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Global Foot-and-Mouth Disease Research Alliance

GFRA

NEWSLETTER

Fighting Foot-and-Mouth Disease

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Global Foot-and-Mouth Disease Research Alliance

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The socio-economic impact of FMD

Theo Knight-Jones

FMD in endemic countries causes a global economic impact, due to production losses and vaccination costs, of around US$6.5 to 21 billion per year [1]. This does not include the impact of loss of trade or restricted development of the livestock sector, which are potentially enormous. Much of this burden falls on Asia, with China and India the worst affected. Estimated production losses for affected cattle range between US$100 to US$370 per case [2-6]. In addition, losses from FMD outbreaks in free countries average at >US$1.5 billion per year, one of the most notorious being the UK 2001 outbreak with estimated losses of >US$9 billion [7].

Recent studies of FMD incidence in East Africa found that 70–90% of herds experienced clinical FMD each year [8, 9]. This is in line with sero-prevalence studies, which find that in many areas of Africa and Asia about a third of young cattle are infected with FMD each year.

FMD incidence is highest in regions where smallholder farming is commonplace. One study in South-East Asia estimated that outbreaks resulted in a reduction in household income of 4-12% [4], in parts of Laos they may account for 60% of annual household income [10]. The impact of FMD on food security is exacerbated by a reliance on livestock as a means of traction by many smallholders in Asia and Africa [11, 12]. Studies in Laos and Cambodia estimated that affected cattle lose a quarter to three-quarters of their original value [13, 14]. As cattle play an important role as assets that are cashed in at times of need, this has a severe impact on livelihoods.

This high incidence in endemic countries translates to a high risk of virus incursion in free countries. Countries that have eradicated FMD face ongoing costs from periodic outbreaks and the costs of maintaining preparedness. Many countries reduce the impact of the disease with extensive ongoing vaccination programmes. The global scale and costs associated with these programmes are vast with billions of doses administered annuallyThe rewards of successful FMD control are such that some nations vaccinate all cattle as much as five times a year, monopolising the resources of the veterinary services. The costs associated with mass vaccination discourage some countries from attempting to control FMD. Successful FMD control takes many years or even decades, and programmes that are not fully committed are unlikely to reduce disease impact.

FMD losses in India, due to deaths, milk loss, draught losses and treatment costs were estimated to be US$2.7–3.6billion [15] and control is now a priority. Mass vaccination will require billions of doses and biosecurity measures, if effectively implemented, cause additional indirect losses. However, in the long term, such approaches have brought significant returns in South America and elsewhere.

The impact of FMD outbreaks in free countries has been studied in detail and the devastation FMD causes is well understood. Yet our knowledge of FMD impact in endemic countries has, until recently, largely been based on hearsay. From this absence of evidence, FMD control in endemic countries has often been deemed a lesser priority by governments and international funding agencies, particularly for countries without the potential to benefit from FMD free export markets. It has been argued that, in southern Africa, only wealthy farms benefit from FMD free status [16], whilst the control costs are carried by the tax-paying public at large. Others have stated that a significant proportion of the benefits of international export filter down through the supply chain to low-income households [17].

Although mortality from FMD is low, the continued, and widespread, high disease incidence clearly leads to a heavy burden of disease at the population level. However, more studies are needed that measure this impact in order to fill the void of evidence. Benefit-cost analysis requires estimates of the impact of control. Although FMD control measures have proved to be effective in much of the world, efforts to control FMD in developing countries have experienced variable success. Guidance is needed, and control programmes in this setting need to be better evaluated and their impact quantified. As for other areas of FMD research, despite many decades of progress, there is much that needs to be done.

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Development of molecular therapeutics to control early infection during FMD outbreaks

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Current FMD vaccines do not induce a protective response until 7 days post-vaccination. Due to the short incubation period, signs of disease can appear as early as 2 days post-exposure. This situation has underlined the importance of developing antiviral strategies capable of limiting the multiplication of the infective virus and stopping the spread of the disease. To develop therapeutics which can stop the multiplication of FMDV at the early stage of infection we selected two approaches: i) interferon based, and ii) small interfering RNA based.

Interferon based therapeutics

3'UTR RNA was able to induce interferon(IFN) α production when transfected into PK15 cells and inhibited FMDV replication as analysed by qRT-PCR. FMDV 3' UTR transfected PK15 cells were infected with 0.1 pfu/cell FMD type O virus. Viral RNA in the infected cells was quantified by qRT-PCR. Inhibition of viral replication in the cells pre-transfected with UTR RNA was observed. Further, UTR pcDNA plasmid was able to induce IFN α production when transfected into PK15 cells and inhibited FMDV replication as analyzed by qRT-PCR.

The sequence coding for IFN λ was cloned into pShuttle 2, an adenoviral transfer vector, and the presence of the insert was confirmed by PCR. The linearised vector along with the linearised adenovirus was transfected into HEK 293 cells. Recombinant adenovirus with IFN λ was isolated and confirmed by PCR. The recombinant adenovirus expressing IFN λ will be tested for its therapeutic potential.

Small interfering RNA based therapeutics

Small interference RNA (siRNA) is a rapid and effective antiviral approach where double-stranded RNA induces the homology-dependent degradation of cognate mRNA. FMDV 3C protease (3Cpro) is the principal enzyme involved in processing the precursor to a proteome of virus, thereby making it a very attractive target for the design of antivirals. By using bioinformatics computer programs, all FMDV genome sequences in public-domain databases were analysed. Based on the results of homology analysis, four specific siRNAs targeting the conserved 3C region of FMDV were prepared and four siRNA-pSIREN expression vectors under the control of the U6 promoter were constructed. pCDNA3C FMDV and pCDNA 3CFMDV-GFP marker clones were also produced to evaluate the gene silencing efficiency using real-time qRT-PCR, and showed a 4-fold reduction of 3C transcripts in BHK-21 cells. On further analysis of four siRNA's, Si1 and Si2 showed a 100-fold decrease in viral quantity compared to the virus control after 12 hr infection with 1 moi FMDV O. The results demonstrated that transfection of BHK-21 cells with siRNA-expressing plasmids significantly decreased the FMDV O titre. A 3-fold reduction in viral growth was observed when BHK-21 cells were transfected with anti-FMDV siRNAs and treated with 50% tissue culture infective dose ($TCID_{50}$) of FMDV O, compared to control cells.

3ABC antigen based indirect ELISA and a rapid test kit for FMD DIVA

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FMD is major livestock disease of transboundary importance affecting cloven-hoofed animals including cattle, pigs, goats and sheep. Tests based on assaying non-structural proteins (NSP) antibodies in animals, particularly targeting the 3ABC antigen, have been widely used for differentiating infected from vaccinated animals. In our laboratory, an in-house, indirect ELISA was developed for application in multiple species. This is an improvement over the earlier reported ELISA system that was species specific. The test uses recombinant 3ABC protein expressed in insect cells using a baculovirus system. The assay has been validated by testing serum samples representing an endemic situation. Its performance was compared with a similar commercial kit.

