV. Therefore, it was decided to screen 65,000 small chemical molecules from different compound libraries using a high-throughput screening assay with the cell viability reagent CellTiter-Blue® that was implemented and validated (Willems et al., 2011). After a rigorous selection, exploration and optimization process, three compound classes active against the different serotypes of FMDV in the nanomolar range were selected. The chemical, plasma and microsomal stability and the solubility of these compound classes were gradually and substantially improved. The identification of the molecular target by next-generation ultra-deep sequencing of artificially selected drug resistant mutants is ongoing. Explorative safety (toxicity) and pharmaco-kinetic studies in severe combined immunodeficient (SCID) mice and guinea pigs are also ongoing. If serum peak levels well above the EC50 value can be demonstrated, proof-of-concept efficacy studies will be initiated in mice and guinea pigs in the near future. Finally, the most promising antiviral compound will be selected for proof-of-concept studies in natural host species. In contrast to the CHI-compounds, these 3 compound classes are easy to synthesise (in large quantities to treat natural host species) at low cost.

In year one of the FMD-DISCONVAC project, partner 1 implemented and validated an FMDV infection model in SCID mice (Lefebvre et al., 2010). During years two and three, an FMDV infection model was implemented and validated in guinea pigs. In the final year of the project, it was demonstrated that the subcutaneous administration of 2'CMC to SCID mice for 5 days effectively abrogates FMDV infection (Lefebvre et al., 2013). In parallel, it was shown that the oral administration of T-1105 to guinea pigs for 5 days results in significant clinical and virological protection against FMDV infection. Despite excellent potency in laboratory animals, the dose and cost of the synthesis of 2'CMC and T-1105 may still be high for an

economically viable FMD control strategy. Therefore, the further development of the above mentioned 3 compound classes is prioritary.

Work Package 5: Improvement in FMD diagnostics

Task 1. Development of stabilised diagnostic kits

Robust and simple diagnostic kits are essential complements of the measures adopted for disease prevention and control; for FMD, their availability is particularly crucial in endemic countries adopting the Progressive Control Pathway. However, the availability of such kits for FMD diagnosis has been historically very limited, also due to the restrictions in handling of FMD viruses and to not easy accessibility to samples needed for assay validation. To overcome this shortcoming, one main objective of WP5 was the development of next generation ELISA kits for antibodies and antigens serotyping. The objectives were fully achieved.

Development and validation of a set of three ready-to-use kits, stable and user-friendly, for measurement of antibodies to FMDV serotypes O, A and Asia 1 was completed by Partner 4 (IZSLER). The process started with the transformation of in-house Solid-Phase Competitive ELISAs (SPCE) based on monoclonal antibodies (mAbs) in stable and simplified kits. In the kit format the reaction requires only two incubation steps at room temperature: delivery of test sera into plates, supplied pre-sensitized with FMD viruses trapped by mAbs, followed by addition of the homologous conjugated mAb, acting as both detector and competitor. For the validation of the new kits, 2001, 1620, 2384 naive field sera (including bovine, ovine, porcine) were analysed with the SP-O, SP-A, SP-Asia1 kits respectively; results of experimental sera from vaccine potency tests (178) and from vaccinated and or infected animals (410) were compared with those of VNT or liquid phase blocking ELISA (LPBE) obtained against homologous strains. Specificity values, obtained combining results of an internal (Partner 4) and external (Partner 2) validation were: 99.8% for SP-O kit (4 false-positive in 2001 sera), 100% for SP-A kit (1620 sera tested), 99.7% for SP-Asia1 kit (8 false-positive in 2384 sera). Using sera from full dose mono-vaccinated cattle provided by Partner 1 and 5, detection rates were 85% for type O (92% with VNT/LPBE, 87% concordant results in 109 samples), 90% for type A (96% with homologous VNT/LPBE, 86% concordant results in 49 samples), 100% for type Asia1 (20 sera). An external validation performed by Partner 2 with further VNT positive sera from vaccinated and/or infected animals showed sensitivity for type O, A, Asia1 kits of 90% (261 sera), 83% (196 sera), 100% (53 sera) respectively. The lower kit sensitivity for serotypes O and particularly A is also justified by the presence, within each panel, of sera raised against antigenically different strains, which were examined against the homologous antigens by the reference tests and heterologous by ELISA kits. The correlation between the three ready-to-use kits and original in-house ELISAs was evaluated by testing in parallel a set of 310 field sera form a survey in vaccinated regions in Trans-Caucasus countries. Concordance resulted higher than 0,9 with Kappa higher than 0,8 for the three tests, thus both parameters are very high and the very few discordant results were restricted to borderline samples. The concordance between the SP-ELISA kits and VNT was also evaluated on 320 field sera from vaccinated and/or infected population and it was confirmed “good” when the VNT doubtful titres are excluded. Finally, samples of the three kits were provided to five Partners for evaluation; in general they found the kits user friendly and a great improvement with respect to the complex in-house SPCE or LPBE. Additionally, the three kits were supplied to some endemic countries in the framework of EuFMD/FAO projects.

In conclusion, internal and external validation proved adequate performances irrespective of the strains that elicited antibodies. Kits are available upon request and for commercial exploitation.

In addition to the WP5 objectives, an SP-ELISA kit for detection of SAT2 Ab was developed; internal validation using a wide panel of experimental and field sera provided evidence of similar performance and ease of the previous ELISA's, while feasibility studies for a SP-ELISA kit for Ab to SAT1 were undertaken. Therefore, Partner 14 (Merial) provided inactivated purified SAT1 antigen to Partner 4.

The antigen detection ELISA is still an effective tool for the rapid diagnosis of FMD on epithelium vesicle samples. Two novel ELISA kits have been developed by partner 4, one designed for detection and typing of WestEurasia virus pools (serotypes O, A, C, Asia 1) and the second for African virus pools (serotypes O, A, SAT1 and SAT2). Serotype-specificity relies on the use of serotype-specific mAbs coated to microplate wells as catching antibodies, while captured antigens are detected by peroxidase-conjugated, inter-types cross-reactive mAbs; in addition, a pan-FMDV test, detecting any isolate of types O, A, C and Asia1 as well as some isolates of the SAT serotypes, is included in the kits. Type-specific catching MAbs with the broadest intra-typic reactivity for each of the FMDV serotype were selected after preliminary analyses of available panels of MAbs with a wide spectrum of FMDV isolates, representative of antigenic and genetic variability as well as different geographic areas and time periods. In particular, as many as 130 isolates of type O, 108 type A, 53 type Asia1, 33 type C, 32 type SAT1 and 45 type SAT2 were matched against many mAbs. For three serotypes a unique mAb was selected that recognized all or most of the isolates tested, while for three other serotypes a combination of two mAbs, used in tandem or in pool, was identified. Only a minority of strains, in general old historical viruses no longer circulating, were missed by the selected catching MAbs. ELISA plates are provided ready-to-use, i.e. pre-coated with the battery of selected catching mAbs and with positive controls, one for each FMDV serotype, already incorporated into respective wells, so that the test is accomplished with only two incubation steps at room temperature: delivery of samples, followed by incubation of a unique or at most two detector conjugates (depending on the kit); this implies a great simplification compared to the four steps and the several distinct immunological reagents necessary to perform the more complex traditional polyclonal double-sandwich ELISA. Extensive validation of the first kit for serotyping of Eurasian virus pools was achieved by analyzing 298 epithelial suspensions representative of the antigenic and molecular variation within each of the FMDV serotypes. Overall, the results proved evidence that the diagnostic performances of the new ready-to-use kit are similar, or significantly better for type A, than those of the more complex polyclonal ELISA. FMDV antigenic diversity is very well covered by the selected MAbs. Type-specificity resulted strongly improved: cross-reactions between serotypes were not observed, with the unique exception for the partial cross-reactivity of some type O isolates with one of the pan-type A catching mAb.

