# Host-response to foot-and-mouth disease in cattle; possible implications for the development of persistently infected "carriers"

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DTU

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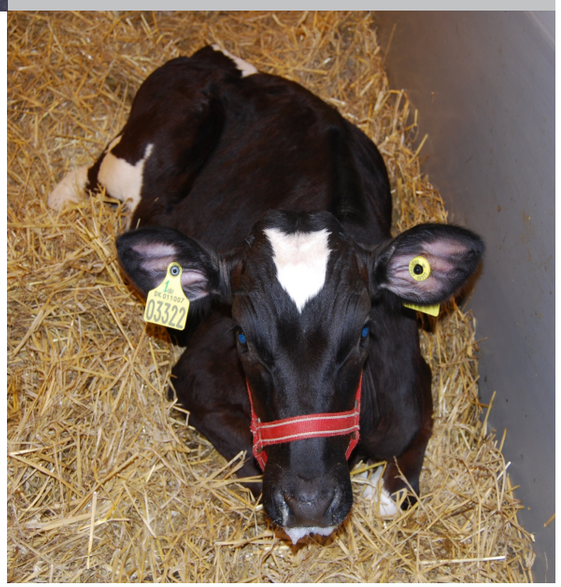

DTU Veterinary
National Veterinary Institute

# Host-response to foot-and-mouth disease in cattle; possible implications for the development of persistently infected “carriers”

**Ph.D. Thesis**

**Carolina Stenfeldt**

**2011**

National Veterinary Institute

Division of Virology

Technical University of Denmark

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# Preface

The work concluded in this thesis has been performed as part of a PhD project, co-funded by the Technical University of Denmark (DTU) and the Faculty of Life Sciences, Copenhagen University, through the Research School for Animal Production and Health (RAPH).
The EU network of excellence "EPIZONE" (FP6-2004-Food-3-A), workpackage 3.2 has contributed to the project by funding a "short term mission" with technical training at the Institute for Animal Health (IAH)-Pirbright in 2009. Drs Bryan Charleston and Nick Juleff are thanked for their assistance during this visit.
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Jeg vil endvidere takke min familie i Sverige for at I altid har støttet mine, ikke altid helt let-opnåelige, drømme og ambitioner.

Til sidst vil jeg rette en stor Tak til alle kollegaer ved DTU-Vet som har bidraget til at gøre min tid ved instituttet såvel underholdende som uforglemmelig.

Carolina Stenfeldt, Vordingborg, April 2011

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## List of abbreviations

| | |
|---|---|
| 3Cpro | 3C-protease |
| Ab | antibody |
| APC | antigen presenting cell |
| APP | acute phase protein |
| B-cell | bonemarrow-derived lymphocyte |
| BTY | Bovine thyroid |
| BVDV | bovine viral diarrohea virus |
| CAT | cloramphenicol transferase |
| CD4 | cluster of differentiation 4 |
| CD8 | cluster of differentiation 8 |
| DC | dendritic cell |
| DNA | deoxyribonucleic acid |
| DPI | days post infection |
| DSP | dorsal soft palate |
| EA-2 | East Africa-2 |
| EBV | Epstein-Barr virus |
| ELISA | enzyme-linked immunosorbent assay |
| Fc | fragment crystallizable |
| FMD | foot-and-mouth disease |
| FMDV | foot-and-mouth disease virus |
| FMDV O | foot-and-mouth disease virus serotype O |
| HCV | hepatitis C virus |
| HE | hematoxylin-eosin |
| HIV | human immunodeficiency virus |
| HP | haptoglobin |
| HPV | human papilloma virus |
| HTCV | human T-cell carcinoma virus |
| IFN | interferon |
| IRES | internal ribosomal entry site |
| Ig | immunoglobulin |

| | |
|---|---|
| kb | kilobases |
| Ln | lymph node |
| Lpro | leader protease |
| Mab | monoclonal antibody |
| MESA | Midle-East South-Asia |
| MHC | major histocompatibility complex |
| mRNA | messenger ribonucleic acid |
| Mx-protein | myxovirus resistance protein |
| NFκB | nuclear transcription factor kappa-B |
| NIPC | natural interferon producing cells |
| NK-cell | natural killer cell |
| OAS | oligoadenylate synthases |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PID | post-infection day |
| PRR | pattern recognizing receptor |
| RNA | ribonucleic acid |
| qRT-PCR | quantitative reverse transcriptase polymerase chain reaction |
| SAA | serum amyloid A |
| SAT | South African territories |
| T-cell | thymus-derived lymphocyte |
| $TCID_{50}$ | tissue culture infective dose 50% |
| $T_H$-cell | helper T-cell |
| TLR | toll-like receptor |
| TNF-α | tumor necrosis factor alpha |
| UTR | untranslated region |

# Summary


### *General purpose and objectives*

Foot-and-mouth disease (FMD) is a viral infection of implicit financial importance for countries, such as Denmark, which rely on a significant trade in agricultural products. The disease is highly contagious with rapid spread amongst susceptible animals, causing substantial economical implications for farmers and live-stock industries of affected countries. The occurrence of persistently infected, so called "carriers" of FMD-virus (FMDV) which may shed infectious virus for prolonged periods of time following exposure to the virus, causes significant complications for effective disease control.

The main purpose of this PhD-project has been to investigate the host response to FMD infection in cattle, with further objectives of elucidating any detectable differences in the measured immune response between animals that developed into FMDV carriers and those that did not.

### *Experimental studies*

The thesis is based on results obtained from seven separate animal experiments with FMDV serotype O, which have been performed at DTU-Vet, Lindholm. In five out of the six experiments that were performed in cattle, animals were infected with FMDV O UKG 34/2001, representing the virus isolate responsible for the FMD outbreak in the UK and northern Europe in 2001. One cattle experiment was performed with an FMDV serotype O isolated from samples collected from a cattle farm in Uganda during an outbreak in 2006, whilst one additional experiment was designed to investigate the clinical course of infection with FMDV O UKG 34/2001 in sheep.

An experimental study design involving endoscopical collection of small biopsies of pharyngeal mucosa from live cattle was developed. This technique enables collection of sequential tissue samples from infected animals, allowing investigation of the local tissue response to infection within this specific anatomical region of individual animals, at different time points following infection.

This sampling system was used to investigate the pathogenesis of FMD infection in cattle through quantification of the levels of FMDV RNA present within the pharyngeal epithelia during early infection. Similar analyses were performed on samples of pharyngeal epithelia

and associated lymph nodes collected during *post mortem* examinations performed at around 32-35 days post infection in order to investigate possible sites of virus persistence.

The early host response to FMDV O in cattle was investigated through measurements of systemic parameters consisting of the acute phase proteins, serum amyloid A (SAA) and haptoglobin (HP), as well as type 1 interferon (IFN). The local tissue response within the pharyngeal epithelia was investigated through measurements of mRNA levels of inflammatory cytokines in sequential biopsy samples.

***Structure of Thesis***

The first chapter contains general background information on the host response to virus infections, as well as characteristics of FMDV and the pathogenesis of the infection. Detailed aims and objectives of the project are stated at the end of chapter 1.

Chapter 2 contains overall descriptions of the animal experiments included in the project. The general concepts of the experimental procedures are described, as well as the clinical characteristics of infection caused by the two different FMDV O isolates in cattle. The clinical description of the experiment performed with FMDV O UKG 34/2001 in sheep includes results of measurements of viremia and the development of specific anti-FMDV O antibodies, as these results are not presented in the included manuscripts.

The third chapter of the thesis contains three manuscripts of research articles for publication in peer-reviewed scientific journals.

The first manuscript is based on serological measurements of the acute phase proteins SAA and HP, together with the bioactivity of type 1 IFN, in three out of the performed cattle experiments. Measurements of the systemic response to early infection with FMDV is related to the observed development of clinical signs of infection as well as the occurrence of viremia and development of anti-FMDV antibodies. Observed variations in the acute phase response of HP between carriers and non-carriers are discussed.

The second manuscript contains results from measurements of mRNA levels of inflammatory cytokines IFNα- and –β as well as tumor necrosis factor –α (TNF-α), in collected biopsy samples. The type 1 interferon response in the analyzed tissue samples is discussed in relation to the previously reported systemic interferon response. The measured cytokine responses, as

well as an observed variation in the TNF-α response between carriers and non-carriers, are discussed in relation to previous publications within the subject area.

The third manuscript deals with investigations of possible sites of virus replication during early and persistent phases of infection. Levels of FMDV RNA was quantified in sequential biopsy samples of pharyngeal mucosa harvested during early infection, as well as in corresponding tissue samples collected *post mortem*.

The final chapter of the thesis contains a general discussion of the obtained results, together with overall conclusions and future perspectives for continued research within the specific area.

# Resumé


### *Formål*

Mund-og-klovesyge (MKS) er en virusinfektion af meget stor økonomisk betydning for lande, der lige som Danmark, er afhængige af en betydelig eksport af animalske produkter fra landbruget. Sygdommen er ekstremt smitsom, og spredes hurtigt fra dyr til dyr. Persistent inficerede dyr (”carriers”) er i stand til at udskille levende virus i lang tid, uden at vise kliniske tegn på sygdom, hvilket gør det meget vanskeligt at kontrollere sygdommen når den bliver introduceret i et sygdomsfrit område.

Hovedformålet med dette PhD-projekt har været at undersøge værtsresponsen ved infektion med MKS virus (MKSV) serotype O i kvæg, med fokus på at udforske eventuelle faktorer i værtsdyrenes respons, der kunne være relateret til udvikling af ”carriers”.

### *Eksperimentelle studier*

Denne afhandling er baseret på resultater fra syv dyreforsøg med MKSV serotype O, udført ved DTU-Vet, Lindholm.
I fem ud af seks forsøg udført i kalve, er dyrene blevet inficeret med MKSV O UKG 34/2001, hvilket er den virusstamme der forårsagede et omfattende udbrud af MKS i England og Nord Europa i 2001. Et kalveforsøg blev udført med en MKS serotype O virus isoleret fra en kvægfarm i Uganda i forbindelse med et MKS udbrud i 2006. Et yderligere forsøg blev udført for at undersøge den kliniske infektion i får med den Britiske virusstamme (O UKG 34/2001).

Der blev udviklet en forsøgsmodel, hvor små vævsprøver (biopsier) blev udtaget fra den pharyngeale slimhinde fra levende kalve ved hjælp af endoskopi. Denne teknik muliggør udtagelse af gentagne vævsprøver fra de inficerede dyr, hvilket gør det muligt at følge det lokale immunrespons, i det enkelte dyr, igennem forskellige stadier af infektionen.

Denne nyudviklede forsøgsteknik blev brugt til at undersøge patogenesen af MKS-infektion i kalve gennem målinger af MKSV RNA i det pharyngeale epithel i de tidlige faser af infektionen. Den mulige lokalisering af det persisterende virus blev undersøgt ved tilsvarende analyse af vævsprøver fra det pharyngeale epithel, samt tilhørende lymfeknuder, der blev udtaget ved obduktion.

Det akutte værtsrespons ved infektion med MKSV O i kvæg blev undersøgt ved målinger af systemiske markører bestående af akut fase proteinerne serum amyloid A (SAA) og haptoglobin (HP) samt type 1 interferon (IFN). Det lokale inflammatoriske respons i det pharyngeale epithel blev undersøgt ved kvantificering af mRNA niveauer af inflammatoriske cytokiner i gentagne biopsiprøver.

***Afhandlingens struktur***

Det første kapitel inkluderer generel information om værtsresponset ved virusinfektioner, samt karakteristika af MKSV og patogenesen ved MKS infektionen. Projektets detaljerede formål er angivet sidst i kapitel 1.

Kapitel 2 indeholder beskrivelser af de dyreforsøg der er blevet udført i projektet. Udover generel information om det udviklede forsøgs-design samt anvendte metoder, er der beskrivelser af de observerede kliniske sygdomstegn ved infektion med de to forskellige MKSV O isolater der blev brugt i kvægforsøgene. Beskrivelsen af forsøget med MKSV O UKG 34/2001 i får, inkluderer, udover kliniske sygdomsbeskrivelser også resultater fra målinger af viræmi og antistof-respons, da disse data ikke er præsenteret i de inkluderede videnskabelige manuskripter.

Det tredje kapitel indeholder tre manuskripter udarbejdet for publikation i videnskabelige tidsskrifter.

Det første manuskript er baseret på målinger af akut fase proteiner SAA og HP, samt bioaktiviteten af type 1 IFN i sera fra tre af de udførte kvægforsøg. Målinger af det systemiske akut fase- og cytokin respons tidligt i infektionen sammenholdes med opståen af specifikke kliniske sygdomstegn, samt udvikling af viræmi og antistof-respons. En observeret forskel i HP respons, mellem carriers og ikke-carriers bliver, endvidere diskuteret.