Though highly sensitive, use of ELISAs requires trained personnel to perform and interpret the test results. Tests that don't need sophisticated instruments and trained manpower are a valuable addition to the available tools for diagnosis of FMD infected animals under field conditions. Keeping this in mind, the institute, in collaboration with M/s ubio Biotechnology, India, developed a rapid test for detection of NSP antibodies. The lateral flow device (LFD) test has the advantage that it does not require cold chain for transport or storage, and results can be read with the naked eye without the need for instruments and trained personnel. This pen-side diagnostic can be performed in 10–15 minutes by veterinarians or farmers.

Rapid LFD Test performance

The LFD assay was compared with an indirect ELISA using a panel of known positive and known negative sera available in the lab. Diagnostic sensitivity and specificity were found to be 87.4 and 97.4, respectively, in comparison to the ELISA.

Sera collected from multiple vaccinated animals that received double the recommended dose of vaccine were also tested to verify the NSP content in the vaccine used. Bull calves received three doses of vaccine within a 2 month period and sera were collected 28 d after the last booster. About 60% of these animals showed reactivity in ELISA while the LFD test showed positivity in 27% animals. The vaccine used here was not certified to be free from NSP contaminants.

The assay has been optimised to screen different FMD target species of animals including sheep, goat, cattle and buffalo, to validate the assay compatibility using either blood or serum (Figure 1). Both the diagnostics have been transferred to M/s ubio Biotechnology, Kochin for commercial scale up. The kit is marketed at a price of less than 1 USD per sample in India by M/s Ubio Biotechnology.

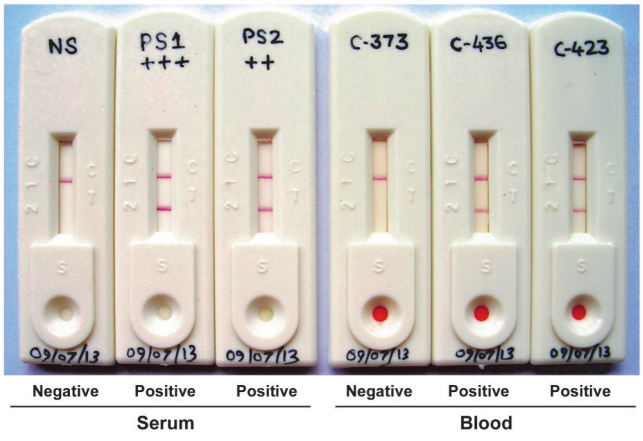

Figure 1: *The test is useful to detect NSP antibodies from either serum, plasma or blood. Blood samples collected from experimentally infected bull calves can be directly applied in the LFD assay. Whole blood collected with anticoagulant in a tube or fresh blood collected from ear puncture (using a lancet) can be collected in a dropper and applied after dilution in the diluent tube provided.*

Realising the potential of simple molecular tools for field diagnosis of foot-and-mouth disease: A report from the field

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Accurate, timely diagnosis is essential for control, monitoring and eradication of FMD. Currently, samples are tested at reference laboratories: a lengthy process which delays critical decision making. Reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) and mobile rRT-PCR provide a realistic option for rapid, sensitive, *in situ* detection. Having adapted [1] and validated a lyophilised RT-LAMP (visualisation by fluorescence or molecular LFD) and lyophilised rRT-PCR [2] in the laboratory, we travelled to Nakuru, Kenya (as part of the EuFMD Commission 'real time' FMDV training course) in November 2013 and Morogoro, Tanzania in June 2014 for the start of field trials.

During the trip to Nakuru, a concordance study was performed *in situ* between the lyophilised mobile rRT-PCR assay (using the Enigma® FL) and antigen LFDs (Ag-LFD). Six cattle, at different stages of clinical FMDV infection, were sampled for epithelium, serum and vesicular fluid. Epithelial samples were processed using antigen extraction kits (Svanova, Sweden) before adding to the mobile rRT-PCR assay; serum and vesicular fluid were added neat. The rRT-PCR correctly detected FMDV in the clinical samples and showed increased analytical sensitivity comparatively to Ag-LFDs. It was of priority throughout the trip to ensure that the Nakuru team were engaged with our activities and were provided with opportunities to partake in the work (Figure 1).

Figure 1: *a) FMDV infected cattle in Nakuru, b) the mobile laboratory, c) Eunice Chepkwony setting up the Enigma® FL*

Figure 2 : *Training the Sokoine staff how to interpret molecular LFDs and Ag-LFDs*

The beginning of the trip to Tanzania (June 2014) was spent in engagement activities with members of Sokoine University staff, particularly focusing on technology transfer of molecular field-based equipment (Enigma® FL for rRT-PCR and Genie® II for RT-LAMP). Training was provided in sample preparation, FMDV detection platforms and result analysis (Figure 2). During this time we tested the Genie® II, molecular LFDs, Enigma® FL and Ag-LFDs on thirteen archived epithelial suspensions, which had been previously characterised as positive or negative for FMDV by rRT-PCR (Tanzania Veterinary Laboratory Agency). Epithelial suspensions were processed by dilution in nuclease free water before adding to RT-LAMP; suspensions were run neat on the Enigma® FL. High concordance was seen between RT-LAMP, molecular LFDs (RT-LAMP-LFD's) and rRT-PCR. Ag-LFDs consistently displayed lower analytical sensitivity compared to RT-LAMP-LFD and rRT-PCR. No amplification was detected in any of the known negative samples.

We then began field trials at two Maasai farms (Morogoro, Tanzania). No acute FMD infection was encountered, therefore eight Ankole crossbred cattle, known to have been infected with FMD one month previously, were selected and sampled for oesophageal-pharyngeal (OP) fluid and serum. Samples were diluted in nuclease free water before adding to RT-LAMP/RT-LAMP-LFD and run neat on rRT-PCR (Figure 3). Good concordance was shown between the Genie® II, molecular LFD, Ag LFD and the Enigma® FL, with FMDV detected in the OP fluid of two cattle. For RT-LAMP/RT-LAMP-LFD, results were obtained in less than 30 minutes from OP collection to result calling; melt curve analysis confirmed LAMP amplicons to be FMDV specific. No FMDV was detected in serum samples.

Figure 3: *a) OP fluid sample preparation with the Maasai, b) Setting up the Genie® II, c) Setting up the Enigma® FL*

Our last day in Tanzania was spent at the Tanzanian Veterinary Laboratory Agency (TVLA), providing a training session in methods including Ag LFDs, rRT-PCR and RT-LAMP/RT-LAMP-LFD (Figure 3). To demonstrate the techniques, four known FMDV positive epithelial were assayed using Ag-LFDs, Genie® II and Enigma® FL. High concordance was seen between all platforms.

The ability of RT-LAMP/RT-LAMP-LFD and mobile rRT-PCR to utilise simple sample preparation, amplification and detection methods offers promise for rapid *in situ* FMD diagnosis. Further trials are planned for testing RT-LAMP on other clinical sample types and acutely infected animals *in situ* in the Serengeti, Tanzania in September.