The two ELISA kits were distributed to many endemic countries in West Eurasia, Middle East and Africa. Feedback proved that the two kits offer satisfying diagnostic performance and the desired ease for countries adopting the Progressive Control Pathway or having poor laboratory facilities. In spite the validation process of the second kit designed for diagnosis in Africa is still in progress and improvement might be achieved, due to urgent and pressing requests the kit was distributed in most African Countries (more than 20) and in six Middle East Countries for diagnostic purposes and field evaluation. Initial feedback reported from users indicates the identification in clinical samples of FMD viruses belonging to all four serotypes covered by the kit and appreciation for its ease and real possibility to create diagnostic capacity. Quite impressive is the number of cases tested and diagnosed in Nigeria, where even SAT1 was detected while the last notification of this virus type was 30 years

earlier. Very good diagnostic performance was confirmed with respect to identification and typing of serotype SAT 2 positive samples, but as expected from the initial internal results, some SAT 1 variants remain undetected, so that further improvement may be required to complete the spectrum of SAT1 detection. In the present format, the kit intended for African virus pools requires use of two different conjugates; studies are in progress to further simplify the kit by incorporation of a unique detector antibody obtained with appropriate mixture of the pan-type O, A, C and Asia 1 mAb with a new pan-SATs mAb recently produced.

As planned in the WP the two kits were also provided to five Partners for evaluation. The samples of the Proficiency Testing Scheme of last two years were correctly detected and identified, as well as other known samples examined.

Task 2. Development of confirmatory DIVA tests and test systems

Partner 3 (CVI) intended to validate a new NS (non-structural proteins) ELISA which could be used in conjunction with the current commercially available Prionics NS ELISA. The initial intention was to develop an NSP ELISA based on polyclonal antibodies, but since it is expected that monoclonal antibodies will produce more reproducible results, it was decided to focus on the development of an NSP ELISA based on monoclonal antibodies. Although several formats were evaluated, none of them showed the same sensitivity and specificity as the Prionics test. There was some indication that the direct coated antigen had a higher sensitivity for swine samples, but since testing in swine is not essential because swine don't become carriers, this was not further pursued. The final set-up of the various NSP ELISA formats showed that they can be used at the same sensitivity as the Prionics ELISA, but with a lower specificity. In this perspective they can be used as screening test, where the positive results have to be confirmed in the Prionics ELISA.

The workshop for validation of new NSP-Ab assays or confirmatory tests was cancelled, in favor of the strategy to perform an initial evaluation, organized by partner 1 and 4, of available assays using two international NSP reference serum panels, described in literature, followed by extended validation only for tests showing promising results. Five new or prototype commercial tests (two penside LF tests and a BioNote competitive ELISA for Ab to 3ABC and two Priocheck ELISAs for Ab to 3D based on two different conjugated competitive MAbs) were evaluated by Partner 4 (IZSLER). Only one 3D-ELISA (conjugate B) showed performances similar to those of the two reference tests: the detection rate was 91.5% (54/59) compared to 90% (53/59) and 93% of (55/59) of the reference NS-Priocheck and IZSLER 3ABC tests respectively. The second 3D-ELISA (conjugate A) resulted less sensitive, with 45/59 infected sera detected, but the two 3D-ELISAs do not miss the same sera and used in combination compensate each other; however, also two repeatedly vaccinated animals reacted positive in both tests. The BioNote ELISA appeared definitively less sensitive (detection rate 39/59, 66%), while both LF devices showed very poor sensitivity (detection rate for UBIO-LFD 54% and only 27% for BioNote-LFD) and specificity resulting definitely unreliable. Since only the 3D-ELISA (with conjugate B alone, or both tests with conjugate 3B and 3A run in parallel) has shown sufficient requisites for an extension of validation, Partner 2 (PIR) has further validated the two new test prototypes using a large number of sera from cattle with different histories of vaccination, infection, disease and carrier status. Although both tests are good for detecting infection in an unvaccinated population, the 3D with conjugate B was less specific. 3D with conjugate A was better than the 3D with conjugate 2B for detecting carrier animals. However, both the tests gave more non-specific results with sera from vaccinated but uninfected animals. Therefore, it is recommended that the 3D test can be used as a non-serotype specific diagnostic test in unvaccinated populations, whereas its use as

a DIVA test is of limited value. In addition, Partner 2 has developed and validated 2B and 3B peptide tests and 4 recombinant non-structural protein tests (3ABC, 3CD, 3D, 2C). The previously reported 2B test was optimised to reduce the non-specificity of the assay with different blocking buffers and by mutating the peptide antigen. Specificity was checked using 991 naïve cattle sera. Sensitivity was checked with 470 cattle sera from direct contact challenge experiments and needle challenge experiments. ROC and Bayesian analysis revealed a good sensitivity, specificity and area under curve (AUC) for these tests except for the 2C NSP test. The tests were found to be comparable to the Prionics 3ABC NS test and could be used in conjunction with it for confirmatory testing.

Partner 2 (PIR) has been working for several years to develop and validate tests for FMDV-specific IgA antibody in upper respiratory tract secretions, as a means of identifying ruminants that are FMDV carriers. After improving the test specificity through the use of a control antigen, results showed that the most sensitive mucosal sample is nasal fluid and not saliva, as had been previously reported. It has been shown that whereas DIVA serology using non-structural protein ELISAs is a sensitive measure of previous infection, the IgA test is particularly relevant for detection of carrier animals. This is a significant breakthrough, since high sensitivity/specificity detection of residual carrier cattle is still perceived as a major stumbling block to the use of vaccination-to-live policies for FMD control. Earlier work had focused on serotype O, but extensive validation for serotype A was more recently carried out using several thousand saliva and/or nasal samples from vaccinated and subsequently infected cattle that had been collected together from various archives. The samples originated from 7 homologous and 5 heterologous potency tests of A serotype viruses carried out at the laboratories of Partners 2 (PIR), 3 (CVI) and 6 (IIL). 581 naïve cattle saliva/nasal fluids were tested in the IgA test to check the assay's specificity. 250 saliva samples from the field originated from cattle known to have been vaccinated with serotype A. As with the O serotype test, the A serotype IgA test detected FMD virus carriers amongst FMDV infected cattle. ROC analysis revealed that the IgA assay has good area under curve (AUC), Specificity (Sp) and Sensitivity (Se).