Det andet manuskript indeholder resultater af målinger af mRNA niveauer af de inflammatoriske cytokiner IFN-α og –β, samt tumor necrosis faktor –α (TNF-α) i de udtagne biopsiprøver. Det tidlige respons af type 1 IFN i det lokale væv bliver diskuteret i forhold til det tidligere publicerede systemiske IFN respons. Det målte cytokinrespons i vævet, samt en observeret forskel i TNF-α respons mellem carriers og ikke-carriers bliver diskuteret i forhold til tidligere publikationer indenfor det specifikke område.

Det tredje manuskript omhandler den mulige lokalisering af virus replikation i de tidlige, samt persistente faser af MKSV infektion i kvæg. Niveauer af MKSV RNA bliver kvantificeret i biopsier fra pharynx, udtaget i de tidlige faser af infektionen, samt i tilsvarende vævsprøver udtaget ved obduktion af dyr i den persistente fase.

Afhandlingens afsluttende kapitel indeholder en generel diskussion af de opnåede resultater, samt konklusioner, og muligheder og perspektiv for fremtidlige studier indenfor det aktuelle emne.

# 1. Introduction

***Background***.

Foot-and-mouth disease (FMD) is an economically devastating viral infection which affects cloven-hoofed animals including cattle, pigs and sheep. The disease is highly contagious, spreading rapidly amongst susceptible animals, with vast financial implications for farmers and agricultural industries of affected countries.

FMD is endemic in large parts of the world, including major parts of South America, Africa, the middle-east and Asia. Most developed countries, including the EU, USA and Australia, are kept free of FMD by means of restrictions on trade and strict controls on import of agricultural products from endemic areas. An outbreak of the disease in normally disease free areas can result in huge consequences for the agricultural industry due to export bans and limitations of trade. As an example of this, the direct costs for handling of the outbreak of FMD in the UK in 2001 have been estimated to have reached £2,75 billion, whilst indirect costs in the form of combined losses related to agricultural export and the tourist trade are believed to amount to an additional £5,75 billion[6].

The general strategy for controlling FMD outbreaks in disease free areas is based on a "stamping out"-policy, with culling of all infected and susceptible animals within an area surrounding the affected farms. Furthermore, larger restriction zones with bans on transport and trade of animals and animal products, including transport of animals destined for slaughter, are maintained for prolonged periods. The stamping-out strategies imply huge demands on control agencies in the form of logistics and financial resources. In addition to this, the ethical dimension related to the destruction of vast numbers of both infected and healthy animals gives rise to considerable public debate.

It is possible to vaccinate animals against FMD, indeed the huge number of outbreaks that occurred in Europe in the 1950/60's were controlled through this approach. Furthermore, during recent years the application of emergency vaccination has proven to be an effective measure in order to control dissemination of FMD during outbreaks[36]. However, protective immunity acquired from available FMD-vaccines will only last for up to six months, and vaccination strategies are further complicated by the fact that there are seven different serotypes of the virus, with little or no cross-protection between serotypes.

### *Characteristics of the clinical FMDV infection*

The characteristic clinical picture of FMD infection in cattle involves a transient increase in body temperature accompanied by the typical clinical signs of lameness, excessive salivation and inappetence. Vesicular lesions develop in areas covered by cornified stratified squamous epithelia such as the oral cavity and coronary bands. Infected animals develop a significant viremia, with high levels of FMDV detectable in the blood for about 2-3 days. The viremia is effectively counteracted by the development of high titres of specific neutralizing antibodies within 4-7 days following the appearance of clinical disease.

Disease severity varies greatly between different virus isolates, as well as between different host species[2]. Mortality rates are generally very low in adult animals, whereas juvenile animals may develop fatal myocarditis as a consequence of the infection. The clinical course of the disease is usually over within 14 days after infection, but the occurrence of persistently infected, so called "carrier-animals" within ruminant species causes further complications for disease control.

## Host response to virus infections

The host immune system functions to protect the individual against infectious disease by means of recognizing the presence of potentially harmful agents within the body and eliminating these through the actions of highly regulated effector mechanisms. Subsequent immunological memory is generated in order to enable a stronger and more efficient immune response upon repeated exposure to a specific pathogen.

### *The Innate Immune Response*

The innate immune response is the first line of host defense. This branch of the immune system acts in a non-specific manner in order to eliminate any substance or agent not belonging to the body itself. Microorganisms that cross the epithelial barriers of the skin or the mucosa of the gut or respiratory tract are likely to be detected by phagocytic cells present in the submucosa[55]. Macrophages are mature phagocytes of the monocyte lineage that recognize common features present on several classes of pathogens through the action of pattern recognizing surface receptors (PRRs). Encountered pathogens are internalized leading to destruction of the pathogen itself, as well as the production of signaling molecules in the

form of inflammatory cytokines which function to activate and recruit other effector cells of the immune system[55]. Macrophages are also involved in "clearing up" the debris from damaged cells as well as removal of inactivated pathogens which have been neutralized by other branches of the immune response.

Another class of phagocytes are the granulocytes, or polymorphonuclear leukocytes, which include neutrophils, eosinophils and basophils. These cells are mainly involved in the defense against bacterial and parasitic infections, and their functions will therefore not be discussed in further detail.

The third class of phagocytic cells is constituted by the dendritic cells (DC). Immature cells of the DC lineage migrate through the bloodstream from the bone marrow to enter various tissues of the body. DCs take up particulate matter that they encounter and display peptide fragments of these, bound to major histocompatibility (MHC) complexes on the cell surface. DCs are stimulated through activation of PRRs or by the action of the inflammatory cytokines secreted by macrophages leading to further secretion of cytokines by the DCs themselves, as well as the expression of co-stimulatory molecules on the cell surface[29,57]. Activated DCs act as antigen-presenting cells (APCs) with the main function of activating immature T- lymphocytes through presentation of specific antigens bound to cell-surface MHC-complexes. DCs thereby constitute a crucial link between the functions of the innate host response and the subsequently initiated adaptive immune response[54]. Plasmacytoid DCs belong to a specific lineage of DC which in addition to functioning as APCs also have an adjunct role in modifying the immune response through the production of large amounts of type 1 interferons (IFNs) in response to viral infection[54].

***The Adaptive Immune Response***

The initial actions of the innate immune response are soon followed by the induced adaptive response. The adaptive immune response acts through clonal expansion of lymphoid cells expressing surface receptors capable of high affinity binding to specific pathogens that are encountered[54].

T- and B-lymphocytes are produced in the central lymphoid tissues within the thymus and bone marrow respectively. Immature lymphocytes migrate to peripheral lymphoid organs such as the spleen and lymph nodes following a stringent process of clonal deletion of any cells expressing receptors with potentially self-reactive binding capacities. The specificity of the variable region displayed on surface receptors of B- and T-lymphocytes is randomly

generated through serial events of gene rearrangement during generation of the lymphocyte precursors[53]. Each lymphocyte displays numerous copies of receptors of identical specificity on their surface. Immature lymphocytes will only become effective after activation through interaction with an APC presenting antigen matching the receptor binding site together with necessary co-stimulatory molecules.

APC displaying bound antigen migrate to peripheral lymphoid organs upon stimulation by inflammatory cytokines and the activation of PRRs. Immature lymphocytes with receptor specificity matching the presented antigens are selected for clonal expansion leading to the subsequent generation of a large number of activated B- and T-cells expressing receptors with specific binding capacities[56,57].

The surface receptors of activated B-lymphocytes are secreted as specific antibodies (immunoglobulins) as the B-cells develop into mature plasma cells. The main function of secreted antibodies is to neutralize pathogens through the generation of immune complexes consisting of pathogens bound by several antibody molecules. Immune complexes of opsonized pathogens are subsequently removed by phagocytes[56]. This specific mechanism of clearing infections by the adaptive immune response is of high importance in viral infections, as will be discussed in more detail further on.

There are several subsets of mature T-lymphocytes with varying functions within the adaptive immune response. T-cells are commonly divided into subsets with similar functions as defined by the expression of different surface molecules. $CD8^+$ T-lymphocytes are cytotoxic cells that are capable of killing virus infected cells following interaction with antigens displayed by MHC class I on the surface of infected cells. This class of T-cells is therefore generally considered to be of high importance for the host response to viral infections[57].

$CD4^+$ T-cells are also known as helper T-cells ($T_H$-cells), and can be further subdivided into $T_H1$ and $T_H2$-cells as well as the more recently discovered $T_H17$ and regulatory T-cells. $T_H1$ and $T_H2$ cells are important for the activation of antibody production and subsequent class shifting in B-lymphocytes. $T_H1$ and $T_H2$ cells induce production of immunoglobulins of different isotypes and the nature of the T-cell response will therefore have a direct effect on the antibody response. $T_H1$ cells are also involved in providing activating signals to cytotoxic T-cell subsets, as well as interacting with MHC class II complexes expressed by macrophages in order to induce destruction of ingested pathogens[57].

The development of the different subsets of $CD4^+$ T-cells is directed by the mixture of inflammatory cytokines produced by cells of the innate immune system, most importantly by the DCs, which will favour the induction of either a $T_H1$ or a $T_H2$-cell response. A predominating $T_H1$ response will induce a cellular response through activation of cytotoxic $CD8^+$ T-cells, as well as the production of opsonizing antibodies of IgG isotype. $T_H2$-cells will instead promote humoral immunity through induction of IgM, IgA and IgE. Although both subsets of helper T-cells are usually induced during the acute phase of infection, one or the other of the $T_H1$ and $T_H2$ subsets is often found to predominate in infections that become chronic[57].

Another type of lymphocyte displaying cytotoxic activity are the natural killer (NK) cells. NK cells function as a feature of the innate immune response as they do not express surface receptors of specific binding capacities such as other lymphocytes, but are instead activated to kill any cell displaying epitopes detected as "non-self" [43].

***Cytokines and other InflammatoryMarkers of specific interest***

*Type 1 Interferons*

IFNs are a family of cytokines which have been named according to their ability to interfere with the replication of intracellular pathogens. There are several sub-classes of IFNs; type 1 IFNs include the predominant types IFN-α and –β, whilst type II IFN is constituted by IFN-γ. Type I IFNs are induced through signaling pathways involving activation of nuclear transcription factor κB (NF-κB) through either one of two complementary receptor systems[65]. The first pathway of activation is through a class of ubiquitously expressed cytosolic receptors that detect viral nucleic acids in infected cells. These receptors are functional in almost all types of nucleated cells with the function of inducing type I IFN in response to viral infections. The second class of receptors is the Toll-like receptors (TLRs) which belong to a germline-encoded family of PRRs that has evolved to detect molecular features shared by entire classes of pathogens[29]. Of high importance in viral infections are TLR-3 and-7 that are located within endosomes of specialized cells, such as DCs, and which are activated upon detection of double- and single stranded RNA respectively[29,65,72]. As these TLRs are located in the membranes within intracellular compartments of phagocytic cells, this pathway of IFN induction does not require that the cell itself is actively infected by the virus. The restricted distribution of TLR-3 and-7 to intracellular compartments will provide access to material

internalized by the phagocytic cells, and at the same time prevent self-recognition as the binding sites of the receptors are kept isolated from the cytosol.

Type I IFNs interact with a common cell surface receptor leading to transcription of several kinds of IFN- inducible gene products, all with the common function of protecting the stimulated cell against virus infection. As examples of this, the IFN induced 2'-5' oligoadenylate synthases (OAS) functions to activate the nuclease RNAse L, leading to degradation of viral RNA, whilst the Mx proteins have proven to be effective in protecting cells against influenza and vesicular stomatitis virus[65].

Type 1 IFNs induce expression of co-stimulatory molecules necessary for activation of lymphocytes by macrophages and DCs[55], and have been shown to facilitate cross presentation of viral antigens to cytotoxic $CD8^+$ T-cells by DCs[46]. The cytotoxic activity of $CD8^+$ T-lymphocytes is further enhanced by the induction of an increased expression of cell-surface MHC class I on IFN stimulated cells[47,55]. It has furthermore been shown that cells stimulated by type 1 IFN become sensitized to undergo apoptosis upon subsequent viral infection[64] whilst IFN also functions to directly stimulate cytotoxic activity in Natural Killer (NK) cells[65]. These functions may serve important roles in limiting the spread of infection as virus infected cells will be rapidly eliminated.

*Interferon-γ*

IFN-γ is an important cytokine produced by mature T-lymphocytes and NK cells. This cytokine has various regulatory functions associated with the adaptive immune response. It serves as a highly potent activator of macrophages and stimulates antigen presentation through both MHC class I and II[16]. IFN-γ acts through a cell-surface receptor which is distinct from the type 1 IFN-receptor[65]. The downstream effects of IFN-γ involve a general modulation of the immune response through stimulation and repression of a large number of genes associated with cytokine production as well as events related to cellular proliferation and apoptosis[16]. The expression of IFN-γ is in itself regulated through interactions with other cytokines such as IFN-α and-β and tumor necrosis factor (TNF)-α[16].