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***Figure 4: a**) Demonstration of field-based molecular tests, b) Staff from TVLA and The Pirbright Institute*

Articles of Interest

Influence of antibodies transferred by colostrum in the immune responses of calves to current foot-and-mouth disease vaccines. Bucafusco D, Di Giacomo S, Pega J, Juncos MS, Schammas JM, Pérez-Filgueira M, Capozzo AV. Vaccine. 2014 Jun 23. pii: S0264-410X(14)00857-3. doi: 10.1016/j.vaccine.2014.06.056.

Infection dynamics of foot-and-mouth disease virus in pigs using two novel simulated-natural inoculation methods. Stenfeldt C, Pacheco JM, Rodriguez LL, Arzt J. Res Vet Sci. 2014 Apr;96(2):396-405. doi: 10.1016/j.rvsc.2014.01.009.

Transmission of foot-and-mouth disease virus from experimentally infected Indian buffalo (Bubalus bubalis) to in-contact naïve and vaccinated Indian buffalo and cattle. Madhanmohan M, Yuvaraj S, Nagendrakumar SB, Srinivasan VA, Gubbins S, Paton DJ, Parida S. Vaccine. 2014 May 14. pii: S0264-410X(14)00485-X. doi: 10.1016/j.vaccine.2014.03.094.

In vitro surrogate models to aid in the development of antivirals for the containment of foot-and-mouth disease outbreaks. Osiceanu AM, Murao LE, Kollanur D, Swinnen J, De Vleeschauwer AR, Lefebvre DJ, De Clercq K, Neyts J, Goris N. Antiviral Res. 2014 May;105:59-63. doi: 10.1016/j.antiviral.2014.02.009.

Application of baculovirus expressed virus-like particles in liquid-phase blocking ELISA for sero-monitoring vaccinated animals

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Liquid-phase blocking enzyme-linked immunosorbent assays (LPB-E) are accepted tests for post-vaccination monitoring. The current method of LPB-E utilises chemically inactivated FMDV as the diagnostic antigen. However, this practice is risky due to the need for handling live FMDV during antigen preparation.

Empty virus-like particles (VLPs) generated by baculovirus expression systems retain the antigenicity of the whole virus antigen. Considering the fact that LPB-E is extensively employed for sero-monitoring in FMD control programmes, the baculovirus expressed empty capsid antigens are valuable reagents. The developed technology would make consistent antigen availability without batch to batch variation. Further, the non-infectious nature of empty VLPs may widen the acceptability of empty capsid reagents for diagnostic assays across geographical barriers. We have demonstrated that the test performance of VLP based LPB-E was comparable with conventional LPB-E, qualitatively as well as quantitatively, thus emphasising the diagnostic application of recombinant FMDV VLPs. Analysis of the LPB-E results showed that titres obtained using empty capsid antigen were significantly similar with those obtained by inactivated virus antigen. The recombinant empty capsid antigen when evaluated against sera with a broad range of antibody titres, from low to high, showed good correlation with inactivated virus antigen.

***Table:** Comparative performance of empty-capsid-antigen-based LPB-E relative to inactivated-virus-antigen-based LPB-E with sera collected from unvaccinated and vaccinated cattle*

Type of cattle serum	Number of samples	Empty capsid antigen LPBE		Inactivated virus antigen LPBE	
		Titre (Log_{10}) ≤1.5	Titre(Log_{10}) ≥1.8	Titre (Log_{10}) ≤1.5	Titre(Log_{10}) ≥1.8
Unvaccinated	25	25	0	25	0
Vaccinated	127	8	119	10	117
Total	152	33	119	35	117

Baculovirus expressed VLPs of FMDV Type O as new generation antigen

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Vaccination is a well-accepted strategy for control of FMD in endemic countries. Currently, chemically inactivated virus antigens are used for preparation of FMD vaccine. To develop a non-infectious and safe recombinant vaccine, we expressed the structural polypeptide of FMDV (O/IND/R2/75) using a baculovirus expression system.

We showed that inclusion of mutated viral 3C protease in frame with the polypeptide (P1-2A) enhanced the yield of structural proteins. The structural proteins retained antigenicity and assembled into empty virus-like particles (VLPs). Immunisation of guinea pigs with purified fractions of the VLPs resulted in comparable humoral and relatively higher cell-mediated immune responses by 4 weeks, when compared to conventional vaccine in guinea pigs. Further, up to 70% of the VLP immunised guinea pigs were protected against challenge with homologous guinea pig-adapted virus. Our results highlight the application of recombinant FMDV VLPs in FMD vaccination.

Baculovirus-expressed FMDV O VLPs reacted in a sandwich-ELISA indicating antigenicity which could be exploited further for use as an alternative antigen in diagnostic assays.

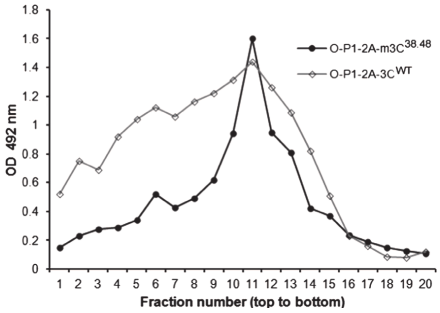

Figure: *Result of sandwich ELISA showing reactivity of purified fractions of baculovirus expressed proteins, with homologous anti-146S sera.*

Table: *Protection in guinea pigs immunised with either inactivated conventional vaccine or capsid antigens derived from recombinant baculoviruses, upon challenge with FMDV type O (1000 $GPID_{50}$) on 28 dpv.*

Group	No. of guinea pigs without clinical disease/challenged	% protection
Control (PBS)	0/10	0
Conventional vaccine	9/10	90
O-P1-2A-3CWT	5/8	62
O-P1-2A-m3CG38S F48S	7/10	70

Development and evaluation of adenovirus vectored FMD vaccine using Indian vaccine strains of FMDV

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FMD is considered a major threat to animal health globally. Inactivated vaccine is being used worldwide for the control of the disease. New vaccine platforms have also been explored in the last few years with the human adenovirus 5 vector (Ad5) platform being the most successful molecular vaccine to date. One such vaccine developed by scientists of USDA has been licensed for manufacture.

In order to test the efficacy of Ad5-FMD vaccines in endemic settings like India, a collaborative project was initiated between the Indian Veterinary Research Institute Bangalore, Project Directorate on FMD, Mukteswar and Plum Island Animal Disease Center, New York. Using the adenovirus vector platform developed by USDA, recombinant Ad5-FMD constructs expressing capsid proteins of Indian FMD vaccine strains (O IND R2/75, A IND 40/2000 and Asia 1 IND 63/72) were made. The Western blot experiments confirmed that all the recombinants tested were expressing FMDV capsid proteins. The recombinant viruses were also tested for FMDV type specificity using sandwich ELISA (Fig.1 & 2).