A large-scale comparison has also been made between the sensitivity and specificity of the IgA test and that of serum ELISAs for non-structural protein antibodies. Also the impact on the IgA test of substituting the inactivated FMDV antigen in the test with a recombinant FMDV capsid was investigated. In the serotype O IgA test, replacing the homologous inactivated antigen with purified empty capsid increased the sensitivity of the test. Out of 32 known carrier animals, this capsid-based IgA ELISA could detect 31 carrier animals whereas using unpurified inactivated antigen a maximum of 29 carrier cattle were detected.

Publication planned: Detection of persistent infection in serotype O FMD vaccinated and subsequently infected cattle using IgA assay. Authors: Biswal J., Paton D, Taylor G, Parida S.

Publication planned: Validation of IgA assay for the detection of A serotype FMDV carrier animals. Authors: Parekh K., Haas B., Dekker A., Paton D., Parida S.

Task 3. Development of a multiplex immunoassay

Partner 9 (ANSES) intended to develop a multiplex immunoassay for FMD diagnosis by using the Luminex liquid array technology and allowing (i) simultaneous detection of antibodies against vesicular disease viruses and FMDV structural and non-structural proteins and (ii) serological discrimination between FMDV-infected and vaccinated animals in a single reaction.

First stage in the development of this multiplex immunoassay was to produce FMDV antigens compatible with coupling reactions. These antigens were expressed as recombinant 6HisTag viral proteins in *E. coli*, purified and then coupled to luminex magnetic fluorescent microspheres. Such purified recombinant FMDV proteins (3D, 3ABC, VP1 serotype O, A or Asia1) were used to produce five antigen-coupled bead sets. The 3ABC recombinant protein was kindly provided by the IDvet Company. The attempt to produce 3A and 3AB proteins was without success. Their expression and purification remain to be improved. Simplex immunoassays were then carried out to (1) assess coupling performances (background and specificity) of each bead set using specific monoclonal antibodies or control serums, as well as to (2) set up and optimize the concentration of each reagent. In order to improve performances of the test, another supplier for the biotinylated anti-species antibodies with undeniable benefit in term of MFI (mean fluorescence Intensity) ratios was found. Preliminary luminex experiments have been performed using polystyrene carboxyl beads, but in order to improve precision and thus performance of the test, it was decided to switch to magnetic bio-plex carboxyl beads (Bio-Plex pro magnetic COOH Beads), allowing automated and more accurate washing steps. Two different beads coupling strategies have been assayed: (i) indirect coupling and (ii) direct bead/FMDV antigen coupling. Indirect coupling requires to couple beads with commercial mAb anti-His tag and then to incubate the coupled beads with the antigens of interest. Finally, the direct coupling strategy on magnetic beads was retained. Antigen-bead coupling efficiency was monitored in each experiment by anti-his (mAb) reaction. Luminex immunoassays in different combination from single to 4plex were then assessed by using well characterized control bovine serum samples from uninfected, infected, or vaccinated cattle. So far, the development of this bead-based immunoassay gave promising results. A Duplex immunoassay allowing the simultaneous detection of anti-NSP 3D and 3ABC antibodies (Ab) and a 4plex test allowing detection of anti-3D Ab and O, A, Asia1 serotyping were developed. Both prototypes were evaluated using a standardized bovine serum panel (36 samples), initially assembled for evaluation of the relative sensitivity of new NSP tests. All the 36 sera were found positive with the duplex anti- 3ABC & anti-3D Luminex test. According to these results, this duplex immunoassay appears as the most sensitive serological NSP method among those compared. Both Luminex detection of anti-3ABC and anti-3D Ab (published results or done at ANSES) gave similar results. For the detection of anti-3D and anti-VP1 Ab in a 4plex assay, results of two independant 4plex experiments revealed that all sera are positive for anti-3D Ab (as previously obtained with the duplex immunoassay). Encouraging serotyping results were also obtained but cross reactivity is observed (A/Asia; SAT2/Asia; A/O) and a loss of sensitivity for detecting anti-VP1 Ab against serotype O. Nevertheless, cross-reactivity is also observed using commercially available Type O Elisa. Future work will include further experimentations to determine sensitivity and improve specificity of the multiplex test. A 5plex including 3ABC coupled beads as well as multiplex immunoassays including NSP and SP from other vesicular diseases (SVDV, VSV) in order to provide differential rapid diagnosis will be developed as well.

Work Package 6: Improving knowledge on FMDV transmission between species and in recently vaccinated animals

Task 1. Transmission experiments in vaccinated and non-vaccinated cattle one week post vaccination

Partner 3 (CVI) performed transmission experiments in vaccinated and non-vaccinated cattle one week post vaccination to quantify the contribution of a contaminated environment to

FMDV transmission and the influence of vaccination hereon. Several direct and indirect contact experiments were performed. In the direct contact experiments, contact calves were exposed to infected donor calves. In the indirect contact experiments, contact calves were housed in animal rooms that had held infected calves from either 0-3 days post infection (dpi) or from 3-6 dpi. Oropharyngeal fluid (OPF) swabs, urine, faeces and blood samples were collected daily and were tested for the presence of FMDV by virus isolation and RT-PCR. Virus isolation results from both experiments were used to quantify FMDV transmission. FMDV transmission was quantified using a generalized linear model based on a 2 route (2R) i.e. contact and environment, stochastic SIR model. The model included information on FMDV survival in the environment. The results of this study indicate that roughly about 60% of transmission occurs through the environment as indicated by the estimated partial $\square_0$ of 4.7. The fact that a contaminated environment contributes largely to the transmission of FMDV indicates that disinfection is an essential tool to reduce FMDV transmission via the environment. Vaccination 1 week prior to infection of donor calves conferred protective immunity against FMDV infection.

Task 2. Transmission experiments between vaccinated and non-vaccinated sheep and cattle

FMDV infected sheep might be a high risk for transmission of FMDV to cattle because sheep can secrete and excrete considerable quantities of FMDV for a long period of time while the clinical signs in this animal species are often unapparent. Partner 3 (CVI) performed an experiment to quantify transmission between infected sheep and in-contact calves was performed. Transmission of FMDV was studied in 10 animal rooms, each housing 2 infected donor sheep and 1 in-contact calf. During the experiment, OPF swabs, probang, blood, urine and faeces samples were collected and were tested for the presence of FMDV by virus isolation (VI) and/or RT-PCR. Serum was tested for the presence of antibodies against FMDV. VI, RT-PCR and serology results were used to quantify FMDV transmission. FMDV transmission was quantified using the transient state method and the final size method. FMDV transmission occurred in 4 out of the 10 groups. Of the 4 infected calves, only 1 calf showed clinical signs of FMD, was positive by means of VI and RT-PCR and developed antibodies against FMDV. The other 3 calves became subclinically infected; two of them were positive by RT-PCR and all 3 developed antibodies against FMDV. Using the transient state method, a partial R0 was estimated to be close to 1 (estimates varied from 1 -1.4). Using the final size method, R0 was estimated at 0.87. The study shows that transmission of FMDV from sheep to cattle does occur but that the expected number of secondary cases in cattle produced by infected sheep is relatively low. It was concluded that the infectivity of sheep to cattle is probably less than the infectivity of cattle to cattle and that the fact that sheep are long-term se-/excreters of FMDV is not of high risk for transmission of FMDV to cattle.