*Tumor necrosis factor-α*

Tumor necrosis factor--α (TNF-α) is a common pro- inflammatory cytokine which is secreted by cells of the innate immune system in response to various kinds of infectious agents and tissue damage. It functions by stimulating a local inflammatory reaction in order to contain

the infection and by stimulating the recruitment of a large number of immune cells[55]. The local inflammatory reaction serves to modulate expression of surface molecules on endothelial cells while increasing the permeability of small blood vessels in order for cells of the immune system to gain access to the site of infection. Similar functions make TNF-α a highly potent actor in the pathogenesis of septic shock. A systemic release of this cytokine in response to a pathogen present within the bloodstream will cause general vasodilation with subsequent loss of blood pressure and multiple organ failure. The importance of TNF-α in the host response to viral infections has been investigated in relation to its possible involvement in control and potential reactivation of latent virus infections[22,44].

*The Acute Phase Proteins*

A characteristic property of the inflammatory cytokines induced during the innate immune response is induction of what is known as the acute phase response which involves a shift in proteins synthesized and secreted by the liver. The acute phase proteins (APPs) constitute a broad family of proteins whose serum levels are either up- or down regulated in response to infection. Some of the APPs have distinct functions in the host response to infection. C-reactive protein and mannose binding lectin are important APPs in humans and are capable of opsonizing pathogens by binding to certain molecular features found on the surface of various pathogens[55]. Other APPs have less well defined functions but may serve as easily measurable markers indicative of the presence of a systemic response to inflammation or other kinds of physical stress or trauma[11,61].

The value of different APPs as markers of disease is different between different host species[40]. In cattle, serum amyloid A (SAA) and haptoglobin (HP) have proven to be reliable markers that are significantly induced in many types of both bacterial and viral infections[33,39,40]. HP is a protein that binds free hemoglobin in the circulation. It is present at very low concentrations in serum of healthy cattle, but there can be a several thousand-fold increase within a few days in response to infection[32]. SAA is a precursor of amyloid A protein. It is present at measurable levels in healthy cattle, but will react by a very rapid up-regulation upon acute inflammation and can be found at levels of approximately 5 to 10 times the normal serum concentrations in acutely infected animals[32].

Both the innate and adaptive immune responses are highly important when discussing the host response to FMD. The virus has developed several strategies of circumventing the immune system[34,62] in order to evolve into an effective and extremely contagious pathogen.

Further knowledge of the mechanisms of interaction between FMDV and the different functions of the host immune system may be useful when developing strategies for prevention and control of the disease.

## Foot-and-mouth disease in cattle

### *The virus*

FMD-virus (FMDV) exists in seven distinct serotypes; O, A, C, Asia-1 and South African Territories (SAT) 1, 2 and 3, with a large number of subtypes within each serotype[45]. It is a non-enveloped, positive stranded RNA virus belonging to the *Aphthovirus* genus of the picornavirus family. The virus capsid consists of 60 copies each of four structural proteins; VP1, VP2, VP3 and VP4[13]. The main function of the capsid is protection of the RNA genome as well as attachment to host cell surface receptors. Within live animals, the interaction with cells is achieved primarily through binding to αv integrin receptors, most importantly αvβ3 and αvβ6[58,67]. Following attachment to host cells the viral genome is delivered to the cytoplasm where viral replication takes place, after which subsequent release of viral progeny occurs through complete lysis of the host cell.

The viral genome consists of a single copy of infectious RNA (approximately 8,5 kb in length), and contains a single large open reading frame encoding a polyprotein which is cleaved, via multiple intermediates, by virus encoded proteases during and following translation.

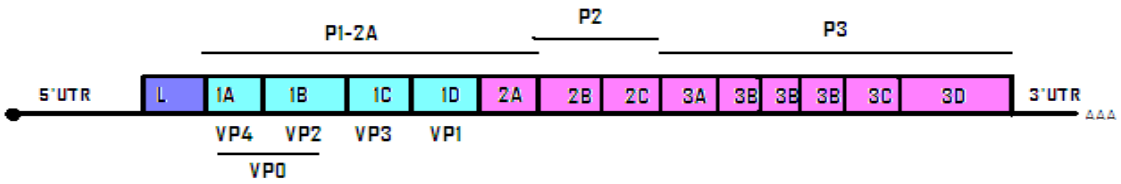


*Figure 1: Organization of the FMDV genome.*

The 5'-terminal region of the FMDV genome contains an extensive untranslated region (UTR) of more than 1300 nucleotides. This part of the genome is essential for FMDV replication and pathogenesis as it includes an internal ribosomal entry site (IRES) which enables translation of the viral RNA even though all cap-dependent translation of host cell mRNA is blocked in infected cells[14]

Host cell protein synthesis is efficiently shut off by the action of the FMDV leader protease (Lpro), which is a papain-like cystein protease located at the N-terminus of the polyprotein (Figure 1). The Lpro has the function of inducing the cleavage of the translation initiation factor eIF4G, following its self-cleavage from the growing polyprotein during translation of the FMDV genome[13]. As FMDV initiates translation in a cap-independent fashion through the action of the IRES, the virus is not dependent on intact eIF4G for translation, and can thus freely make use of the host cell machinery for protein synthesis[14,48].

The assembled capsid proteins constitute the sites of antigenic recognition, and also facilitate attachment and entry of virus to the host cells. Genetic variations in the regions encoding these proteins are therefore important determinants of FMDV characteristics.

The proteins encoded in the P2 and P3 regions of the FMDV genome are nonstructural proteins involved in replication of the viral genome as well as processing and assembly of the structural viral proteins. The 3C protease (3Cpro) acts on the majority of cleavage sites within the FMDV polyprotein and has also been shown to be capable of cleavage of several proteins involved in host cell protein synthesis[15]. The 3D protein is the virus encoded RNA-dependent RNA polymerase, which is highly conserved between different sero- and subtypes of FMDV. During FMDV replication the 3D protein initially transcribes a negative strand copy of the RNA genome, which is then used as template for the transcription of new copies of the positive strand genome.

### *FMDV pathogenesis*

FMDV pathogenesis has been studied in detail by several research groups. The initial site of FMDV replication has been suggested to be located within epithelia and lymphoid associated tissues of the pharyngeal area[3,6,21,51,59] or, alternatively, within the lungs[18,19].

An experimental study of the early pathogenesis of FMDV in cattle performed by Pacheco et *al*[59] showed that FMDV RNA could be isolated from tissue samples from both the upper and lower respiratory tracts at 24 hours post aerosol exposure to FMDV serotype O. Infectious FMDV could be isolated from tissue samples collected from the upper respiratory tract at a

significantly higher level than from similar samples derived from the lower respiratory tract, although samples from both sites were positive for FMDV genome by qRT-PCR. It was proposed that FMDV would reach the lower respiratory tract following aerosol exposure but that the pulmonary tissues were only poorly permissive to FMDV replication whilst primary virus replication was found within respiratory-associated lymphoid tissues of the upper airways[59]. In a subsequent publication, the same group went on to define the initial site of virus replication as being located within the epithelial cells overlying the lymphoid crypts of the pharyngeal mucosa. It was concluded that substantial viral replication took place within pulmonary cells of epithelial histiogenesis as early as 6 hours post aerosol exposure, with a decrease in viral load in pharyngeal tissues detected at 48 hours post exposure[8]. It was thus, proposed that the early pathogenesis of FMD in cattle involves primary replication in pharyngeal epithelia during the "pre-viremic" phase of infection, with the highest viral load found within the epithelia of the dorsal soft palate (DSP). It was further concluded that there is a subsequent widespread FMDV replication within pulmonary pneumocysts which is accompanied by a significant decrease in viral load within pharyngeal tissues[8].

In contrast to these recent findings, previous work of other authors have failed to find supportive evidence for FMDV replication within the lungs[6,21]. Burrows *et al* performed an extensive study of the pathogenesis of FMDV in cattle in 1981[21]. Results from this study were based on investigations of sites of viral replication through isolation of infectious virus from tissue samples collected from 56 cattle that had been subjected to varying routes of FMDV exposure. It was concluded that large amounts of live virus could be isolated from the pharyngeal region, whereas there was little or no evidence of FMDV replication within the lower respiratory tract[21]. These findings are also in line with what have been concluded by other research groups in subsequent studies. Zhang and Alexandersen measured the RNA-loads in different tissues during acute and persistent stages of FMDV infection in cattle[75]. It was in this study proposed that the initial phase of viral replication took place within the pharyngeal epithelia, whilst substantial viral amplification was found within vesicular lesions in the epithelia of the oral cavity and coronary bands. It was possible to detect FMDV RNA in the lungs of viremic animals, but at levels that were much lower than what was found at sites of secondary viral replication, and thus, the lungs were not considered to play an important role in the pathogenesis of FMDV in cattle[75].

Thus, there is still no clear consensus regarding the early pathogenesis of FMD, and comparison of published results is confounded by variations in experimental design with the use of different virus strains and varying routes of initial administration of the virus.

Following primary replication, the virus spreads systemically via the lymphatics and vascular system to the peripheral sites of secondary replication which are characterized by the presence of stratified cornified squamous epithelia, such as the coronary bands and oral cavity[3]. Infected cattle develop transient viremia lasting for about 2-3 days, which is counteracted by the development of circulating anti-FMDV antibodies. Clearance of viremia is achieved by the action of phagocytic cells, most importantly macrophages, which are effective in detecting and internalizing opsonized FMDV[49].

As indicated above, the clinical disease follows a rapid time course and is typically manifested by a sudden rise in body temperature and development of vesicular lesions at peripheral areas of viral replication. The degrees of salivation, inappetence and lameness observed in affected animals may vary in correlation to the severity of lesions. In animals housed under normal conditions of modern farming facilities, ruptured vesicles often become the site of bacterial infections. These secondary infections may have severe implications for the welfare and usability of dairy cattle as a result of chronic injuries to the feet and udders. The viral infection in itself may also have a direct effect on the milk yield of infected cattle, as well as on growth rates and the overall welfare and ability to withstand other infections in all animals affected. These long-term effects of FMDV infection are of high relevance to areas of the world where the disease is endemic and infected animals are not culled.

In the absence of secondary bacterial infections at the sites of FMDV lesions, the clinical course of the infection usually subsides within 7-14 days, although ruminants may develop into persistently infected “carrier-animals”.

***FMDV carriers***

FMDV carriers are defined as animals with asymptomatic, intermittent, presence of infectious virus in oropharyngeal fluid beyobd 28 days after infection[62]. It is believed that in these animals (approximately 50% of infected cattle), FMDV is capable of persisting, at a low level of replication within pharyngeal epithelial cells[20,80], or as intact, but largely quiescent, viral particles within germinal centers in pharyngeal tissues[42]. These animals are a source of infectious virus, and their presence is therefore unacceptable in areas free of FMD.

The carrier state can develop in animals which have been immunologically naïve at the time of exposure to the virus, as well as in animals that are protected against the clinical disease by the presence of adequate levels of circulating antibodies due to vaccination or previous exposure to the virus[20,30,62]. The duration of the carrier state varies between species, with the longest duration recorded in African buffaloes (5 years), followed by cattle (2 years) and sheep (9 months)[25,50,52,66]. Pigs do not become carriers of FMDV[5].

FMDV carriers have high titres of circulating anti-FMDV-antibodies, and as viral excretion may be intermittent and of a low level, it may be difficult to detect carriers by screening of animals at herd level. As a direct consequence of this, according to current regulations in areas kept free of FMDV without vaccination, any animal with anti-FMDV antibodies in the circulation is a potential carrier of the virus, and is thus, not accepted. These circumstances have profound implications for the control measures applied in order to handle FMDV outbreaks in disease free areas. Although emergency vaccination has proven to be an effective measure to prevent spread of FMDV[36], all animals that have been vaccinated against FMD will subsequently have to be eliminated in order for the country to regain a completely FMD-free status, with fully lifted restrictions on international trade.

***Host response to FMDV***

As previously discussed, viral infections of the respiratory tract are usually sensed by effector cells of the innate immune system, such as mature cells of the monocyte lineage, which are present in the respiratory mucosa. Activation of pattern recognizing cellular receptors leads to production of an array of pro-inflammatory cytokines, such as tumor necrosis factor–α (TNF-α) and type 1 IFN.

Recent studies have demonstrated that the Lpro of FMDV is capable of down-regulation of transcription of IFN-β through interaction with NFκB[27,73]. Clinical experiments with an attenuated FMDV mutant lacking the Lpro have indicated a decrease in the ability of this virus to spread from the initial site of replication compared to the corresponding wild-type strain[17,19]. It has been proposed that the decreased pathogenicity of this leaderless mutant may be a direct consequence of the virus's inability to block host cell IFN expression, which would normally leave neighboring cells more resistant to virus infection[17].

It has been shown that uptake of FMDV by macrophages is possible without the aid of opsonizing antibodies, although the phagocytic activity of these cells becomes much efficient as a result of the interactions of cellular Fc receptors with virus bound immunoglobulins[49].