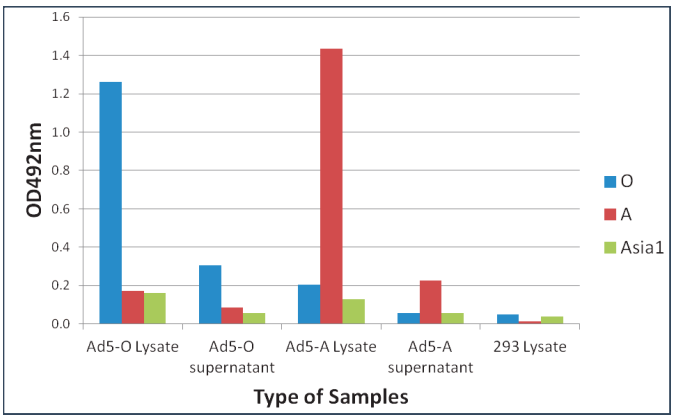

Figure 1: *Confirmation of FMDV type O and A capsid expression by Sandwich-ELISA*

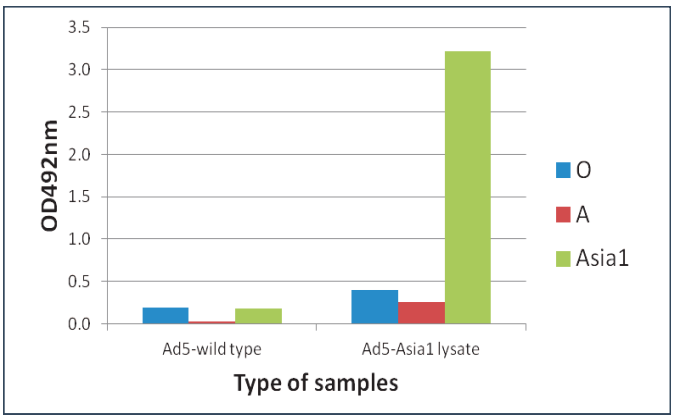

Figure 2: *Confirmation of FMDV Asia-1 capsid expression by Sandwich-ELISA*

Vaccination trial in cattle

The proof of concept vaccination trial in Indian cattle was carried out as follows:
Six months to one year-old indigenous breed male calves purchased from a local animal market were screened for antibodies against three serotypes of FMD virus (O, A and Asia1) and hAd5 virus. Animals that tested negative (<0.9 $\log_{10}$) for FMD and hAd5 antibodies were used for a vaccination trial in a six animals per group format. The animals were inoculated subcutaneously with Ad5-FMDV monovalent or Ad5-FMDV trivalent vaccine in 2 ml phosphate buffered saline (PBS). Ad5-Blue virus was used as a vector control and inactivated oil adjuvanted trivalent vaccine obtained from a commercial vaccine manufacturer was used as positive vaccine control (inoculated intramuscularly). Monovalent Ad5-FMD vaccine groups were inoculated with $5x10^9$ pfu per animal. Trivalent Ad5-FMD vaccine was prepared by mixing $5x10^9$ pfu of each of the monovalent vaccine in a final volume of 2ml per dose. Vaccines were inoculated at two sites on either side of the neck. All the animals used in the experiment were co-housed.

To assess the humoral immune response in the vaccinated animals, post-vaccinate sera was collected on days 7, 14, 28 and 63. Serum samples were collected on 14 and 28 days after booster vaccination. Neutralising antibody titres were estimated by virus neutralisation test as well as liquid phase blocking ELISA (LPBE).

The results of the cattle immunisation study can be summarised as follows:

- In the monovalent Ad5-FMD type O group, five of six animals (83%) sero-converted. Out of five responders, peak antibody titres ($\log_{10}SN_{50}$) were 2.1 in two animals and in the range of 1.36 to 1.65 in the remaining three animals. Except one animal which maintained peak titre of 2.1, antibody titres declined in all the responders and by week 9, titres declined below 1.65 (Fig.3). In LPBE, all six animals (100%) showed detectable antibody titres with peaks ranging from 1.65 to 2.56.

- In the monovalent Ad5-FMD type A group, four of six animals (67%) developed a specific antibody response. These four responders showed peak antibody titres ($\log_{10}SN_{50}$) of 1.5, 1.65, 1.8 and 2.4. Except one animal which maintained peak titre of 1.95, antibody titres declined in all the responders and by week 9, titres declined below 1.65 (Fig.4). In LPBE, three out of six animals (50%) showed detectable antibody titres with peak titres ranging from 1.65 to 2.26.

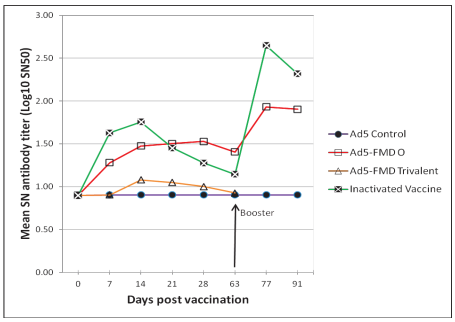

Figure 3: *Mean neutralising antibody titre in Ad5-FMD (type O) vaccinated cattle*

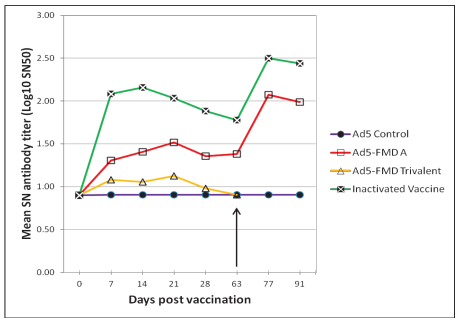

Figure 4: *Mean neutralising antibody titre in Ad5-FMD (type A) vaccinated cattle*

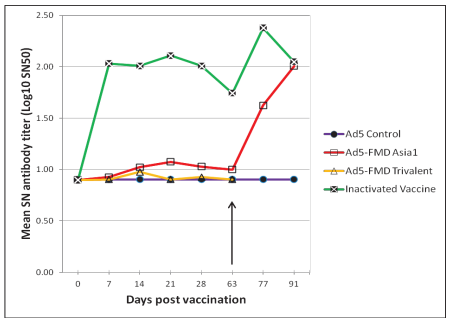

Figure 5: *Mean neutralising antibody titre in Ad5-FMD (type Asia-1) vaccinated cattle*

- In the monovalent Ad5-FMD type Asia-1 group, two of six animals (33%) developed a specific antibody response with a peak antibody titre ($\log_{10}SN_{50}$) of 1.36. Except one animal which maintained a peak titre of 1.95, antibody titres declined in all the responders and by week 9, titres declined below 1.65 (Fig.5). In LPBE, five of six animals (83%) showed detectable titres with a peak titre ranging from 1.65 to 1.96.

- In the trivalent Ad5-FMD group, detectable levels of antibodies were found in one of six animals (16%) for type O (Fig.3) and two of six animals (33%) for type A (Fig.4). None of the animals responded for type Asia-1 (Fig.5). By LPBE test, a titre of ≥1.65 was detected in four of six animals (67%) for type O, two of six animals (33%) for type A and three of six animals (50%) for type Asia-1.

- All animals vaccinated with standard inactivated trivalent vaccine sero-converted against all the three types of FMDV (Fig. 3, 4 & 5).