Task 3. Transmission experiments in vaccinated and non-vaccinated buffalo

India holds the world's largest cattle and buffalo population and according to the 2007 animal census, 105 million buffalo in India constitute 57% of the total population of buffalo in the world. However, no detailed study has been carried out yet relating to the role of Indian buffalo in FMD epidemiology and transmission and there is also little information on the effectiveness of FMD vaccines in this species. Partner 6 (IIL) designed a study to test FMDV transmission between buffalo and the ability of FMD vaccines to prevent this. The usual cattle

dose of O Manisa vaccine was administered to buffalo that were subsequently challenged with the homologous virus that had been prepared and titrated in buffalo. The vaccinated buffalo developed good antibody responses, similar to those seen in cattle. Challenge was affected by exposing vaccinated and control unvaccinated buffalo to donor animals that had been previously inoculated with the O Manisa challenge strain. Although the donors developed signs of FMD, a 72 hour exposure period did not transmit infection to either vaccinated or unvaccinated control buffalo. To follow-up on this failure of transmission, it was decided to revert to using a different challenge virus that had been previously shown to transmit between buffalo under experimental conditions (O/HAS/34/05). The vaccine was also changed to the strain used throughout India (O/IND/R2/75). Donor buffalo that had been inoculated with O/HAS/34/05 were used to challenge vaccinated and control unvaccinated buffalo and cattle at 28 days post-vaccination, but with a 5 day direct contact exposure period. All six vaccinated cattle (100%) and four vaccinated buffalo (66.6%) were protected from clinical disease whereas two vaccinated buffalo (33.4%) and all of the in-contact unvaccinated buffalo and cattle were unprotected. All the vaccinated buffalo and cattle showed medium to high neutralizing antibodies at the time of challenge. The study indicates that FMDV can be transmitted from infected buffalo to susceptible in-contact naïve buffalo and cattle by direct contact. The antibody response elicited in buffaloes was comparable with antibody responses induced in cattle and gave rise to protection of some of the vaccinated animals. These findings support the notion that Indian buffalo could have a role in FMDV transmission that may have an impact on future control strategy.

Task 4. Field studies

Partner 11 (KVI) performed a survey of structural and NSP antibodies in vaccinated herds in intermediate endemic areas in Israel. During outbreak periods, the number of NSP positive animals at unaffected farms was limited, which indicates that (i) virus spread via aerosol is very limited and (ii) it is possible that the level of protection by the vaccine was able to prevent virus multiplication. Similarly, sampling in previously affected regions indicates that there is only low exposure to FMDV among domestic animals in Israel during non-outbreak periods.

In a study at an FMDV affected farm in Israel, the younger animals (which received fewer vaccinations) were less well protected than the older animals. From results of another study, it was concluded that emergency vaccination was highly effective in preventing clinical and sub-clinical infection, whereas routine vaccination with the same vaccine formulation provided only limited protection, probably due to poor longevity of the elicited immune response.

Moreover, the role of wildboars and gazelles in FMDV spread and transmission in Israel was investigated by Partner 11. A total of 638 wild boar samples, collected in the period from 2009 to 2011 were tested for the presence of NSP antibodies. All sera were NSP negative. A total of 13 gazelle serum samples were collected in 2011. Two out of 13 samples were NSP positive, but the numbers were too low to draw definite conclusions. Therefore, another serum collection of wild boars (79) and gazelles (32), collected during the years 2007-12 were tested. Of these, 14 wild boar samples were found to be NSP positive (17.7%). Most of these samples (12) were collected in parallel to an outbreak which occurred in 2007 and involved clinically affected wild animals. Two out of 32 (6.25%) of the gazelle samples were NSP positive as well. The main conclusion is that there is variability in the involvement of wildlife in FMD outbreaks in Israel. Also, all the positive samples were collected adjacent to the borders, suggesting introduction of FMD by wildlife from neighbouring countries.

Task 5. Quantification of FMDV in animal excretions and secretions

Literature data about the quantification of FMDV in animal excretions and secretions were analysed by partner 3 (CVI). The maximum titres of FMDV reported in different secretions and excretions and the experimental conditions in which they occurred, were recorded in a database. Highest FMDV amounts were reported in upper respiratory tract samples of cattle. Next, the data were analysed using a stepwise regression procedure. The model demonstrated that: (i) in general cattle secrete and excrete the highest amounts of FMDV compared with other animal species, (ii) during the clinical stage of the disease more virus is se- /excreted and (iii) less virus is excreted later after infection. The amounts of FMDV that are released in the different types of secretions and excretions depend on: (i) the animal species, (ii) the FMDV serotype that caused the infection and (iii) the stage of disease of the infected animal. The data collected in the experiment reported under task 1 of this work package were also analysed and the findings correlated to the analysis of the literature data. Higher quantities of FMDV were found in upper respiratory tract samples and lesser quantities in blood, urine and faeces samples of the calves. The obtained results could be useful as a first step to study which materials, contaminated with secretions and excretions from FMDV infected animals, are risk factors for transmission of FMDV.

Work Package 7: Development or adaptation of computerised FMD spread models to optimise vaccination schemes

Partner 13 (DTU) and 3 (CVI) focussed on investigating how different models can predict the spread of FMD in different countries, and whether a model designed for a specific country can be used to model the spread of FMD in another country.

Three stochastic simulation models (InterSpread Plus (ISP), the Davis model (DADS), and the North American Animal Disease Spread Model (NAADSM)) were adjusted to simulate the spread of FMD in Denmark (DK) by partner 13. The DADS model was modified to DTU-DADS and was also used to simulate the spread of FMD in the Netherlands (NL) are explained in the subsequent section. At the CVI (Partner 3), the DKM (Dutch Kernel Model) was used to siulate the spread of FMD in Denmark; as presented beneath. Several control scenarios were identified and simulated including vaccination in a vaccine to kill or vaccine to live settings. Partner 13 (DTU) in collaboration with Partner 1 (CODA-VAR), organized a 2-days workshop to reduce the gap between virologists and epidemiologists. Virologists were invited to teaching session on simulation modeling and thereafter, they were introduced to a practical session on simulation modeling, in which they used the ISP model. This gave them the chance to understand hwo models work and what data is needed to feed the models.

DTU (partner 13) modeling of the Dutch situation: The DTU-DADS, which is a stochastic and dynamic spatial simulation model of disease spread between herds, was used to simulate the spread of foot-and-mouth disease (FMD) in the Netherlands using Dutch data. Colleagues from the CVI provided herd data, including number of animals, herd type, and location (coordinates). Data on contact structure between herds was obtained from Velthuis and Mourits (2007) and were adjusted to fit the model. Other input data were retained in the model based on the Danish simulation project (Boklund et al., 2013).