In contrast to this, cell culture based experiments have demonstrated a necessity for FMDV to be bound by specific antibody in order to induce transcription of IFN by naturally IFN producing cells (NIPC) of the DC lineage[35]. This would imply that a significant release of IFN by plasmacytoid DCs in response to FMDV infection would not occur until the concentration of anti-FMDV antibodies in the circulation has reached adequate levels.

There has been some varying reports on whether FMDV is capable of actively infecting cells of the DC lineage[67]. Bautista *et al*[12] concluded that porcine skin DC were refractory to infection by FMDV, possibly due to high levels of IFN-α expression. It has also been reported that other lineages of DCs are susceptible to FMDV infection, albeit resulting in an abortive infection, which does not lead to cytopathic effects or subsequent release of viral progeny[37]. Regardless of the possible mechanisms of cellular uptake and infection, production of type 1 IFN seems to be an important consequence of the interaction between FMDV and DCs[12,35,67].

FMDV replication in cell culture is highly sensitive to the inhibitory effects of type 1 IFNs[24] and it has also been demonstrated that IFN-γ is capable of clearing persistent FMDV infection in cultured bovine epithelial cells[81]. Other studies have demonstrated the potential of both IFN-α[23] and IFN-γ[74] to provide protection against the development of clinical disease in pigs challenged with FMDV. The exact mechanisms by which FMDV may interact with cells of the innate immune system as well as the nature of interactions between the virus and the hosts IFN response represent areas of research that deserve further study.

***Aspects of specific interest in FMDV immunology***

Neutralizing anti-FMDV antibodies may be detected in the circulation of infected animals as early as 3-4 days following natural infection. Although the immune response induced by FMDV is rapid, the window between antigen exposure and the development of adaptive immunity may be crucial when applying emergency vaccination as a control measure in the event of an FMD outbreak. Previous studies have demonstrated a protective immunity following vaccination against FMDV which preceded achievement of what has normally been considered as being protective levels of specific antibody in the circulation[26,30,31]. It has been postulated that this early protection achieved by high potency emergency vaccines against FMDV may be a consequence of activation of the innate immune response[10], with further suggestions of the specific involvement of a cellular response of γδ-Tcells[7,69] and NK cells[71].

FMDV has developed several mechanisms for effective evasion of the host immune response[34]. In addition to a general suppression of host cell protein synthesis[15,48] and a more specific interference with the host cell IFN response through degradation of NFκB[27], FMDV is also capable of interfering with surface expression of MHC class I molecules[63]. Continuous expression of peptide epitopes derived from the interior of the cell by cell surface MHC complexes plays an important role in the cellular defense mechanisms against viral infections. Cytotoxic $CD8^+$ T-cells are activated to kill virus infected cells upon detection of "non-self" peptides presented by MHC class I molecules. By preventing surface expression of viral antigen, FMDV is capable of circumventing this specific branch of the cellular immune response. However, it has also been shown that a general down regulation of MHC class I expression may trigger NK cell cytotoxicity[41], and may thus mediate clearance of virus infected cells through this mechanism. The critical point about down regulation of MHC class I may be that it provides the time required for the virus to replicate and then spread efficiently to another host. Investigations of early cellular mechanisms involved in the response to FMDV infection could be useful for the development of effective emergency vaccines against FMD.

***Other persistent virus infections***

There are several viruses that are capable of causing persistent infections, with or without apparent clinical disease. Many of these viruses are tumor causing agents such as the DNA viruses human papilloma virus (HPV) and Epstein Barr virus (EBV), which are capable of persisting through interference with the proliferation and lifespan of infected cells[81]. Human immunodeficiency virus (HIV) and Human T-cell carcinoma virus (HTCV) have an RNA genome, but belong to the retrovirus family. These viruses encode an RNA-dependent DNA polymerase and are capable of latent persistence by incorporating a DNA copy of the viral genome into the host cell DNA. A virus of more relevance to the pathogenesis of FMDV could be the Hepatitis C virus (HCV), a positive stranded RNA virus belonging to the *Flavivirus* family. HCV causes a persistent chronic infection in 80% of infected humans, whereas the remaining 20% will suffer a more acute infection, which is subsequently cleared. Research on HCV persistence has been focused on how the virus is capable of circumventing the immune system, proposing the involvement of a blunted $CD8^+$ response in addition to interference with DC functions[38].

Within the veterinary field, the non cytopathic version of the bovine viral diarrhea virus (BVDV), another member of the *Flavivirus* family, is capable of causing persistent infection

in fetuses that are exposed to the virus during early gestation. These animals develop an immunological tolerance towards the virus, and are capable of shedding large amounts of BVDV throughout their life time[60].

As FMDV is an RNA virus, it would not be expected to be capable of persisting latently within cells as would be the case for some more stable DNA viruses such as different members of the herpes virus family. The instability of the RNA genome would require FMDV to maintain a low level of replication in order to persist within infected cells. Whilst acute FMDV infection is of a highly cytolytic nature, resulting in complete destruction of infected cells, the virus does not cause any lesions or clinical signs of disease during persistent infection. The high titres of neutralizing antibodies found in the blood indicate an intracellular localization of the virus. How this virus is capable of persisting without being targeted by the cellular immune response, and which factors that are involved in determining if an animal will develop into a persistently infected carrier or not, remain to be elucidated.

## Project Aims

It is clear that the innate immune response plays an important role in FMDV infection in cattle. Further investigations of the acute host response to this infection may provide information that could help elucidate possible mechanisms responsible for the variations in outcome of the clinical infection. Understanding of immunological events involved in the development of persistently infected subclinical carriers of FMDV may be useful in the development of effective intervention strategies for control and prevention of FMD outbreaks.

The general aims of this project have been:

- Development of an experimental model for FMDV in cattle that enables collection of tissue samples from the pharyngeal mucosa of live animals at sequential time points during the infection.
- Investigation of the systemic immune response to FMDV infection through measurements of markers of the acute phase response in the blood of infected animals.
- Investigation of the local host response to FMDV infection by characterization of the expression of relevant genes in samples of pharyngeal mucosa collected at sequential time points during infection.

- Investigation of FMDV pathogenesis through analysis of the level of viral replication in the pharyngeal mucosa during acute and chronic stages of infection.

## 2. Clinical studies of infection with FMDV serotype O in cattle and sheep

The contents of this thesis is based on the results of seven separately performed clinical experiments with FMDV serotype O. Six of the experiments were performed in cattle with one additional experiment investigating the clinical course of FMDV infection in sheep. One of the cattle experiments was performed as part of another project investigating certain serological aspects related to epidemiology and surveillance of FMDV in endemic areas of eastern Africa. In this experiment vaccinated and naïve animals were challenged with a field strain (FMDV O UG 312/2006) isolated from samples collected from a cattle-farm in Mbarara district, Uganda, during an outbreak of FMD in 2006. In all of the remaining experiments, the animals were inoculated with a serotype O isolate originating from the FMD outbreak in the UK in 2001 (FMDV O UKG 34/2001).

| **Experimental ID** | **Animal Species** | **Infected Animals (Inoculated : contact)** | **Control animals** | **Virus Isolate** | **Duration of experiment (PID)** |
|---|---|---|---|---|---|
| **FMD P1C 2008** | Cattle | 8 (4:4) | | FMDV O UKG 34/2001 | 65 |
| **FMD 2S 2008** | Sheep | 20 (12:8) | | FMDV O UKG 34/2001 | 140 |
| **FMD 3C 2008** | Cattle | 12 (6:6) | | FMDV O UKG 34/2001 | 100 |
| **FMD 4C 2009** | Cattle | 12 (12:0) | | FMDV O UG 312/2006 | 32 |
| **FMD 5C 2010** | Cattle | 12 (6:6) | 3 | FMDV O UKG 34/2001 | 32 |
| **FMD 6C 2010** | Cattle | 12 (6:6) | 3 | FMDV O UKG 34/2001 | 14 |
| **FMD 7C 2010** | Cattle | 12 (6:6) | 3 | FMDV O UKG 34 /2001 | 36 |

*Table 1. Overview of animal experiments included in the project.*

| Experimental ID | Alternative ID |
|---|---|
| FMD 3C 2008 | FMD 2008 |
| FMD 5C 2010 | FMD2010a |
| FMD 6C 2010 | FMD 2010b |
| FMD 7C 2010 | FMD 2010c |

*Table 2. Alternative experimental IDs, as referred to in manuscript 1-3.*

## Cattle Experiments

The initial experiment performed as a part of this project (FMD P1C 2008) was the first animal experiment to be performed within the newly built BSL-3+Ag research facilities at DTU-Vet Lindholm. The purpose of this experiment was, apart from introducing the concepts of working with FMDV in the natural host species within biosecure research facilities, to produce a batch of virus which could be used in subsequent experiments. For this reason, four calves (1 inoculated and 3 contact infected) were euthanized during the acute phase of infection in order to harvest epithelial samples from which infectious virus could be harvested.

As the definition of an FMDV carrier is an animal that excretes live virus for more than 28 days after infection[62], the remaining animals from the first experiment and animals of the following experiments were kept for observation and sampling for periods exceeding this to allow determination of individual animal's carrier status. One experiment (FMD 6C 2010) was, however, terminated at PID 14 due to technical reasons. The duration of the individual experiments (see Table 1) refers to the number of days from inoculation to termination. Animals were generally allowed a period of approximately 14 days for acclimatization and sampling prior to inoculation.

### FMDV O UKG 34/2001

FMDV O UKG 34/2001 belongs to the Middle-East South Asia (ME-SA) topotype of FMDV serotype O. It is a well characterized isolate, which has been utilized in several clinical studies of FMDV infection in both cattle, pigs and sheep[1,6,75,77].

The inoculum used in the majority of the experiments included in the project (see Table 1.) was prepared from a virus isolate obtained from World Reference Laboratory for FMD at the Institute for Animal Health (IAH) Pirbright, UK. This virus was originally isolated from an abbatoir sample collected from a pig during the FMD outbreak in the UK in 2001. The isolate had been passage once in cattle at the IAH-Pirbright (from bovine VI31 17/12/04, $10^{7.0}$ $TCID_{50}$), before being transferred to DTU-Vet, Lindholm.

*Virus Inoculum*

Prior to the first experiment, the inoculum obtained from the IAH-Pirbright was tested in primary bovine thyroid (BTY) cell culture in order to determine the current titre. A volume of 10µl of the original suspension was used for titration (starting with a dilution of 1:100) showing a titre of $10^{8.0}$ $TCID_{50}$/ml. A subsequent 10 fold dilution in phosphate buffer resulted in an estimated titre of $10^{7.0}$, and each of the 4 inoculated animals recieved 0.5 ml of the diluted inoculum, resulting in an individual dose consisting of $10^{6.7}$ $TCID_{50}$.

The inoculum used for subsequent experiments with FMDV O UKG 34/2001 was obtained through pooling of virus harvested from the tongue epithelia of two of the contact infected animals that were euthanized during the acute phase of infection in the first experiment. The titre of this batch of inoculum was determined to be $10^{8.5}TCID_{50}$/ml in primary BTY cells following the previously described procedure. The inoculum was stored in aliquots at -20$^{o}$C with added glycerol (50%) and was subsequently diluted 10-fold in phosphate buffer prior to administration. Each animal inoculated in subsequent experiments received 0.5 ml of the diluted inoculum, corresponding to a single dose of $10^{6.9}$ $TCID_{50}$.

*Experimental Procedure*

All animals were pre-treated using a broad spectrum antibiotic (enrofloxacin; Baytril©; 2.5 mg/kg or tetracycline; Aquacyclin® 5mg/kg) for four days upon arrival in order to treat any existing bacterial infection and were further treated with β-lactam penicillin (streptocillin©; 5ml/100kg) for four days around inoculation to avoid interference caused by any bacterial infections.

In all of the cattle experiments performed with FMDV O UKG 34/2001, one half of the animals included in the study were directly inoculated through sub-epidermal injection in the tongue[6]. In the experiment FMD 4C 2009, where animals were infected with a Ugandan field

isolate, all 12 animals included in the study were directly inoculated in the tongue. Animals that were not subjected to direct inoculation gained the infection through continuous contact with inoculated animals, with two inoculated and two contact animals in each pen, and the stable facilities also allowing for physical contact between animals in separate pens.

Animals that were randomly selected for inoculation were sedated through intravenous injection of Xylazin (Rompun® 2%; 1 ml/100kg) before receiving the inoculum administered at 5-6 separate injection sites at the base of the tongue (Figure 2). Following the inoculation, the animals were revived through intravenous injection of atipamezol (Antisedan® vet; 0.4 ml per ml of Rompun previously administered).

The method of subepidermo-lingual inoculation[6] is easily reproducible, it allows for control of the amount of infectious virus that each inoculated animal receives and the method has previously been used in numerous experimental studies of FMD in cattle[4,75,78]. Although representing an artificial route of viral entry, direct inoculation in the tongue may simulate viral entry through damaged epithelia such as could occur through abrasions present within the oral cavity or in the skin. It can further be argued that the animals included in the contact infected groups in these experiments will receive the virus under what would resemble natural conditions through direct transmission of virus from one animal to another.