- Following booster immunisation in monovalent Ad5-FMD groups and inactivated vaccine groups, a significant increase in neutralising antibody response was observed when sera collected on days 14 and 28 were tested by VNT (Fig.3, 4 & 5) and LPBE.

From the observations, we can conclude that:

- The vaccination trial in Indian cattle showed that after primary immunisation, the animals responded comparatively better in monovalent Ad5-FMD O & A groups than Ad5-FMD Asia-1.
- Trivalent Ad5-FMD vaccine did not induce significant antibody responses compared to monovalent Ad5-FMD vaccines.
- Neutralising antibody titres increased significantly after booster immunisation.
- Ad5-FMD vaccinated cattle had lower titres than the animals immunised with inactivated oil adjuvanted trivalent FMD vaccine.
- Neutralising antibodies against Ad5V appear to peak early and decline drastically (Fig.6).

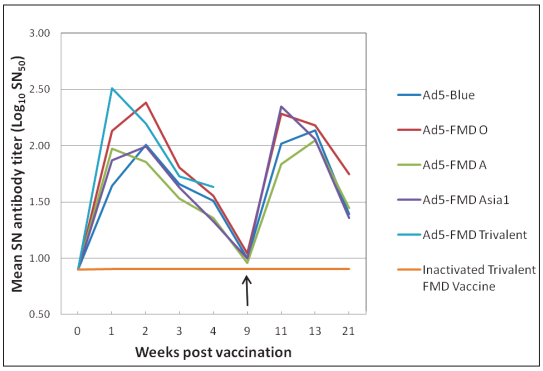

Figure 6: *Mean neutralising antibody response against Ad5 virus in cattle following vaccination with recombinant Ad5-FMD viruses*

Establishing critical diagnostic capability for FMD in deer

Collaborative project between IDC (New Zealand) and the NCFAD (Canada)

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Introduction

FMD has never occurred in New Zealand and an incursion would have a major economic impact on the country, with agriculture being an important contributor to the economy. Whilst most of New Zealand's agriculture involves sheep (30 million) and cattle (10 million) farming, deer farming is also important. More than 1 million deer are farmed, predominantly red deer (*Cervus elaphus elaphus*). There is also a substantial feral deer population present. Furthermore, deer and other livestock species are often farmed alongside each other, which may lead to spread of the virus between them.

It has been reported that FMDV infected deer often do not show clinical signs[1)]. This means that laboratory testing is required in order to identify infected deer during an incursion and the subsequent proof-of-freedom phase. A large number of test methods for FMD are available[2)] but their appropriateness for deer is currently unknown, as these tests had been developed for other livestock species, namely cattle, sheep, goats and pigs. The aim of this project was to evaluate a number of FMD test methods for use in red deer.

The FMD in Deer Project is a collaborative project between the Animal Health Laboratory (AHL), Ministry for Primary Industries (MPI), New Zealand, and the National Centre for Foreign Animal Disease (NCFAD), Canadian Food Inspection Agency (CFIA), Winnipeg, Canada. The project consists of two components: the Winnipeg Component and the New Zealand Component.

Winnipeg Component

The Winnipeg Component was completed in November/December 2013. Ten red deer were experimentally infected with an O-strain FMDV. Samples were regularly collected over a 4 week period: nasal swabs, oral swabs, probang samples, whole blood and serum. In addition, lesion samples were collected. Samples were tested by IRES and 3D real-time RT-PCR, virus isolation, virus neutralisation test (VNT), two types of SP O ELISAs, four types of NSP ELISAs and an NSP antibody penside test. Some samples were also tested by two antigen penside tests and in the O-serotype antigen ELISA. Experimental infection was the only way to obtain samples from infected red deer, as field samples do not exist worldwide.

New Zealand Component

In the New Zealand Component, which is currently underway at the AHL, the diagnostic specificity will be determined for those test methods that performed well with experimentally infected animals. A total of 1000 serum samples will be tested in SP O- and NSP ELISAs and a penside test, and another 200 serum samples with matching nasal swabs will be tested in both RT-PCR assays. Furthermore, with inactivated serum samples obtained from the experimentally infected animals from the NCFAD, another SP O ELISA that has recently become available will be evaluated at the AHL. In addition, a proportion of the RNA samples from Winnipeg will be used for evaluating sensitivity and specificity in AHL's own RT-PCR assays.

Findings and Discussion

Not unexpectedly, one outcome of this project was that deer are difficult to infect[3,4)]. Of the 10 experimentally infected red deer, only one animal became clinically affected, despite a relatively high viral dose used for inoculation. Six more animals developed a humoral immune response after re-inoculation. At this stage, it can be said that one of the commercial SP O ELISAs and one of the commercial NSP ELISAs performed comparably well to the reference methods used at the NCFAD, which were virus isolation, VNT, and the two in-house antibody ELISAs, an NSP ELISA and an SP O ELISA. Both RT-PCR assays performed well. We would like to emphasise that not every commercially available test method worked satisfactorily for red deer.

Detailed results of this project will be published in due course in a scientific journal.

Acknowledgments

This project is jointly funded by MPI Operational Research and the CFIA. We would like to thank all staff involved at the NCFAD, Winnipeg, for their excellent work during the experimental infection of animals. A special thank-you goes to the New Zealand veterinarians who collected samples at slaughter houses throughout New Zealand. We are very grateful to all staff at the AHL who are performing the testing of New Zealand samples and samples received from the NCFAD. The advice and collaboration given by the project team at the Investigation and Diagnostic Centre, Wallaceville, New Zealand is much appreciated.

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Cellular bases of the development of the anti-FMDV local antibody response in cattle parenterally vaccinated and infected by aerosol route

Collaborative project between PIADC-ARS (USA) and the Institute of Virology-INTA (Argentina)

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FMD has been consistently considered a major threat on a worldwide scale for livestock industries and international trade of meat and meat derivatives. Work proposed here is aimed at addressing current gaps in basic knowledge related to immunity to FMDV in cattle, focusing on the mechanisms underlying the induction of memory responses by FMD vaccination upon infection via the oronasal route and its impact on the further protection to the disease.

Research conducted in collaboration between PIADC and the Institute of Virology at INTA, have clearly indicated that primary immune reactions after oronasal infection or parenteral vaccination generate antibody responses at mucosal and systemic levels (J Virol **87:**2489-2495). Similarly, induction of secondary responses in FMDV vaccinated cattle after aerogenous infection is evident at both systemic and local levels. However, vaccination/infection-related factors (viral antigen persistence, recruiting signals, etc) that trigger this FMDV-specific B-cell anamnesis, as well as its origin and time-course, remain unclear. Whether the vaccine antigen reached the local lymph tissues (alone or associated to antigen-presenting cells) or if FMDV-specific B-lymphocytes migrated from the vaccination site to other lymphoid tissues is also unknown. In the same way, contribution to protection of these mucosal FMDV-specific antibody responses is yet to be elucidated. We hypothesise that parenteral vaccination promotes generation of resident B-cell memory cells in mucosal lymph nodes and that the induction of local secondary responses at the respiratory tract of vaccinated cattle after oronasal infection play a role in preventing the virus to gain access to blood stream and generalise the infection to secondary replication sites in foot (and other) epithelial tissues.