The model simulated spread of infection between herds through different spread mechanisms: 1) direct animal movement between herds, 2) abattoir trucks, 3) milk tankers, 4) medium risk contacts, 5) low risk contacts, 6) markets, and 7) local spread, which include potential airborne spread within 3 km zone. Following the detection of the first infected herd, a set of default control strategies was applied representing the EU and national regulations. These included: 1) depopulation, cleaning and disinfection of detected herds, 2) a 3 days national stand still on animal movements in the country, 3) a 10 km radius zone (surveillance zone) around the infected herds for 30 days in which movements between herds and out of the zone were restricted and herds were surveyed one time before lifting the zone, 4) a 3 km radius zone (protection zone) around the infected herd for 21 days, in which movements between herds and out of the zone were restricted and herds are surveyed during the first week, meaning that herds within the protection zone would be surveyed 2 times, 5) backward and forward tracing of contacts from and to detected herds. If a herd had received animals from a detected herd, the receiving herd was also depopulated and disinfected, while in case of other kind of contacts, the herd was surveyed. When a herd was subject to surveillance, the animals were inspected for clinical signs of FMD. In case of sheep herds, the animals were also sampled for serological analysis.

The model simulated 3 scenarios of disease control: 1) the basic scenario as described above, 2) the basic scenario plus pre-emptive depopulation of susceptible herds in 1 km radius around detected herds (Cull-1km), with the pre-emptive depopulation starting on the same day of first disease detection, and 3) the basic scenario plus emergency vaccination of susceptible herds in 2 or 5 km radius around detected herds. The vaccination started 1 week following the detection of the first infected herd. During this week, pre-emptive depopulation of susceptible herds in 1 km radius around detected herds was carried out. Emergency vaccination was applied as suppressive (vaccine to kill: VTK) or protective vaccination (vaccine to live: VTL).

CVI (partner 3) modelling of the Danish situation: During the project, three models of FMD transmission were compared with each other: DKM, ISP (Interspread Plus) and DTU-DADS, which is a modified version of the DADS (Davis Animal Disease Spread Model). Partner 3 used their DKM model, described by Backer et al. (2012a, 2012b). This model is individual-based using a module for within-herd transmission and a module for between-herd transmission of FMD. The model is spatial in that it uses location coordinates of each FMD susceptible farm (cattle, sheep and pigs) in the country. An important difference with the other two models ISP and DTU-DADS is that the DKM model simulates the between-herd transmission in a parameter-sparse way (i.e. only a small number of input parameters are needed). The between-herd transmission is modelled by a kernel: a probability for a neighbouring farm to get infected by a source farm (infectious farm) as function of the between-farm distance. All transmission pathways between farms occurring during an epidemic (i.e. under transport regulations) are included in this one probability. This between-farm kernel was estimated by using observed data of the FMD epidemic in The Netherlands in 2001. The kernel was also estimated (by others) for the UK, using observed data of the FMD epidemic in the UK in 2001. The two kernels did not differ much.

During the current project, Partner 3 used the DKM model to simulate FMD epidemics in Denmark. For this, Partner 13 (DTU, Denmark) delivered location coordinates of all FMD susceptible farms in Denmark. A between-farm kernel could not be estimated for Denmark, as no observed data are available of an FMD epidemic in Denmark. So the Dutch kernel was used in the model. Surprisingly, with the DKM model (using the location coordinates of farms in Denmark and with the Dutch kernel) no large epidemics could be simulated for Denmark.

The reason for this was found to be the more-evenly distribution of farms in Denmark compared to The Netherlands. The average between-farm distance in Denmark is larger than in The Netherlands. This was verified by comparing density maps of Denmark and the Netherlands, and by comparing risk maps for the two countries. In the risk map for Denmark, no areas were found where the between-farm reproduction ratio R_0 was larger than 1. R_0 is the average number of new infected farms caused by one infectious farm in a population of susceptible farms. R_0 is characterized by a threshold value of 1, below which no large epidemics can occur. The DKM model did not simulate large epidemics for Denmark, and the risk map for Denmark did not show areas where R_0 was larger than 1.

On the contrary, the ISP model and the DTU-DADS model did simulate epidemics for Denmark. We then decided to use one simulated epidemic for Denmark by the model ISP (iteration 720) as if it was an observed (real) epidemic, and subsequently estimated the between-herd kernel for Denmark. This kernel for Denmark was then compared to the Dutch kernel (based on the Dutch epidemic in 2001) and it was much higher than the Dutch kernel: the probability of getting infected at close distances to a source farm was about 6 times higher. At very long distances (> 10 km), the probability of getting infected was lower than by the Dutch kernel.

Moreover, all 1000 simulated epidemics (iterations) of the ISP model were used to estimate the kernel for Denmark again, to check if iteration 720 was an exception in these 1000 iterations. This was not the case.

It was shown that with the ISP-based kernel for Denmark as input, the DKM model does simulate large epidemics in Denmark, and the simulation results of the DKM model could be compared to those of ISP and DTU-DADS. Also, the risk maps for Denmark now showed areas where R_0 was larger than 1.

DKM model results were first compared with those of ISP (for Denmark). The ISP model simulates longer and larger epidemics than the DKM model. For 1 km vaccination, the median duration was 60 days (ISP) vs 40 days (DKM), but the number of detected farms was a factor 2 higher (ISP). However, *ranking* of the evaluated control scenarios was the same in both models: 1 km ring culling yields shorter and smaller epidemics than 1 km ring vaccination.

Then, DKM model results were compared with those of DTU-DADS (for the Netherlands). The Dutch DKM simulations were published by Backer et al. (2012a and 2012b). DTU-DADS was used by partner 13 using location coordinates of each farm in The Netherlands. The DTU-DADS model simulates shorter and smaller epidemics than the DKM model. Simulated epidemics with 1 km cull are a factor ½ shorter, lasting only 30 days according to the DTU-DADS model, when started in the high-density-livestock-area Gelderse Vallei (in the Netherlands). The number of detected farms was also smaller by DTU-DADS, but not that much (not a factor ½). However, in the VTK-5km and VTL-5km, the difference was rather small. Again, the *ranking* of evaluated control scenarios was similar in both models: 1 km ring culling yields the shortest and smallest epidemics; 2 km ring vaccination yields epidemics a bit longer and larger than 1 km cull; and 5 km ring vaccination is similar to 1 km cull (although small differences were seen between the models here).

Furthermore, the DTU-DADS model simulated shorter and fewer epidemics than the Dutch ISP model (Velthuis and Mourits, 2007) especially in the basic scenario.

In conclusion, the FMD simulation models cannot be used to predict the exact size and/or duration of a FMD epidemic. Comparison between models from one or different countries can

be hampered due to difficulty to compare input parameters, and the effect of differences in modelled processes. The use of models should focus on the epidemiologic and economic assessment of the disease effects and on comparing alternative control strategies, in order to evaluate the most effective strategy. The use of different models, simultaneously, gives insight into modelling processes and reduces coding errors.