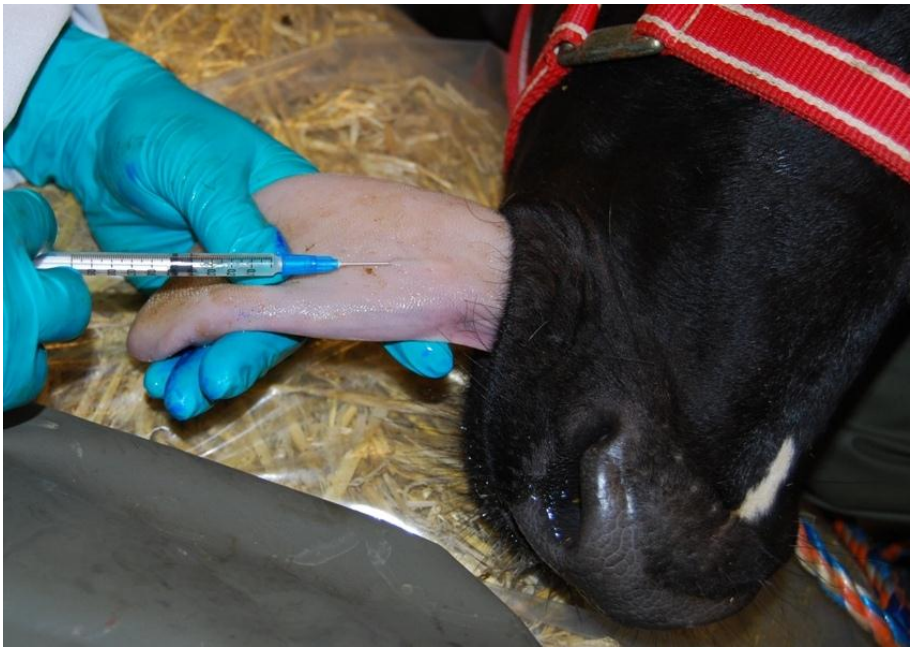

*Figure 2. Sedated calf receiving FMDV inoculum through subepidermal injection in the tongue.*

*Collection of routine samples*

A similar protocol for routine sampling was used in all of the performed experiments. All animals were monitored daily, with measurements of rectal temperature and observation of clinical signs. Serum samples were collected daily from 3 days prior to inoculation and throughout the first two weeks of the experiments but then on a weekly basis throughout the remaining part of the experiment.

Samples of oropharyngeal fluid (probang samples)[68] for quantification of virus excretion were collected prior to inoculation, once daily during the first week after inoculation, then every other day throughout the second week and on a weekly basis subsequently. Additional probang samples were collected after PID 28 so that the carrier status of individual animals was based on the analysis of a minimum of four samples. The uninfected control animals which were included in the last three experiments performed were handled and sampled following a protocol similar to that used for infected animals. These animals were kept in a separate isolation unit and were subjected to sedation and "mock-inoculation" using phosphate buffer, in order to mimic the procedure used for inoculation and sample collection in the test groups.

Results from quantification of viremia and development of anti-FMDV antibodies as well as measurements of acute phase proteins and type 1 interferon in serum are reported in manuscript number 1. Results from measurements of viral excretion in probang samples, as well as the prevalence of animals that developed into FMDV carriers are reported in manuscript number 3.

*Development of method for collection of biopsy samples of pharyngeal mucosa*

During the second cattle experiment (FMD 3C 2008) a novel technique for endoscopical collection of small tissue samples from the pharyngeal mucosa of the dorsal soft palate (DSP) of live animals was introduced. This experimental model was developed in order to allow collection of sequential tissue samples from this specific anatomical region, which has been reported to be an important site for FMDV replication in both early and persistent stages of infection[3,6,21,59,75,80]. Obtained samples were used to investigate the presence of FMDV RNA (manuscript 3), as well as the expression of selected inflammatory cytokines (manuscript 2) at different stages of infection. The original idea was to harvest biopsy samples, which would enable monitoring of the progression of infection within individual animals, with minimal

interference to the natural course of infection. The study-design allowed for a comparison of possible differences in virus replication and the local tissue response to infection between animals that would subsequently develop into FMDV carriers and those that would not.

Animals were sedated through intravenous injection of xylazine (Rompun vet© 1.5 ml/ animal) and positioned in ventral recumbency. A fiberoptic endoscope (Vet-vu 2FSb) was introduced through one nostril and positioned to allow clear visualization of the nasopharynx, with the entrance of the trachea in central view (Figure 3). Tissue samples were collected from the ventral surface (constituting the dorsal soft palate), just to either side of the mid plane, rostral to the epiglottis. The site of sampling is believed to house a high density of diffuse lymphoid tissue, which has also been corroborated through standard hematoxylin-eosin (HE) staining of randomly selected biopsy samples (Figure 4). Two pieces of tissue, approximately 1x1x1 mm in size, harvested from slightly different locations within the sampling area were included in each sample.

The first biopsy sample from each animal was collected one week prior to inoculation. The second biopsy was collected during the acute phase of infection, defined as the first day where the animals showed a rectal temperature of above 40$^{o}$C, with a third sample collected 7 days later. In experiment FMD 3C 2008, a final collection of biopsies was performed at PID 28. In subsequent experiments, final collection of tissue samples was performed during post-mortem examinations performed at the termination of the experiments at PID 32-35.

*Figure 3. Endoscopical examination with collection of biopsy samples from sedated calf.*

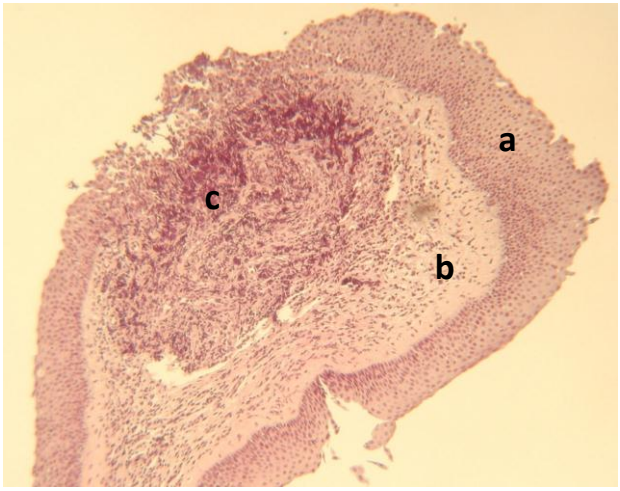


*Figure 4. Hematoxylin-eosin (HE) staining of randomly selected biopsy sample harvested form an uninfected calf. The picture shows intact non-cornified stratified squamous epithelia (a) with underlying sub-mucosa (b) and lymphoid tissue (c).*

***Clinical appearance of infection with FMDV O UKG 34/2001 in cattle***

All animals included in the test groups developed mild to moderate clinical signs of FMD (Figures 5-8), with an increase in body temperature and vesicular lesions in the oral cavity being the most prominent findings occurring in all individuals. Less than half of the animals developed vesicular lesions on the feet and only two individuals (out of 56) developed clearly visible lameness (Figure 8). Inoculated animals developed clinical signs of FMD at PID 1-2, whilst onset of clinical disease in contact animals was observed from PID 2-5. The animals showed no significant loss in appetite and all animals recovered from clinical disease within approximately seven days, without further complications, and without the need of supportive medical treatment.

Vesicular lesions within the oral cavity were most commonly found on the dental pad (Figure 5) and gums in addition to the tongue (Figure 6), which was most affected in animals that were infected through direct inoculation. The epithelia overlying the lesions appeared thickened and whitish, before being sloughed off within few days. Lesions became covered by fibrinous exudate, and healed from below within approximately 5-7 days after initial appearance.

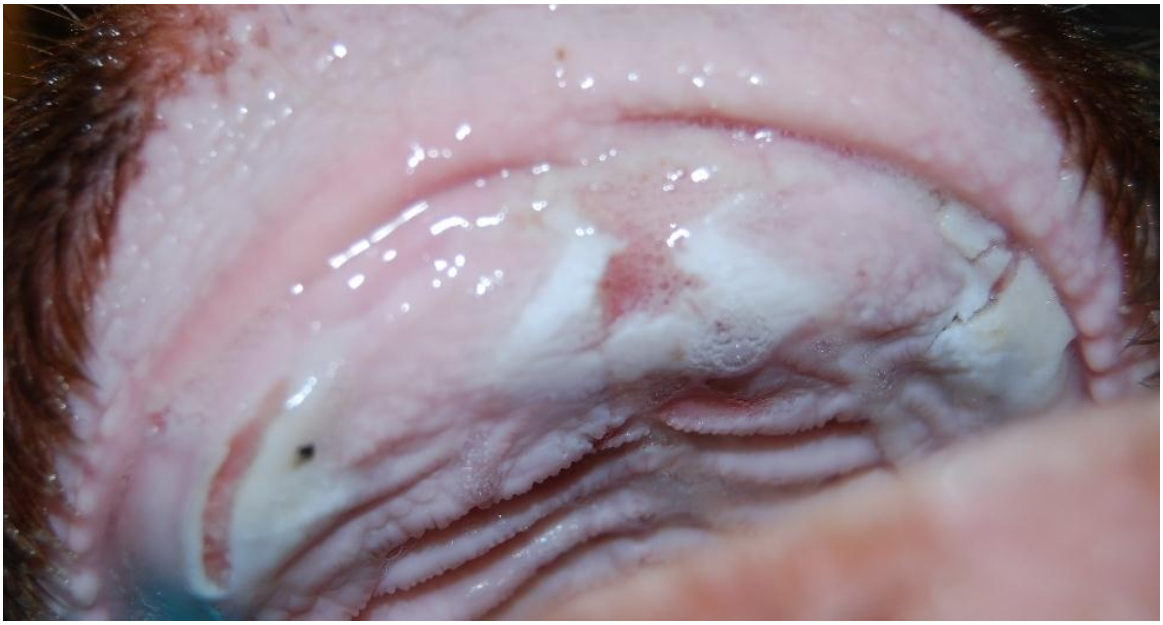

*Figure 5. Ruptured vesicles located on the dental pad observed at the first day of appearance of clinical symptoms.*

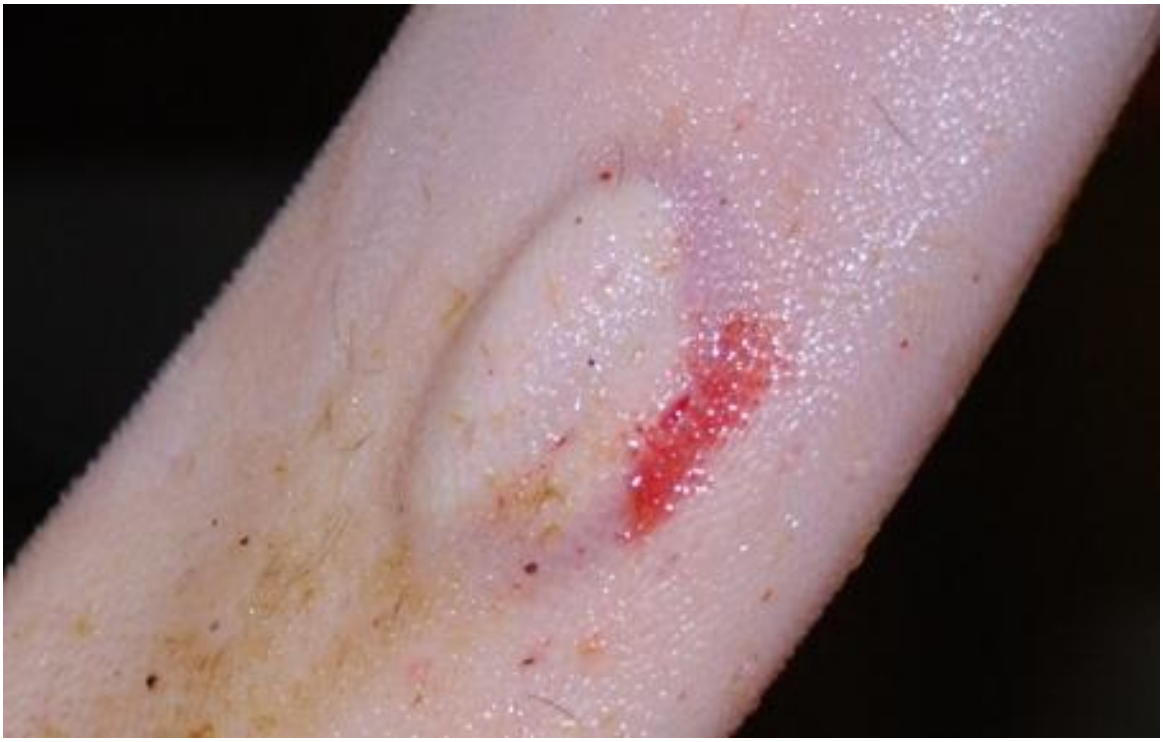

*Figure 6. Intact vesicle on the tongue of a contact-infected calf during the early phase of infection.*