The Foot-and-Mouth Disease Risk Management Project – AAHL, Australia

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Australian Animal Health Laboratory, Geelong, Australia

Australia is in the enviable position that it has not suffered an outbreak of FMD since 1872. This freedom from disease, together with the absence of other diseases such as BSE, has provided the country with trade advantages worth billions of dollars. In fact, it is estimated that a widespread outbreak of FMD could lead to losses of up to 50 billion Australian dollars over a 10 year period, mostly due to trade embargoes and market losses. FMD is therefore a severe risk that warrants investment in risk mitigation and research. However, despite the presence of a national state-of-the-art high containment facility, the risk of working with live FMD virus is deemed too high, and any such research has to be done offshore in collaboration with partners. The FMD Risk Management Project, started in 2010, is funded by Meat and Livestock Australia and Animal Health Australia with support from all major livestock industries, and is managed by the CSIRO-Australian Animal Health Laboratory (AAHL). AAHL has relied heavily on the GFRA partners to perform a number of vaccine efficacy studies using vaccine strains held in the Australian vaccine bank against viruses currently circulating in South East Asia, using cattle, sheep and pigs. The outputs from this project aims to ensure the Australian livestock industries, and the Australian community, will be better prepared to minimise the inevitable disruption to trade that would be caused by an outbreak of FMD.

Some of the highlights of these collaborations include testing a newly developed vaccine developed from the 2010 outbreak virus in South Korea in collaboration with Merial and The Pirbright Institute. This vaccine was compared with O1 Manisa for efficacy in cattle and pigs. In collaboration with the National Centre for Foreign Animal Diseases, Canada, we tested the protective capacity of high potency vaccination (>6 PD_{50}) with O1 Manisa 4 days prior to challenge with O/SKR/2010 by direct contact with infected sheep. A second trial was of similar design but used the A22 Iraq vaccine and the A/VIT/15/2012 strain as challenge. Further studies were done in sheep at the Plum Island Animal Diseases Centre, USA where different routes of infection (intra-nasopharyngeal instillation, aerosol exposure, coronary band inoculation and direct contact) and different vaccine doses were tested using the O1 Manisa vaccine and challenging with O/SKR/2010. Although not a GFRA partner, collaborative studies were done in Vietnam as part of capacity building and also to introduce GFRA activities to the region. The O1 Manisa and A Malaysia 97 vaccines were tested against the type O and A viruses, respectively, currently circulating in SEA by vaccinating pigs and challenging at early time points post-vaccination. In addition, the experiments were designed to determine whether vaccination lowers virus excretion thereby preventing infection of pigs in close, but not direct contact with vaccinated and infected animals. As part of the vaccine efficacy testing in pigs, the use of cotton ropes as a diagnostic tool for FMDV in pigs was investigated (Vosloo *et al.* Transbound Emerg Dis. 2013 Dec 11. doi: 10.1111/tbed.12196.). The ease in which oral fluid samples from ropes were collected and extracted makes rope sampling an extremely useful, non-invasive method of sample collection that may complement FMD monitoring efforts in pig populations. Upcoming collaborations are currently planned with the Central Veterinary Institute, the Netherlands, and SENASA, Argentina, to examine vaccine efficacy of serotype O and A viruses in cattle.

GFRA has ensured that FMD research could continue for Australia, if not in country, then abroad where resources could be shared. It is AAHL's vision that these collaborative projects will continue into the future and that the FMD field at large will benefit more and more from collaborations such as these.

Viral determinants of pathogenesis and virulence of FMDV

Collaborative project between PIADC-ARS (USA), the CBMSO (Spain) and the Institute of Biotechnology-INTA (Argentina)

Mariano Perez-Filgueira

INTA, Institute of Virology, Buenos Aires, Argentina

The FMDV strains A/Argentina/2000 (A/Arg/00) and A/Argentina/2001 (A/Arg/01) were isolated from infected bovines during the 2000–2001 FMDV epidemics that occurred in Argentina. While strain A/Arg/00 was said to produce mild lesions in the affected animals, strain A/Arg/01 caused severe lesions in cattle in the field as well as in experimental challenge trials. In order to identify viral genetic determinants of the differential virulence displayed by both FMDV isolates, a molecular clone of A/Arg/01 strain and viral chimeras containing the S-fragment or the internal ribosome entry site (IRES) of A/Arg/00 in the A/Arg/01 backbone were constructed (A2001clone) and characterised in terms of plaque size and replication kinetics in cell culture. The IRES appeared as a determining factor of the lower level of A/Arg/00 replication in BHK-21 cells. High-throughput RNA probing revealed structural differences between both IRESs, which in turn were differentially modulated by the viral 3´untranslated region (3´UTR), as determined by *in vitro* and *in vivo* translation experiments. These data support a role of the IRES-3'UTR modulation in determining the level of FMDV replication in field strains (García Núñez et al., Virology 448:303-313, 2014).

More recently, we demonstrated that FMDV strain A/Arg/01 was more pathogenic than A2001clone in mice, in spite of the fact that these viruses shared similar growth properties in cell culture. Sequence comparison studies showed 6 amino acid changes in the polyprotein (in viral proteins VP2, VP1 and 2C) and 26 synonymous changes throughout the viral genome between the parental FMDV strain and A2001clone. Experiments are in progress in order to evaluate the role of each amino acid change on the lethality of FMDV in adult mice.

FMDV ecology in South East Asia

Collaborative project between DAH-Vietnam and ARS-USDA-Plum Island

Jonathan Arzt & Luis Rodriguez

Foreign Animal Disease Research Unit, Agricultural Research Service, Plum Island Animal Disease Center, United States Department of Agriculture

In 2011, a unique collaboration was established between two GFRA partners: the Vietnam Department of Animal Health (DAH) and the Agricultural Research Service (ARS) at Plum Island Animal Disease Center, USDA. The project entitled "Molecular epidemiology, surveillance, and predictive tools for FMD control in Vietnam" is aimed at elucidating the ecology of FMDV in Vietnam with emphasis on determining the potential role(s) of persistently infected cattle and Asian buffalo as carriers of FMDV and sources of new outbreaks. These goals were closely aligned with the core vision and mission of GFRA, as the activities clearly represented a global research partnership with the intention to contribute towards the progressive control and eradication of FMD. The establishment of this project was facilitated by another GFRA partner: the Australian Animal Health Laboratory, who were already working in Vietnam.