Potential impact (including socio-economic impact and the wider societal implications of the project so far) and main dissemination activities and exploitation of results (not exceeding 10 pages)

SOCIO-ECONOMIC IMPACT AND WIDER SOCIETAL IMPLICATIONS

The current version of the OIE Terrestrial Animal Health Code makes provision of zoo-sanitary measures (i.e. disinfection, decontamination and cleaning of the animal holding facilities, their surroundings, transport vehicles and equipment), movement restrictions, "stamping out" (i.e. culling and disposal of all infected and susceptible "contact" livestock) and/or vaccination to control outbreaks and regain FMD-freedom. Until recently, most FMD-free countries/zones not practising routine vaccination favoured a "stamping out" policy accompanied by zoo-sanitary measures and movement restrictions. As a result of the mass slaughter of animals during the 2001 epidemic, however, and accelerated by the availability of reliable "marker" vaccines and DIVA tests, both the World Organisation for Animal Health (OIE) and European guidelines were amended to facilitate the use of emergency vaccination (Council Directive 2003/85/EC) and to reduce the waiting period from 12 to 6 months when applying such vaccination.

The FMD-DISCONVAC project was dedicated to develop, enhance and complement the animal-sparing, foot-and-mouth (FMD) disease vaccine-based control strategies for free and endemic regions.

In order to improve these FMD vaccine based control strategies, the work developed under the FMD-DISCONVAC project aimed to fulfil the following objectives:

- Improve FMD vaccine quality (efficacy-potency, purity, safety)
- Investigate heterologous protection by FMD vaccines
- Develop novel vaccines and antiviral agents
- Improve FMD diagnostics
- Improve the knowledge on FMDV transmission
- Develop computerised FMD spread models and vaccination schemes

These objectives were organised in six work packages and were outlined in the work plan providing a logical process for improving of knowledge on critical aspects of FMD vaccine control strategies.

By including several National Reference Laboratories (NRL) and the World Reference Laboratory (WRL) for FMD, two associated countries (Switzerland and Israel) and three ICPC partners (Argentina, China and India), the project reinforced the European network of experts for European research in vaccinology, diagnostics and epidemiology in FMD.

Several outcomes of the project emphasize its successful contribution to improve FMD vaccine based control strategies:

Improve FMD vaccine quality (efficacy-potency, purity, safety)

In light of the 3R principle, it is expected to replace the current in vivo 'Gold Standard' tests for vaccine efficacy, purity and safety, by validated in vitro laboratory tests. To correlate serology to protection, a serology based logistic regression model for a FMDV A Iran 96 and O Manisa vaccine and an improved FMDV-specific bovine IFN-γ ELISA was determined. A

predictor based on *in vitro* assays was developed that on average performs better than current in vivo tests for determining vaccine potency. A cell viability assay was evaluated as VNT high throughput test. Proof of concept was established that a simple ELISA for detection of NSP in FMDV antigen preparations is feasible. A Western Blot assay detecting the presence of NSPs was developed to check the purity of FMD vaccines. A set of MAbs-based sandwich ELISAs were established to discriminate full/empty capsids from 12S sub-particles. An antigen payload quantification method was developed based on chromatography. In a heterologous vaccination/challenge setting a better correlation to the vaccine dose was observed with opsonising antibody levels compared to neutralizing titres. These results made it possible to determine the quality of in vivo animal challenge tests and set the standard for alternative serological tests, strengthening the position of the EU on the global market when it comes to the implementation of the 'vaccinate-to-live' policy, which will have a serious impact on the economic situation and the social acceptance of control measures whenever a future FMD epidemic will occur.

Investigate heterologous protection by FMD vaccines

The availability of high quality vaccines is extremely important to implement the vaccinate-to-live policy by predicting how well these vaccines protect against a field virus of another strain within the same serotype. The work focused on serotypes O and A. Highly potent (multivalent) vaccines to serotype A may offer a reasonable degree of cross-protection which, in general, correlates with serology, although not necessarily with the r-value. Achieving heterologous protection with serotype O appears more difficult and the correlation of heterologous protection with serology requires further studies. Advanced mathematical techniques have the potential to significantly reduce the confidence intervals for estimates of r1-values, avoiding future in vivo cross-protection studies. This will help decision-makers in their difficult choice on which vaccine to use in future outbreaks and in their responsibility in updating and reinforcing FMD vaccine/antigen banks.

Develop novel vaccines and antiviral agents

Development of new generation FMD vaccines by using three different approaches:

(1) prevent FMDV infection at the oro-pharynx by reinforcing the mucosal immune response. FMD capsids delivered by adenovirus elicit good immune responses in mice whether given intramuscularly or intranasally. However, when the adenovirus construct was tested in cattle only the intramuscular route of vaccination proved effective in providing protection against intranasal challenge. Moreover, nanoparticles containing chitosan coated PLGA loaded FMDV DNA have potential as a nasal delivery system for vaccines. Although intranasal delivery with FMDV antigen mediated by nanoparticles could not provide complete clinical or virological protection, this reduced the severity of the disease and the virus excretion, and delayed clinical symptoms.

(2) avoid the need for virus culture by using other efficient new generation vaccines, making the production of FMD vaccines environmentally safer. Cav2-based vectors can express immunogenic FMDV antigens in rodents, suggesting that Cav2-P1/3C can be considered to be a potential marker vaccine against FMD. Results obtained in a mouse model using recombinant baculovirus as adjuvants or as antiviral agents for FMDV, could not be repeated in a reduced proof-of-concept experiment performed in cattle. Intravenous administration of a recombinant baculovirus carrying additional CpG motifs could not prevent development of the disease when applied 6 or 24 h before intranasal infection of virulent O1 Campos strain FMDV.

(3) decrease the post vaccination immunity gap by using potent and selective anti-FMDV antiviral compounds. Three different compound classes that are characterized by easy synthesis (in large quantities to treat natural host species) and low cost of goods are active against the different serotypes of FMDV in the nanomolar range. These 3 compounds classes are readily available for pharmaco-kinetic and antiviral studies in laboratory animals in the near future. Proof-of-concept of antiviral activity in vivo was demonstrated for the 3-oxo-3,4-dihydro-2-pyrazincarboxamide derivative T-1105 in guinea pigs and for the ribonucleoside analogue 2'-*C*-methylcytidine in severe combined immunodeficient mice.

All three approaches will supplement the existing control tools to combat FMD and allow the enhancement of emergency contingency plans enabling a better, quicker and animal sparing response to FMD outbreaks.

Improve FMD diagnostics

Improvement in FMD diagnostics by an increased availability of FMD diagnostics, the improved standardization and harmonization of FMD diagnostic results and new, possibly better, diagnostic tools for confirmatory tests and/or test systems for NSP-serology will help the OIE/EU to better interpret the dossiers submitted to demonstrate/substantiate FMD-freedom and, by facilitating and accelerating the development and distribution of the most effective diagnostics for FMD in Europe and in the rest of the world, could contribute to the vision of the ETPGAH.