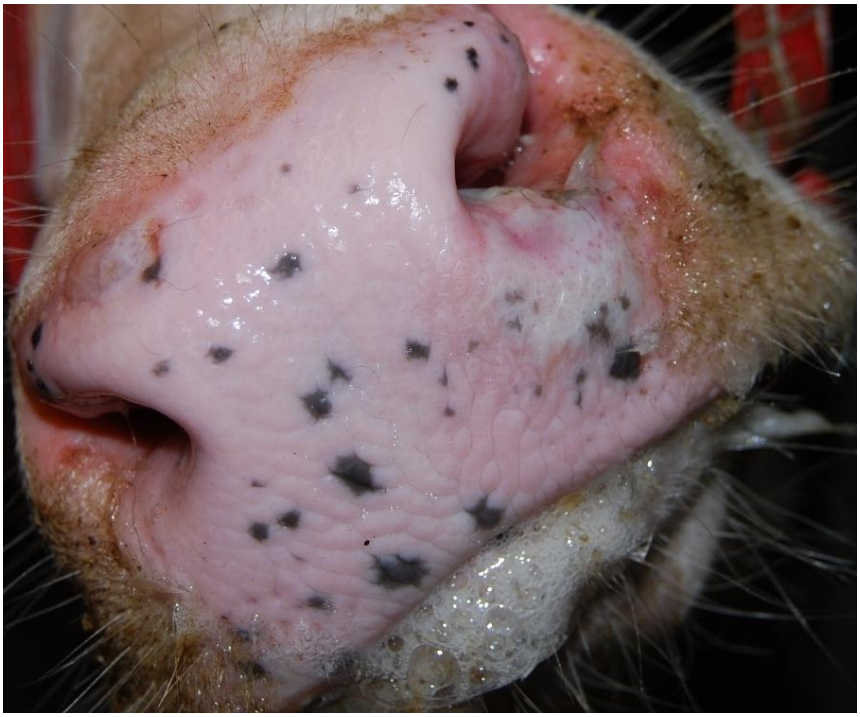

*Figure 7. FMDV-infected calf showing characteristic clinical signs of excessive salivation as well as a vesicular lesion on the muzzle during the acute phase of infection.*

*Figure 8. Acutely infected calf displaying bilateral front limb lameness due to vesicular lesions within the hoof-clefts.*

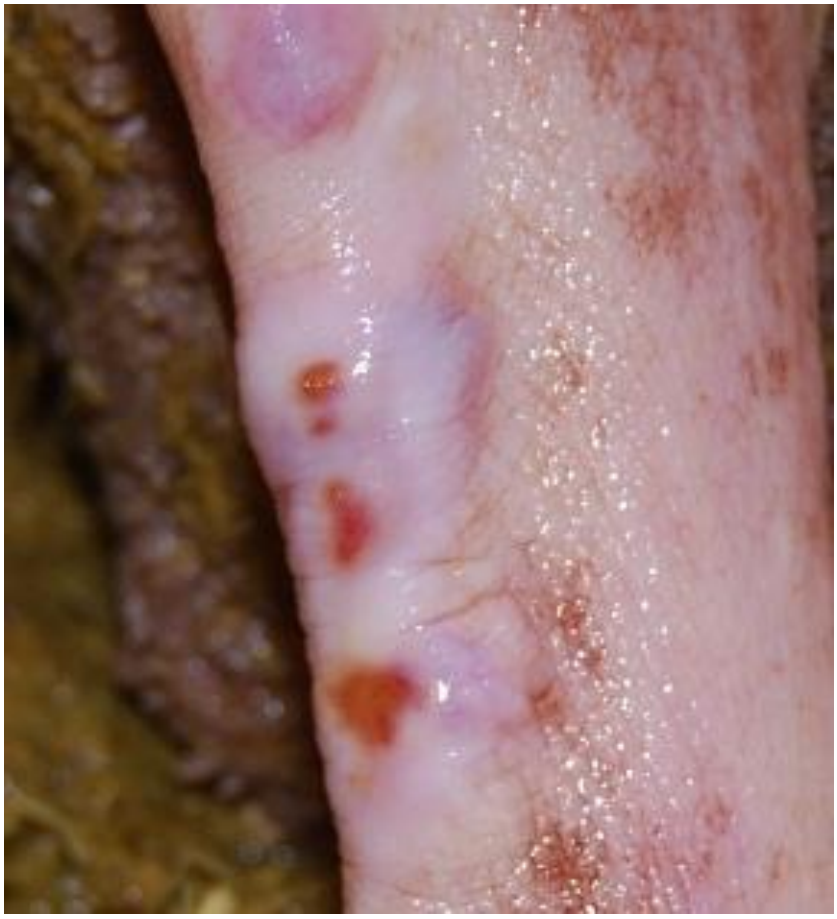

*Figure 9. Vesicular lesions on ruminal septa in a calf which was euthanized during the acute phase of infection. This region of the rumen is covered by cornified stratified squamous epithelia which makes it susceptible to FMDV replication.*

**FMDV O UG 312/2006**

This Ugandan FMDV isolate was derived from a probang sample collected from a cow in Mpigi village, Mbarara district, Uganda, in 2006[9]. The sampling and subsequent characterization of the virus was performed as a part of the project "Livestock and wildlife diseases in East Africa", funded by the Danish International Developments Agency (DANIDA). Sequencing of the structural VP1 region showed that the specific isolate belonged to the East Africa -2 (EA2) topotype of FMDV serotype O[9].

*Virus Inoculum*

The inoculum used for the experiment was prepared through isolation in BTY cell culture, within the FMD facilities at Lindholm Island. The infectious titre was determined as $10^{6.4}$

$TCID_{50}$/ml, and each inoculated animal received a single dose of $10^{6.1}TCID_{50}$ through subepidermal injection in the tongue.

*Experimental procedure*

The experimental protocol was designed with the aims of reproducing the diverse serological profiles which are often found in domestic animals and wildlife in East Africa, with combinations of antibodies against several different FMDV serotypes. Within this region, domestic animals are generally not subjected to prophylactic FMDV immunizations. Vaccination campaigns are, however, often implemented as a measure of combating the FMDV outbreaks that occur within specific areas. Domestic livestock are therefore often subjected to repeated immunizations with vaccines containing antigens of the FMDV serotypes which are currently believed to be in circulation. Serological surveillance is made difficult as most animals will have developed antibodies against a mixture of different FMDV serotypes, both as a consequence of repeated vaccinations and due to possible exposure to the infection.

By using an immunization protocol with FMDV vaccines produced and commonly used in the area, and then subsequently challenging the animals with a locally derived FMDV isolate, the purpose of the experiment was to produce sera from animals with a known history of FMDV exposure. The sera would subsequently be used for validation and optimization of serological assays used for epidemiological surveillance.

In the experiment (FMD 4C 2009), 12 calves were directly inoculated with the FMDV O isolate derived from Uganda. 6 of the animals had been through a six week vaccination protocol consisting of three vaccinations, with two different vaccines. Vaccinated animals had received one dose of either of two monovalent vaccines with antigen components of either serotype SAT-1 or serotype SAT-2, followed by two additional administrations, separated by 18 days, of a trivalent vaccine against serotypes O, SAT-1 and SAT-2. Challenge with the type O virus was performed 47 days after the animals had received the last of the three vaccinations.

The general design of the experiment, with observations of the clinical progression of the infection and collection of routine samples, was similar to what has been described above for cattle experiments with FMD O UKG 34/2001.

***Clinical appearance of infection with FMDV O UG 312/2006***

The vaccination scheme did not protect the animals from developing severe clinical disease when subsequently challenged with the Ugandan FMDV O-isolate. The clinical appearance of infection resembled that previously described for infection with FMDV O UKG 34/2001, although with a significantly increased severity of lesions. Several of the animals received supportive treatment with analgesics and anti-inflammatory drugs (Torbugesic 10mg/100kgs and Metacam; 0.5 mg/kg) as well as antibiotics (Streptocillin; 5ml/100kgs) in order to alleviate the clinical symptoms. The clinical course of infection was, however, quickly overcome and all animals recovered fully from the infection within approximately 7 days. Results from serological investigations from this experiment have been presented elsewhere[70].

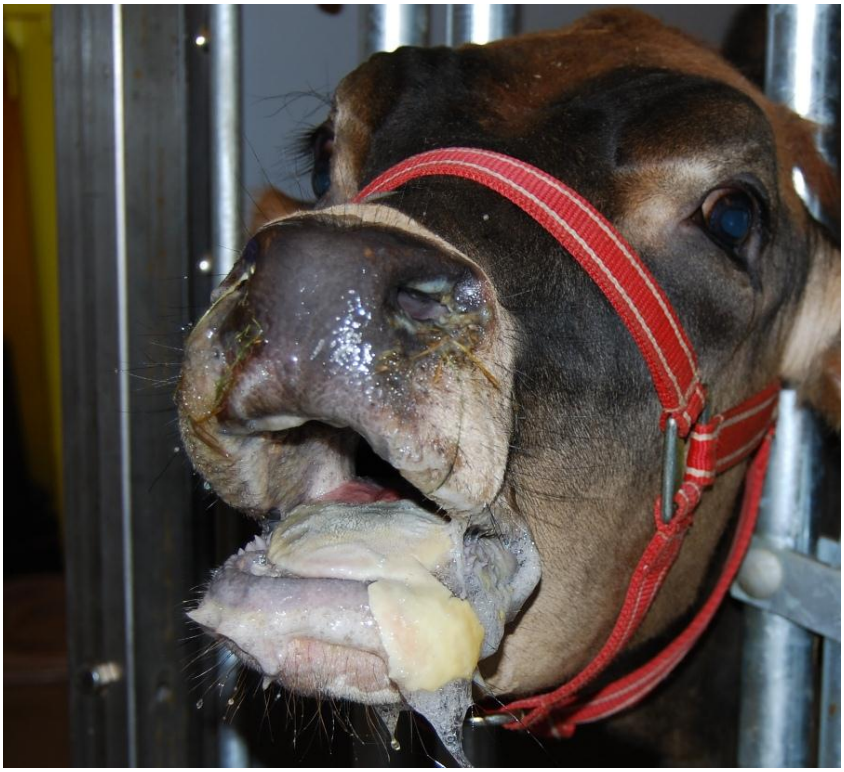

*Figure 10. Profound sloughing of epithelia in the oral cavity of calf infected with FMDV O UG 312/2006. This calf lost all superficial epithelia from the tongue within 1 day after this picture was taken. The animal received antibiotic treatment due to a suspected (secondary) bacterial infection in the oral cavity. The lesions healed very efficiently, the animal was able to eat normally within few days, and there was no chronic damage caused by the infection.*

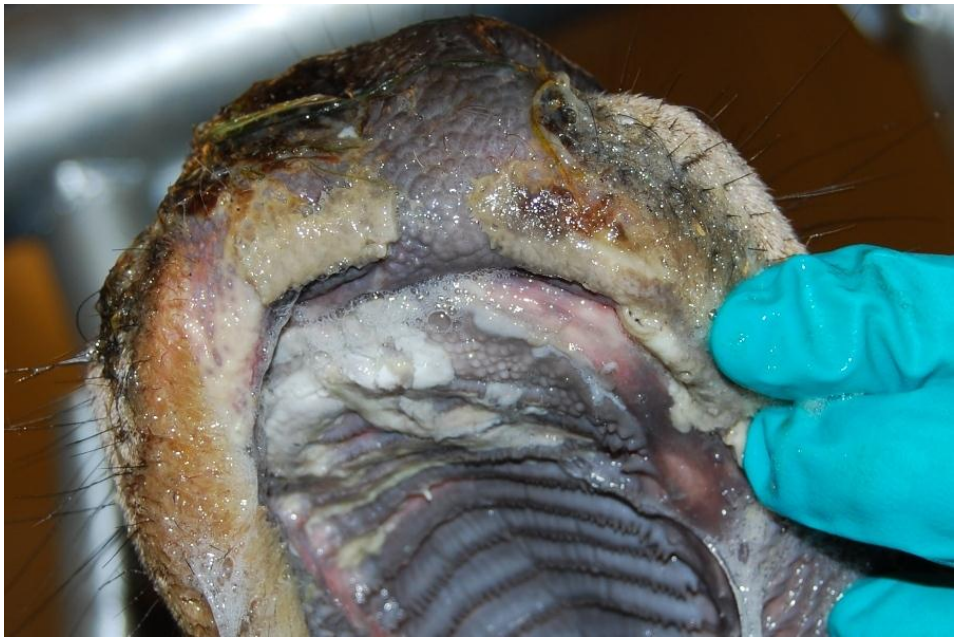

*Figure 11. Significant sloughing of epithelia from lips, dental pad and gums in calf infected with FMDV O UG 312/2006. This is the same animal as seen in Figure 10.*