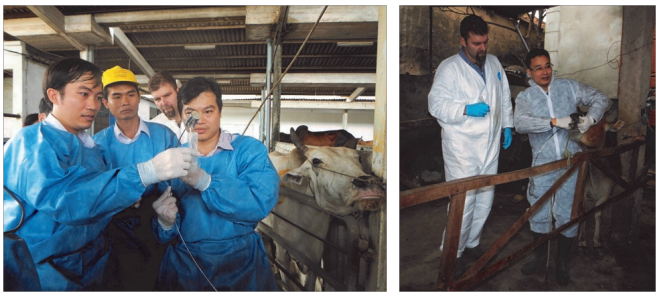

Figure 1: *Probang training sessions at slaughterhouses in Ho Chi Minh City (left) and near Hanoi (right)*

Scant resources were available to tackle this ambitious goal, but with the oversight and authority of DAH Headquarters, including Principal Investigator Dr. Do Huu Dung, and study design input from ARS-USDA, two teams of field investigators were assembled. One team would be led by Dr Nguyen Tung and based out of National Center for Veterinary Diagnostics (NCVD), Hanoi. The other team would be led by Dr. Ngo Thanh Long at Regional Animal Health Office No.6 (RAHO6) in Ho Chi Minh City. The teams would engage in activities that had overlapping themes, but some operational differences.

Both teams set out to conduct targeted surveillance in regions of known recent outbreaks. The goal was to identify carriers for various downstream research activities. The NCVD team focused on buffalo which are present in greater abundance in northern provinces whereas the RAHO6 team focused on cattle. Confirmed carriers were enrolled in longitudinal analyses, transmission studies, and/or necropsy studies. Although data analysis is still ongoing, initial results demonstrated important differences in disease occurrence and circulating FMDV strains between locations in northern and southern Vietnam.

Figure 2: *Two donor-sentinel pairs from longitudinal transmission study*

Various forms of data from these activities are intended to be available in a handful of manuscripts and meetings in coming months and years. But, in short, the collaboration has been a great success in various manners. Most importantly, the relationship between the two institutions has been established and fortified to facilitate ongoing and continuing collaboration. Joint design and execution of the sub-projects by DAH and ARS scientists was the foundation for the relationship; but, the highly successful output delivery cemented the positive experience. The productivity of this new relationship is reflected in a newly awarded grant to support another four years of follow-up research. Additionally, several new technologies were delivered from ARS to DAH which have now been expertly incorporated into routine diagnostics and research practices. These new practices combined with the dedication of DAH and ARS scientists ensure the future success and productivity of this partnership.

Figure 3: *Carrier buffalo in Lang Son province*

LAB PROFILES

National Centres for Animal Disease, Canadian Food Inspection Agency Winnipeg-Manitoba and Lethbridge-Alberta, Canada

Soren Alexandersen

In the past year, the National Centres for Animal Disease (NCAD, Winnipeg and Lethbridge) have focused their efforts on developing and/or improving specific FMDV diagnostic tests. In a recent study, a panel of monoclonal antibodies (mAbs) was produced and used in an antigen detection ELISA for the characterisation of new isolates of FMDV serotype O. The mAbs were characterised using peptide array and mAb resistant mutant selection. Seven out of nine mAbs reacting with 5 known antigenic sites and two other non-neutralising mAbs were identified. The mAbs were then evaluated by antigen detection ELISA for the detection of 46 FMDV serotype O isolates representing 7 of 10 known topotypes. This panel of mAbs is useful for monitoring the emergence of antigenically distinct strains and determination of relevant antigenic site differences.

A panel of FMDV serotype A specific mAbs has also been developed. These were examined for their ability to compete with a polyclonal anti-serum from cattle experimentally inoculated with FMDV serotype A. The aim was to develop a sensitive and specific serotype A competitive ELISA. Two mAbs were selected and are currently being evaluated in the competitive ELISA.

Additionally, the mAbs generated at the NCAD have been used in the development of rapid lateral flow strip tests for pen-side diagnosis of FMDV and the development of a rapid immunoassay method for quantitatively measuring FMDV particles in infected cultures. Some mAbs have also been supplied to other laboratories for various purposes.

The NCAD is also investigating the feasibility and suitability of a poly-specific virus capture Apolipoprotein H-enzyme-linked immunosorbent assay (ApoH-ELISA) for detection of FMDV and other vesicular disease viruses. FMDV and SVDV particles were shown to bind to ApoH-coated plates based on real-time reverse transcription polymerase chain reaction amplification of RNA extracted from contents of the plate. Preliminary results show that using ApoH-ELISA for FMDV detection is comparable to the conventional FMDV antigen detection.

A multiplex assay for simultaneous detection of seven swine viruses (FMDV, SVDV, VESV, CSFV, ASFV, PCV2, PRRSV) and an FMDV typing assay were developed for a user-friendly electronic microarray system that integrates and automates capture probe printing, amplicon hybridization, washing and reporting. The user friendly multiplex assay successfully detected 60 strains of the seven targeted viruses, including 23 strains of FMDV representing all seven serotypes. No reactivity was observed with a panel of bacteria and viruses associated with livestock and oral swab material from healthy pigs. The assay was also able to detect the presence of viral nucleic acids of all seven viruses in clinical samples from experimentally infected animals or biological material inoculated with the target viruses. The FMDV typing assay accurately identified the serotype of 23 FMDV strains representing all seven serotypes. These assays are being adapted to new instrument platforms that can fully automate the entire work flow so that no user handling is required after sample introduction.

Other projects in the NCAD labs include enhancing the replication of FMDV in cell-lines by genetic engineering and development of multiplex bead-based assays for simultaneous diagnosis of vesicular diseases in swine.
The NCAD continues to collaborate with other laboratories within the GFRA. Specifically, there was collaboration with the Investigation and Diagnostic Centre, Ministry for Primary Industries, New Zealand on the evaluation of diagnostic tests for FMD in red deer. The multiplex assay for simultaneous detection of seven swine viruses was developed in collaboration with the Pirbright Institute, UK, the EU-Reference Laboratory for CSF, Germany and Plum Island Animal Disease Center, USA. There is ongoing collaboration with CSIRO, Australian Animal Health Laboratory on the evaluation of FMD vaccines in sheep. Reagents and samples generated at the NCAD have been shared with other laboratories, including APHIS/USDA, Laboratório Nacional Agropecuário, Brasil, and industry partners. A test method for FMD developed at the NCAD is in the process of being licensed.

CODA-CERVA, Belgium

David Lefebvre

New projects:

- 'Rapid Antiviral Containment of Epidemics of Classical Swine Fever and Foot-and Mouth Disease' (EraSME-RACE), started September 1st 2013, funded by the Flemish Agency for Innovation by Science and Technology, beneficiary Aratana NV, research partners CODA-CERVA and Synovo GmbH
- 'Mechanism of action and barrier to resistance of antiviral drugs for foot-and-mouth disease virus and classical and African swine fever virus' (ANTIVIRES), started January 1st 2014, funded by the Belgian Federal Public Service for Health, Food chain safety and Environment, beneficiary CODA-CERVA, research partners Aratana NV and the University of Leuven, Belgium

International collaborations:

- An OIE Twinning project between CODA-CERVA and the NVRI from Vom, Nigeria, was officially approved in June 2014 by the OIE.
- In July-August, CODA-CERVA will provide a 2-week training course to a person from BVI, Botswana in the framework of a bilateral collaboration on quality control of methods used for diagnosis and for vaccine evaluation.