A portfolio of next generation ELISA kits for FMD diagnosis was developed, characterised by simplicity, stability and standardization. The portfolio of ready-to-use and all-inclusive kits includes both kits for detection of SP-antibody to serotypes O, A, Asia 1, SAT2 and for detection of NSP antibodies. It also includes kits for FMDV antigens detection and serotyping in clinical samples. Thanks to their properties, these kits can be used also in poorly equipped laboratories to improve diagnostic capacity and FMD control in endemic countries. Exploitation of the foreground has already started; kits were distributed through EUFMD/FAO projects to more than 40 countries (in Middle East, WestEurasia and Africa) for evaluation and diagnosis, and to several project partners for evaluation. Extensive validation of mucosal IgA tests and NSP tests based on novel proteins and peptides was performed that could be able to help detect infected animals, especially carriers, within a vaccinated population. A competitive 3ABC antibody ELISA was developed as an alternative to the 3ABC commercial test for pre-screening of sera during an outbreak, with positive samples to be confirmed by a more specific NSP tests. A Duplex immunoassay, allowing the simultaneous detection of anti-NSP 3D and 3ABC, a prototype of a triplex immunoassay based on Luminex technology (3D, 3ABC, VP1 Asia1) and a 4plex test, allowing detection of anti-3D antibodies and O, A, Asia1 serotyping, were developed. A 5plex serological test including 3ABC protein is underway.

Knowledge on performance characteristics of the available DIVA diagnostics on a global scale helps to understand the FMD situation in other regions of the world, resulting in an increased awareness of the potential threats to the European Union.

Improve the knowledge on FMDV transmission

Obtaining quantified knowledge on FMDV transmission within and between different species in the period shortly after applying emergency vaccination will enhance our knowledge on FMDV spread and transmission following the use of high-potency monovalent or multivalent vaccine. Studying transmission dynamics in real-time outbreak situations will set-up early warning systems for FMDV penetration.

Transmission experiments in non-vaccinated cattle and vaccinated cattle one week post vaccination, show that the contribution of the virus contaminating the environment was much higher than expected. Vaccination one week before challenge protected cattle against clinical disease and virus shedding. The between species transmission from infected cattle to sheep and from infected sheep to cattle was quantified. FMDV could be transmitted from infected Asian buffalo to susceptible in-contact naïve buffalo and cattle by direct contact. FMD vaccination of buffaloes could prevent the transmission of disease from infected buffaloes to susceptible in-contact cattle and buffaloes. Therefore, Indian buffalo could play a role in FMDV transmission that may have an impact on future control strategy. Analysis of quantitative data from different publications show that species, type of ex-/secretion, clinical disease and serotype has a significant effect on the amount of virus excreted.

Knowledge on (1) the vaccination efficacy to prevent the ability of the Asian buffalo to transmit FMDV infection, (2) the role of wildlife in FMDV maintenance and transmission and (3) quantified knowledge on the presence of FMDV in viral secretions and excretions in different species is now available and can be used to improve FMD control measures.

Develop computerised FMD spread models and vaccination schemes

Development or adaptation of computerized FMD spread models to optimize vaccination schemes by studying the applicability and feasibility of modifying existing simulation models (InterSpreadPlus, Davis, NAADS and other models within the Consortium) for FMD spread to suit the exploration of vaccination strategies in the EU and other countries where FMD is considered an exotic threat. During a workshop data within the Consortium were identified and made available. The InterSpread Plus model and the Davis model were described and compared with other models within the Consortium. The ranking of the simulation models according to their likely sensitivity to weak or missing information from participating countries/regions were submitted. An inventory of different relevant vaccination strategies for Denmark and the Netherlands has been prepared. Computerized FMD spread models developed within this project enable now the design of vaccination strategies for high-risk regions within countries and are relevant step-stones to model vaccination strategies for endemic regions of the world.

MAIN DISSEMINATION ACTIVITIES AND EXPLOITATION OF RESULTS

Exploitation and dissemination of the results was considered by the consortium to be vital. Dissemination of the results of the project was fulfilled through a series of internal and external dissemination activities.

Internal dissemination

The consortium was composed by a multidisciplinary scientific team from 14 partners based in 11 different countries. The ability of the consortium to successfully complete the project was attributed to the scientific expertise in different fields including vaccinology, vaccine development, virology, diagnostics, molecular biology including sequencing, immunology, epidemiology and disease surveillance/control, conduct of animal experiments and field trial experience.

Internal dissemination between partners was achieved through the organisation of yearly project meetings, teleconferences, communication via e-mail and on the project website, training of scientific staff and exchanges of personnel between institutes; transfer of

laboratory tests, samples and test protocols between partners, detailed reporting including full materials and methods in the periodic reports to the EC.

External dissemination

The project management team kept in close contact with (i) EC DG RESEARCH and DG SANCO, (ii) the ETPGAH with special attention to ERA-Net as the CVOs are involved in the DISCONTOOL project, (iii) international organisations such as FAO EUFMD, OIE and informed them of any outcome of the project that might have an influence on FMD control policies.
External dissemination of results was achieved through the following series of activities:

- Publications in scientific peer reviewed journals;
- Oral and poster presentations at conferences and scientific meetings;
- PhD theses;
- Project website;
- Training of scientific staff
- Workshops, Flyers, video presentation and press release
- Reports to the EC.

Publications in scientific peer-reviewed journals

So far, the following scientific papers have been published or have been accepted/submitted for publication:

1. Lefebvre D.J., Neyts J., De Clercq C. Development of a foot-and-mouth disease infection model in severe combined immunodeficient mice for the preliminary evaluation of antiviral drugs. 2010. Transboundary and Emerging Diseases. Dec., 57 (6), 430-433.
2. Willems T., Lefebvre D.J., Neyts J., De Clercq K. Diagnostic performance and application of two commercially available cell viability assays in foot-and-mouth disease research. 2011. Journal of Virological Methods. April, 173 (1), 108-114.
3. Nagendrakumar S.B., Srinivasan V.A., Madhanmohan M., Yuvaraj S., Parida S., Di Nardo A., Horsingston J. and Paton D.J.. Evaluation of cross-protection between O1 Manisa and O1 Campos in cattle vaccinated with different payloads of O1 Manisa monovalent vaccine. 2011. Vaccine. Feb. 24, 29 (10), 1906-1912.
4. Reeve R., Cox S., Smitsaart E., Perez Beascoechea C., Haas B., Maradei E., Haydon D.T., Barnett P. Reducing animal experimentation in foot-and-mouth disease vaccine potency tests. 2011. Vaccine. Jul 26, 29 (33), 5467-5473.
5. Ferris N.P., Grazioli S., Hutchings G.H., Brocchi E.. Validation of a recombinant integrin avb6/monoclonal antibody based antigen ELISA for the diagnosis of foot-and-mouth disease. 2011. Journal of Virological Methods. Aug 175 (2), 253-260.
6. Carocci M., Cordonnier N., Huet H., Romey A., Relmy A., Gorna K., Blaise-Boisseau S., Zientara S. and Bakkali Kassimi L. Encephalomyocarditis Virus 2A Protein Is Required for Viral Pathogenesis and Inhibition of Apoptosis. 2011. Journal of Virology. Oct., 85 (20), 10741-10754.
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9. Willems T., Lefebvre D., Goris N., Diev V.I., Kremenchugskaya S.R., Paul G., Haas B., De Clercq K. Characteristics of serology-based vaccine potency models for foot-and-mouth disease virus. 2012. Vaccine. August (30), 5849-5855.
10. Oh Y., Fleming L., Statham B., Hamblin P., Barnett P., Paton D.J., Park J.-H., Joo Y.S., Parida S. Interferon-gamma induced by in vitro re-stimulation of CD4-+ T-cells correlates with in vivoFMD vaccine induced protection of cattle againstdisease and persistent infection. 2012. PLoS One. 7(9), e44365.
11. Lavoria M.A., Di-Giacomo S., Bucafusco D., Franco-Mahecha O.L., Pérez-Filgueira D.M., Capozzo A.V. Avidity and subtyping of specific antibodies applied to the indirect assessment of heterologous protection against Foot-and-Mouth Disease Virus in cattle. 2012. Vaccine. Nov 6; 30(48), 6845-6850.
12. Lannes N., Python S., Summerfield A. Interplay of foot-and-mouth disease virus, antibodies and plasmacytoid dendritic cells: virus opsonization under non-neutralizing conditions results in enhanced interferon-alpha responses. 2012. Veterinary Research. aug 30; 43(1), 64.
13. Lefebvre D.J., De Vleeschauwer A.R., Goris N., Kollanur D., Billiet A., Murao L., Neyts J., De Clercq K. Proof of Concept for the Inhibition of Foot-and-Mouth Disease Virus Replication by the Anti-Viral Drug 2'-C-Methylcytidine in Severe Combined Immunodeficient Mice. 2013. Transboundary and Emerging Diseases. Epub ahead of print.
14. Paton D.J., King D.P. Diagnosis of Foot-and-Mouth Disease. 2013. Vaccines and Diagnostics for Transboundary Animal Diseases. Dev Biol. vol 135, 117-123.
15. Lannes N., Summerfield A. Regulation of porcine plasmacytoid dendritic cells by cytokines. 2013. PLoS One. 8(4), e60893.
16. Quattrocchi V., Molinari P., Langellotti C., Gnazzo V., Taboga O., Zamorano P. Co-inoculation of baculovirus and FMDV vaccine in mice elicits very early protection against Foot and Mouth Disease virus without interfering with long lasting immunity. 2013. Vaccine. May 31;31(24), 2713-2718.
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18. De Vleeschauwer A.R., Lefebvre D.J., Willems T., Paul G., Billiet A., Murao L., Neyts J., Goris N., De Clercq K. Proof-Of-Concept for the reduction of Foot-And-Mouth Disease Virus Infection in Guinea Pigs by small chemical Antiviral Drugs. Antiviral Research. Submitted.
19. Bravo de Rueda C., Dekker A., Eblé P., Mart de Jong. Factors associated with high secretion and excretion of Foot-and-Mouth Disease Virus and their possible implications for transmission of the infection. 2013. Preventive Veterinary Medicine. Submitted.
20. Halasa T., Willeberg P., Christiansen L.E., Boklund A., AlKhamis M., Perez A., Enøe C.. Decision on foot-and-mouth disease control informed using model prediction. 2013. Preventive Veterinary Medicine. Submitted.

Another 21 publications are in preparation or planned. A list of these publications is provided in the third periodic report 3 of this project and in the respective Work Package deliverable reports.

Presentations at conferences and scientific meetings

A total of around 84 oral presentations and 27 poster presentations took place at various conferences and scientific meetings over the duration of the project. These also included presentations during the bi-annual Open Session meetings of the EuFMD standing technical committee. These meetings were attended by participants from European Countries and representatives from (i) reference laboratories from all other countries, (ii) international organisations such as FAO, GFRA, DG SANCO and (iii) the industry on vaccine and kit production. Full details are given in the "Dissemination activities" table.

PhD theses

Margot Carocci, Sur la route d'un virus recombinant EMCV-FMDV, 2013, University Paris Diderot – Paris 7, France. (Partner 9, ANSES).

Daryl Borley, Epitope dominance studies in serotype O FMD, 2013, Oxford University, United Kingdom, (Partner 2, PIR). Division of Structural Biology, University of Oxford, The Henry Wellcome Building for Genomic Medicine, Headington, Oxford, United Kingdom.

Two more PhD theses, based on work performed in the framework of this Project are in preparation and will be defended after the final date of the project:

Carla Bravo de Rueda (CVI), Filling knowledge gaps in the transmission of Foot-and-Mouth Disease virus", defence planned in 2014 at Wageningen University, The Netherlands (Partner 3, CVI).

Tom Willems, Improving foot-and-mouth disease vaccine potency estimation, defence planned in 2014 at Ghent University, Belgium (Partner 1, CODA-VAR).

Development and maintenance of the project website

The website for the FMD-DISCONVAC project (www.fmddisconvac.net) was launched in June 2009. The website is composed of a part which can be accessed by the public and a restricted part which can only be accessed by Consortium Members. The open access part of the website includes general information about the project, such as project background, objectives, partners and the description of work packages and news items. The restricted part of the website was used to share documents such as meeting presentations, meeting summaries, milestones and deliverables documents, periodic reports etc.

Training of scientific staff

On three different training sessions, organised on 8-24 April 2012, 10-21 June 2012 and 2-11 November 2010 at IZSLER laboratories, respectively 1, 4 and 9 scientists and other people were trained to practice the new FMD diagnostic kits developed by Partner 4 (IZSLER).

Workshops, flyers, video presentation and press release

- Workshop: “Do we have access to the data needed to model spread of FMD in target regions and countries?”, 19 - 20 May 2010, Copenhagen, Denmark
- Flyer: “open session meeting of the EuFMD standing technical committee: FMD-DISCONVAC scientific meeting”, 27 September – 1 October 2010, Vienna, Austria.
- Workshop and flyer: “FMD Cross-protection, Vaccine matching and Vaccine Banks: Challenges and Opportunities”, 15-16 June 2011, Buenos Aires, Argentina, organised in cooperation with GFRA.
- Flyer: “open session meeting of the EuFMD standing technical committee: FMD-DISCONVAC scientific meeting”, 28 – 31 October 2012, Jerez de la Frontera, Spain.
- Flyer: “Open session meeting of the EuFMD standing technical committee and subsequent occasions: FMD diagnostic kits”, 28 – 31 October 2012, Jerez de la Frontera, Spain and subsequent other occasions
- Video: “Open session meeting of the EuFMD standing technical committee and subsequent occasions: FMD diagnostic kits (antigen detection and serotyping ELISA)”, 28 – 31 October 2012, Jerez de la Frontera, Spain and subsequent other occasions
- Press releases: Symposium - Practical alternatives to reduce Animal testing in quality control of veterinary biologicals in the Americas. ISBN 978-987-25861-0-2: Validation of methods to reduce, refine and replace animal testing for diseases for which antibody is responsible for and predictor of protection. De Clercq K., Willems T.

Reports to the EC.

During the course of the project, three periodic reports were submitted to the EU as well as deliverable reports.

4.1.3. Public website and relevant contact details

Project website

www.fmddisconvac.net

Keywords

Foot-and-mouth disease, disease control, vaccines, diagnostics, transmission, vaccination strategies

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