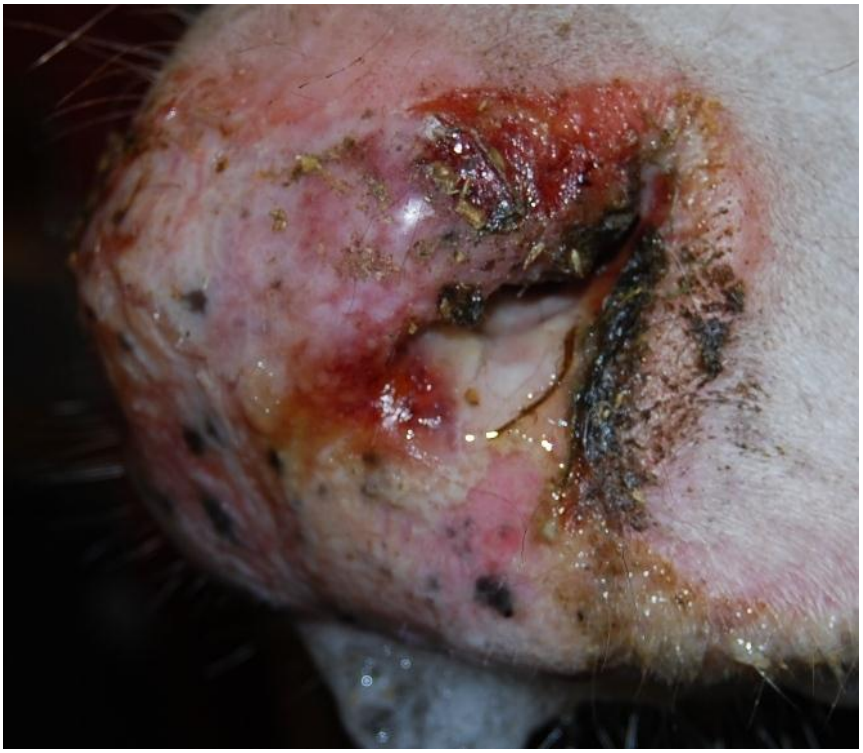

*Figure 12. Severe lesion on the muzzle of a calf during the acute phase of infection with FMDV O UG 312/2006.*

## Clinical experiment with FMDV O UKG 34/2001 in sheep

The experiment FMD 2S 2008 was designed to investigate the clinical appearance of this FMDV isolate in sheep. The experiment was designed to include 20, 4-month old, lambs of which 12 were directly inoculated through injection in the coronary band of one front limb, and remaining animals were infected through continuous contact with the inoculated animals.

The protocol used for routine sampling was similar to what has been described above for the cattle experiments.

### *Clinical appearance of infection*

All animals included in the experiment developed mild and transient symptoms of infection. 19 out of 20 animals developed vesicular lesions in the oral cavity, mainly visible as vesicles located at the dental pad and gums which were covered by thickened epithelia that fell of within a short period of time. The remaining individual was the only lamb which developed a visible foot lesion. This animal, which had not been directly inoculated, developed severe lameness on one hind limb and was unwilling to bear weight on this limb due to pain caused by clearly visible vesicle in the coronary band. The vesicle ruptured, with complete recovery of normal gait within one day of the first appearance of lameness.

The animals showed a significant increase in body temperature, which lasted only for about 1-2 days. There was a transient viremia, which was counteracted by the appearance of anti-FMDV antibodies in the circulation (Figure 16). There was no visible salivation or loss of appetite and with the exception of the one individual that showed lameness, the clinical signs of infection were only clearly visible through direct examination of the oral cavity.

An interesting detail of the clinical appearance of infection observed in this experiment is that during an experiment performed at the IAH-Pirbright, where sheep were infected with FMDV O UKG 34/2001, all animals were reported to have developed lesions in the feet, without any observable lesions within the oral cavity (Ryan Waters, personal communication).

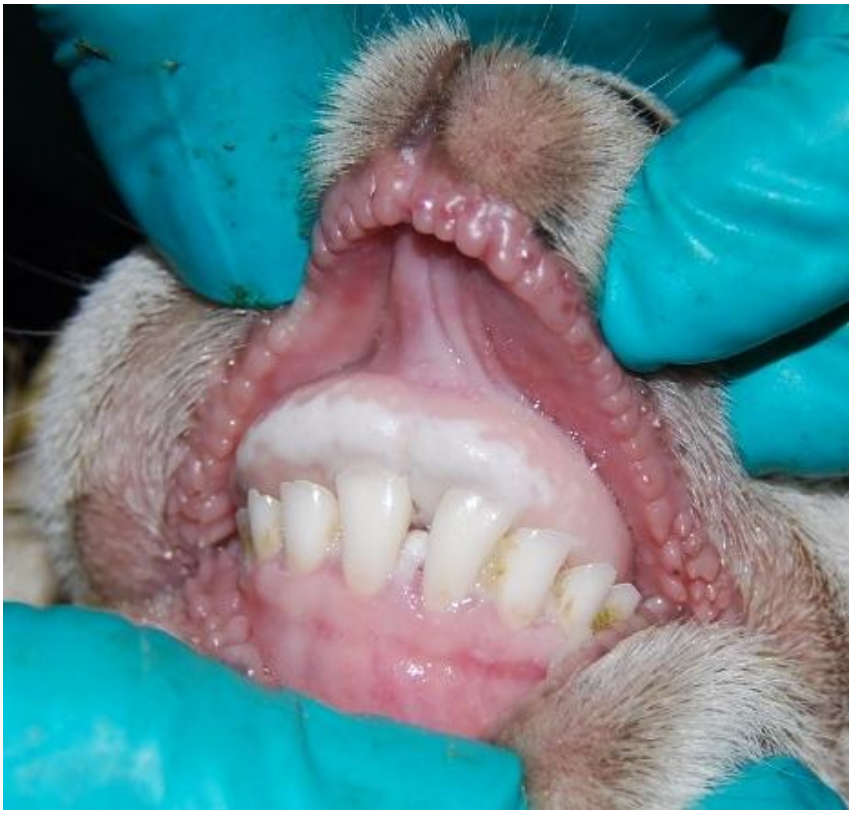

*Figure 13. Vesicular lesion on the dental pad of a lamb infected with FMDV O UKG 34/2001*

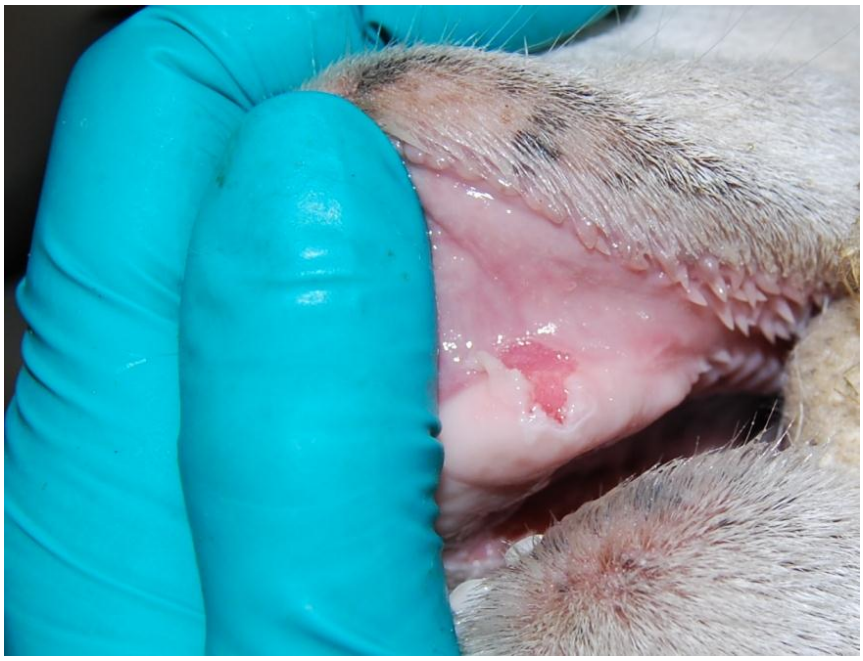

*Figure 14. Ruptured vesicular lesion on the dental pad of a lamb infected with FMDV O UKG 34/2001*

*Figure 15. Vesicular lesion in the coronary band of a lamb infected with FMDV O UKG 34/2001*

***Results of clinical and serological observations.***

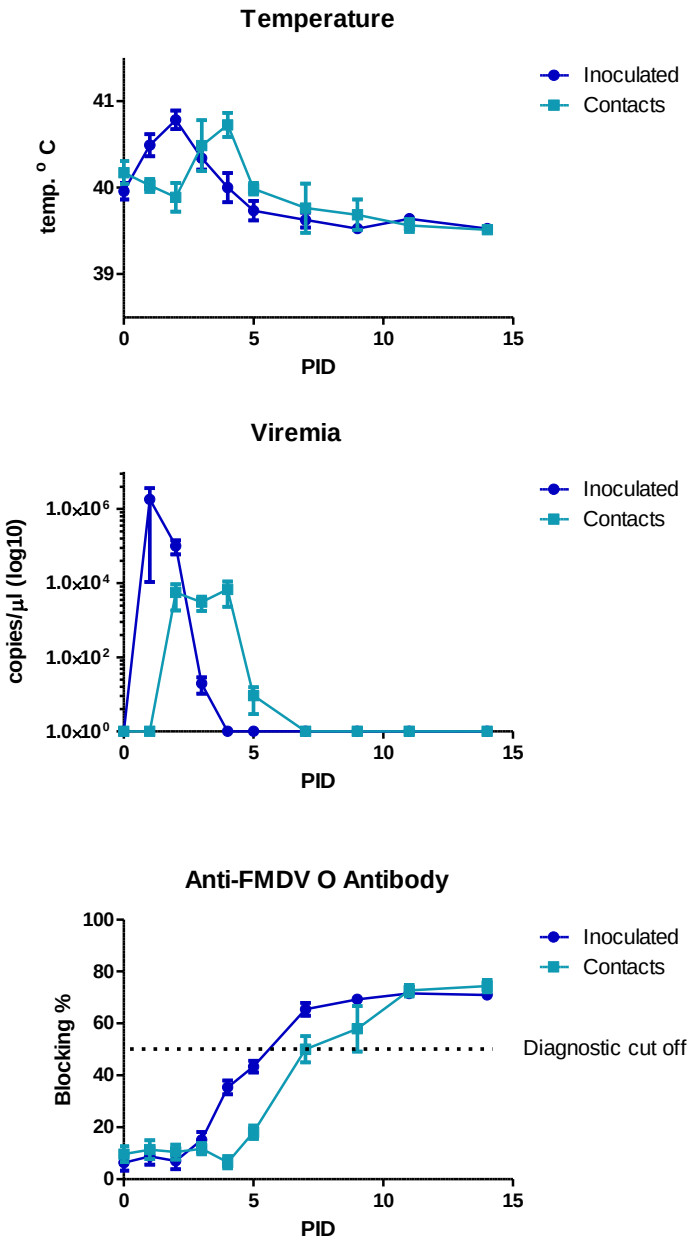


*Figure 16. Results of measurements of temperature, viremia and anti-FMDV O antibodies in sera of 20 sheep (12 inoculated and 8 contact infected) infected with FMDV O UKG 34/2001. The level of viremia was quantified by qRT-PCR and FMDV O antibodies was measured using a solid phase blocking ELISA assay, the methods used are described in detail in manuscript number 1.*

# Manuscript I

**Analysis of the acute phase responses of Serum Amyloid A, Haptoglobin and Type 1 Interferon in cattle experimentally infected with foot-and-mouth disease virus serotype O**.

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**Analysis of the acute phase responses of Serum Amyloid A, Haptoglobin and Type 1 Interferon in cattle experimentally infected with foot-and-mouth disease virus serotype O.**

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## Abstract

A series of challenge experiments were performed in order to investigate the acute phase responses to foot-and-mouth disease virus (FMDV) infection in cattle and possible implications for the development of persistently infected "carriers". The host response to infection was investigated through measurements of the concentrations of the acute phase proteins (APPs) serum amyloid A (SAA) and haptoglobin (HP), as well as the bioactivity of type 1 interferon (IFN) in serum of infected animals. Results were based on measurements from a total of 36 infected animals of which 24 were kept for observational periods exceeding 28 days in order to determine the carrier-status of individual animals. The systemic host response to FMDV in infected animals was evaluated in comparison to similar measurements in sera from 6 mock-inoculated control animals.

There was a significant increase in serum concentrations of both APPs and type 1 IFN in infected animals coinciding with the onset of viremia and clinical disease. The measured parameters declined to baseline levels within 21 days after inoculation, indicating that there was no systemically measurable inflammatory reaction related to the carrier state of FMD. There was a statistically significant difference in the HP response between carriers and non-carriers with a lower response in the animals that subsequently developed into FMDV carriers. It was concluded that the induction of SAA, HP and type 1 IFN in serum can be used as markers of acute infection by FMDV in cattle.