Science reports:

Research articles specific for FMDV:

- A manuscript from CODA-CERVA entitled 'A refined Guinea pig model of foot-and-mouth disease virus infection for assessing the efficacy of antiviral compounds' by De Vleeschauwer et al. has been accepted in June 2014 for publication in the journal 'Transboundary and Emerging Diseases' (TBED).
- An article entitled 'In vitro surrogate models to aid in the development of antivirals for the containment of foot-and-mouth disease outbreaks' with CODA-CERVA as co-authors has been published very recently in the journal 'Antiviral Research' (Osiceanu et al., 2014 May;105:59-63).

- Another proof-of-concept article from CODA-CERVA for antiviral drugs in rodents was published online in 2013: Lefebvre et al., Proof-of-concept for the inhibition of foot-and-mouth disease virus replication by the antiviral drug 2'-C-methylcytidine in severe combined immunodeficient mice. Transbound Emerg Dis. 2013 Mar 11. doi: 10.1111/tbed.12069.

Research articles not specific for FMDV:

- An article entitled 'Application of a cell-based protease assay for testing inhibitors of picornavirus 3C proteases.' with CODA-CERVA as co-authors has been published very recently in the journal 'Antiviral Research' (van der Linden et al., 2014 Mar;103:17–24).
- An article from CODA-CERVA entitled 'False positive results in metagenomic virus discovery: a strong case for follow-up diagnosis' was recently published online: Rosseel et al., Transbound Emerg Dis. 2014 Jun 10. doi: 10.1111/tbed.12251.

INTA, Institute of Virology, Buenos Aires, Argentina

Alejandra V. Capozzo

Our laboratory studies immunological aspects of FMDV, BVDV and *Neospora caninum* infections in cattle. The group is composed of two post-docs, a laboratory manager and three PhD students. Regarding FMD, our research is now focussed in *solving practical issues related to the application of FMD vaccines in endemic settings*.

We are dedicated to three main research lines:

- New serological assessments
- Immunogenicity of current FMD vaccines in the presence of maternal immunity transferred through colostrum
- Simultaneous application of FMD and other important vaccines in cattle

New serological methods

We recognised that in endemic regions where resources are scarce, vaccine efficacy and coverage is not monitored correctly due to the impossibility of implementing currently used assays for serological testing and making their application sustainable over time. These assay are the virus neutralisation test (VNT), and the Liquid and Solid Phase Blocking ELISAs (LPBE and SPBE, respectively). Our contribution to this problem will be to provide new high-throughput tools that developing countries can easily implement for monitoring and evaluating vaccination programs.

These tools are four novel ELISAs, three of them are isotype ELISAs to titrate isotypes of specific antibodies (Ab) against FMDV: IgM, IgG1 and IgG2. The other assay is meant to measure specific Ab avidity. In two recently published studies we applied these techniques to the indirect evaluation of cross protection using type A strains (Lavoria et al Vaccine 2012 Nov 6; 30 (48): 6845. -50 doi: 10.1016/j.vaccine.2012.09.011; Brito et al, Vaccine 2014 Jan 16, 32 (4:433-6 doi: 10.1016/j.vaccine.2013.12.007). We demonstrated that the combined use of these techniques provides more accurate results than measurements made by VNT, the recommended standard technique.

Our current aim is to test how avidity and IgG-subtype ELISAs perform as indirect predictors of protection compared to currently used VNT, SPBE and LPBE. We are now working in collaboration with Dr. Francois Marée at the Onderstepoort Veterinary Institute (OVI) to apply these assays in the assessment of the immune responses induced by SAT-2 vaccination and challenge. It is important to note that we have already started the set-up of the assays at the OVI. Last year, I travelled to the OVI to set up the avidity ELISA using purified virus prepared by Dr Marée´s group and a different peroxidase substrate. Results obtained by the application of this ELISA, describing the avidity of different sera in epitope-replaced SAT-2 mutants, have already been published (Opperman et al J Virol. 2014 May 14. pi: JVI.00470-14). The avidity ELISA was set up in three weeks, meaning that this approach can be easily applied in any other country.

We are open to transfer these protocols and help in the set-up of the assays within each particular laboratory. This kind of joint project will provide information on the kinetics of total Abs, neutralising titres, isotypes and avidity maturation after vaccination, and the behavior of the immune responses measured by all serological assays after experimental infection of naive and vaccinated cattle. This basic information will provide a proof-of-principle on the potential of these assays in endemic settings.

Influence of colostrum in the immune responses of calves to FMD vaccine

The inactivated vaccine is currently applied to adult animals or young animals without maternal antibodies. It has been shown to be effective in these age groups, as inactivated vaccines are well-known to be unable to stimulate neonatal immunity in the presence of maternal antibodies; only cellular responses are obtained with low or no induction of specific antibodies. However, field and experimental data show contradictory results, while some reports show some inhibition by colostral antibodies, others found that purified FMD-oil vaccines are able to overcome the inhibitory effects of maternal antibodies inducing protective levels of antibodies in young animals.

We have recently published a study on the immune responses induced by vaccination and re-vaccination in calves with maternal antibodies (Mat-Abs). Calves with or without colostral antibodies were immunised with commercial FMD vaccine. Humoral responses were assessed, using traditional and novel techniques. We demonstrated that Mat-Abs interfered with the induction of virus neutralising responses, while total antibody assessment did not reveal the same magnitude of interference (Bucafusco et al. Vaccine. 2014 Jun 23. pii: S0264-410X(14)00857-3. doi: 10.1016/j.vaccine.2014.06.056). We are now presenting a paper at the EUFMD open session 2014, where we studied how the presence of maternal immune cells influences calves´ immune responses to vaccination.

Study the effect of simultaneous application of FMD vaccine together with other vaccines in cattle

FMD vaccination in Argentina and other regions within South America is performed under the control of the regulatory authorities and conducted by certified veterinarians. These organised campaigns may facilitate the controlled application of other vaccines against other endemic diseases. There is very little published information on the efficacy of FMD vaccines when co-administered with other vaccines, particularly live vaccines. We started evaluating the simultaneous application of the currently used commercial tetravalent oil FMD vaccine used in Argentina together with the anthrax live vaccine. We measured total antibodies by LPBE and also IgG isotypes. We demonstrated that the combined application of these vaccines did not interfere with the antibody FMDV-booster responses. Antibody titres were similar in both groups and followed comparable kinetics over time. We are now pursuing new studies with other vaccines of regional interest.

UPCOMING EVENTS

September 2014	**The Pirbright Institute Centenary Conference** Guildford United Kingdom, 11–12 September 2014
October 2014	**Open Session of the EuFMD** Cavtat, Croatia, 29–31 October 2014
November 2014	**Reference Lab Network Meeting** Brescia, Italy, 26–27 November 2014
2015	**GFRA Biennial Meeting** Vietnam, TBA

Want to know more?

The Global Foot-and-Mouth Disease Research Alliance (GFRA)

A worldwide association of animal health research organisations to assist the global control and eventual eradication of foot-and-mouth disease.

www.ars.usda.gov/gfra

CSIRO
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Global Foot-and-Mouth Disease Research Alliance

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