## Background

Foot-and-Mouth-Disease (FMD) is a highly contagious viral disease which affects cloven-hoofed animals including cattle, sheep and pigs, with substantial financial implications for affected countries. Severity of clinical disease varies between common domestic species, with pigs developing severe clinical illness, followed by cattle showing obvious but less severe clinical signs, whilst the clinical course of the infection in sheep may be very mild[5].

Foot-and-mouth disease virus (FMDV) is a positive stranded RNA virus. It is the prototype virus of the *Aphthovirus* genus within the picornavirus family. The viral genome includes a single large open reading frame encoding a polyprotein which is cleaved by virus-encoded proteases giving rise to structural and non-structural proteins needed for replication and assembly of new virus particles[11].

The predominant site of initial FMDV replication within infected animals is thought to be located within the epithelia of the pharyngeal mucosa[3,17,34,36], or alternatively within the lungs[14,15]. From here the virus spreads via the lymphatics and vascular system to the peripheral sites of secondary replication, characterized by the presence of stratified cornified squamous epithelia, such as the coronary bands and oral cavity[3]. Infected cattle develop transient viremia lasting for 2-3 days, which is effectively counteracted by the development of circulating anti-FMDV antibodies. The clinical disease follows a rapid time course and is typically manifested by a sudden rise in body temperature and development of vesicular lesions at peripheral areas of viral replication. Affected animals may display varying degrees of salivation, inappetence and lameness corresponding to the severity of lesions. The clinical course of the infection usually subsides within 7-14 days but the potential development of persistently infected carrier-animals creates further complications for disease control. FMDV carriers are defined as animals with asymptomatic, intermittent, presence of infectious virus in oropharyngeal fluid more than 28 days post infection (dpi)[38].

It is believed that in these animals (approximately 50% of infected cattle), FMDV is capable of persisting, at a low level, within pharyngeal epithelial cells[16,52], or as intact, but largely quiescent, viral particles within germinal centers in pharyngeal tissues[31]. Since carrier animals are a potential source of infectious virus, their presence is unacceptable in areas free of FMD.

Development of the carrier state is unaffected by the presence of neutralizing antibodies in the circulation. Thus, both animals that are immunologically naïve at the time of exposure to FMDV, as well as those with circulating antibodies due to vaccination or previous exposure

to the virus can become FMDV-carriers, regardless of pre-occurring clinical disease[16,22,38]. It is known that FMDV carriers do exhibit a measurable adaptive immune-response comparable to that of animals that clear the infection[4] but there is still a significant lack of knowledge regarding the innate immune-response to FMD in cattle. The duration of the carrier state varies between species, with the longest duration recorded in African buffaloes (5 years), followed by cattle (2 years) and sheep (9 months)[19,33,35,43]. Pigs do not become carriers[4]

The innate immune response induced by a viral infection in the upper respiratory tract is characterized by initial activation of peripheral primary effector cells which function to initiate a local inflammatory response, priming and recruiting activators of the cellular immune response. Macrophages present in the respiratory tract produce pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-1 and interferon (IFN) upon stimulation of pattern recognizing surface receptors, causing alterations in local vascular walls, and providing recruitment and activating stimuli to antigen presenting cells and phagocytes[10,47].

Type 1 IFNs are also known as viral IFNs and include interferon-α and -β. These IFNs are secreted by virus infected cells with the function of blocking spread of virus to uninfected cells by inducing alterations in gene transcription and protein synthesis following their interaction with common cell-surface interferon receptors[29,39,47]. It has been proposed that type 1 IFNs may have an important role in the host response to FMDV[4,18,44] and that the ability of the virus to induce an IFN response may be related to the pathogenicity of different isolates of FMDV[13,20].

Locally produced pro-inflammatory mediators also cause alterations in hepatic metabolism, inducing the production of various acute phase proteins (APPs) in the circulation[10]. APPs have been defined as proteins for which serum concentrations are significantly altered in acutely infected animals, compared to that of animals which are clinically healthy[24]. The proteomic pattern of the acute phase response varies between animal species and also depends upon the pathogen responsible for inducing the response[25,37].

Previous studies have shown a correlation between increased serum levels of bovine haptoglobin (HP) and the onset of clinical disease in bovine respiratory disease[24], as well as in infections with bovine respiratory syncytial virus[27]. Increased serum concentrations of HP in cattle acutely infected with FMDV through aerosol exposure, have also been demonstrated[28].

The production of HP and serum amyloid A (SAA) are considered reliable indicators of acute inflammation caused by various infectious agents in cattle[30].

The aim of this study was to investigate the innate immune response to infection with FMDV in cattle by measuring systemic levels of acute phase proteins SAA and HP as well as the biological activity of type 1 IFN in serum. Results from these measurements were evaluated in relation to the timing of the appearance of clinical signs of disease as well as the detection of viremia and circulating antibodies. Measured parameters were further analyzed statistically in order to evaluate whether there was any detectable difference in host response between the animals that developed into persistently infected carrier animals and those that were efficient in clearing the infection completely.

## Materials and Methods

### *Animal experiments and samples*

Animal experiments were performed in high containment research facilities at DTU-Vet, Lindholm Island, Denmark, in accordance with the requirements of the Danish Animal Experiments Inspectorate (License 2003/561-742; 2008/561-1541).

Three independent experiments were included in this study. The animals used were 4-5 month old steers of mixed-Holstein breed. The first experiment (FMD 2008) was performed with 12 animals (six inoculated and six in direct contact). In each of the two subsequent experiments (FMD 2010 a + b), three un-infected control animals, kept in a separate isolation unit, were used in addition to the twelve test animals. Results from this study are thus based on measurements in 36 FMDV infected animals and 6 un-infected controls. Apart from observations of clinical signs of disease and measurements of standard para-clinical parameters such as virus excretion and development of circulating antibodies (see description of protocol below), all three experiments included assays for the serum concentrations of the APPs (SAA and HP) as well as for the bioactivity of type 1 IFN in serum. In the first experiment (FMD 2008) animals were kept under observation for a total of 100 days post infection, whilst the experimental periods for the two subsequent experiments (FMD 2010 a+b) were 35 and 14 days post infection respectively. The following protocol was used in all three experiments.

All animals were pre-treated using a broad spectrum antibiotic (enrofloxacin; “Baytril©”; 2.5 mg/kg) for four days upon arrival in order to reduce the level of any existing bacterial

infection. Animals were subsequently allowed a period of acclimatization of a minimum of 10 days, and were further treated with β-lactam penicillin (”streptocillin©”; 5ml/100kg) for four days around inoculation to avoid interference caused by any bacterial infections.

Six animals were inoculated with FMDV O UKG 34/2001 (original inoculum obtained from the Institute for Animal Health (IAH)-Pirbright, and then passaged once in cattle) using subepidermo-lingual injection, each animal receiving approximately $10^{6.9}$ $TCID_{50}$ in a volume of 0.5 ml administered at 6 to 8 injection sites at the base of the tongue. A standard protocol for sedation consisting of intravenous injection of Xylazinhydrochloride (Rompun© 2%; 1,5 ml/ animal) was used for inoculation and the sedation was reversed through intravenous administration of Antipamezol (Antisedan©; 0,5 ml/ animal). Six other animals were kept in continuous direct contact with the inoculated animals, with two inoculated plus two contact animals in each pen and with the stable facilities allowing direct contact between animals in separate pens.

All animals were monitored daily, with measurements of rectal temperature and observation of clinical signs. Serum samples were collected daily from 3 days prior to inoculation and throughout the first two weeks of the experiments and thereafter on a weekly basis throughout the remaining part of the experiment. Blood samples were stored at 4$^{o}$C overnight, before centrifugation and the sera were subsequently stored at -70$^{o}$C.

Samples of oropharyngeal fluid (probang samples)[45] for quantification of virus excretion, in order to define the carrier status of individual animals[50], were collected prior to inoculation, once daily during the first week after inoculation, then every other day throughout the second week and on a weekly basis subsequently. Probang samples were collected at closer intervals from post infection day (PID) 28 until termination of the experiment so that the carrier status of individual animals was based on the analysis of a minimum of four samples. Control animals were handled and sampled following a protocol similar to that used for infected animals. The animals were sedated and “mock-inoculated” using phosphate buffer, in order to mimic the procedure used for inoculation and sample collection in the test groups.

### *Quantification of Acute Phase Proteins in serum*

#### *Serum Amyloid A*

Serum concentrations of SAA were determined using a sandwich-ELISA kit (Tridelta Developments Ltd. Maynooth, County Kildare, Ireland). Samples were analyzed at a dilution

of 1:500, according to the manufacturer's instructions. A standard dilution of the SAA calibrator included in the kit was added in duplicate to each of the ELISA plates and SAA concentrations in the sera were determined from the standard curves.

*Haptoglobin*

Serum concentrations of HP were determined using a sandwich-ELISA, following a protocol described in detail previously[27] using monoclonal anti-bovine haptoglobin antibodies kindly provided by Dr. Philip Griebel, University of Saskatchewan, Canada.

Briefly, microtitre plates were coated with anti-bovine haptoglobin ascites fluid in carbonate buffer. Serum samples were tested at three dilutions (1:100; 1:300; 1:900), together with a calibration standard of acute phase serum with known concentrations of HP. Bound antigen was detected using biotinylated anti-bovine haptoglobin Mabs, by staining with TMB reagent in citrate buffer. The colour development was terminated by addition of sulfuric acid, and OD-values were read using a standard ELISA reader at wavelengths of 450/650nm.

***Quantification of the bioactivity of Type 1 IFN in serum***

Biological activity of type 1 IFN was quantified with an Mx/CAT reporter gene assay[23], using the transfected MDBK-t2 cell line kindly provided by Dr. Bryan Charleston (IAH-Compton, UK). Briefly, the cells were seeded in 24-well plates and cultured in 1500µl of medium (EMEM with blasticidin (10µg /ml), penicillin (100 IU /ml), streptomycin (100µg /ml) and 10% FCS).

Serum samples, diluted 1:5 in cell culture medium containing 2% FCS, were added to the cells and incubated overnight at 37$^{o}$C. All samples were tested in duplicate, in parallel with standard concentrations (0.125 to 90 IU/ml) of recombinant human IFN-α (Invitrogen). Cells were harvested and CAT-expression was quantified using a commercial CAT-ELISA kit (Roche Applied Science) following the manufacturer's instructions.

***Quantification of anti-FMDV antibodies using a solid phase blocking ELISA***

Serum concentrations of FMD specific antibodies were measured using a serotype-specific solid phase blocking ELISA[9]. In brief, microtitre plates were coated with guinea-pig immune sera raised against FMDV O Manissa before addition of inactivated FMDV antigen. Samples of the bovine sera were then added, incubated overnight and then rabbit anti-FMDV serotype O serum was added and the bound antibodies were detected using horseradish-peroxidase

conjugated porcine anti-rabbit IgG. All serum samples were initially screened at a dilution of 1:5 and positive samples (blocking percentage >50%) were analyzed in a two-fold titration starting at a dilution of 1:10 to allow determination of antibody titers.

***Quantification of viremia and excretion of FMDV RNA in probang samples***

Quantification of FMDV RNA in serum and probang samples was performed using quantitative RT-PCR[50]. Total RNA was extracted using a MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche) with an automated robotic workstation (Roche) from 200µl of sample, according to the manufacturer's instructions. Each RNA sample was eluted in a volume of 50µl and stored at -70$^{o}$C until further processing. Reverse transcription of FMDV RNA was carried out using 6µl of extracted RNA in a total volume of 15µl, using a TaqMan RT kit with random hexamer primers (Applied Biosystems) at 48$^{o}$ C for 45 minutes and 95$^{o}$ C for 5 minutes. Then 7µl of each cDNA was mixed with 18µl of 2x TaqMan universal PCR mastermix (Applied Biosystems) containing 22.5 pmol of each primer and 5 pmol of fluorescently labeled probe. PCR amplification was carried out for 50 cycles, in an Applied Biosystems Model Mx 3005P Thermal cycler and analysed using MxPro qPCR software.

***Statistics***

APP responses were log-transformed and a response measure representing the area under the curve was constructed, where linear interpolation between points of observations was used, and a slight extension at end points to put equal weight on observations evenly spaced in time. APP responses were analyzed with a standard random effects model [8], where the identification of the three sequentially performed experiments ("survey") was entered as a random effect. Fixed effects were Carrier status ("Carrier"), route of infection ("status": inoculated, contact or control), level of viremia and maximum anti-FMDV antibody titre. The effect of the carrier-status of individual animals was modeled as a covariate with the values 1/0 representing carrier/ non-carrier. Tests were performed as likelihood ratio tests, according to the following model,

$$\log(AUC_{ij}) = \beta_A \log(\text{Antibodies})_{ij} + \beta_V \text{Viremia}_{ij} + \beta_C \text{Carrier}_{ij} + \beta_{\text{Status.ij}} + \eta Y_{\text{Survey.i}} + \varepsilon_{ij,}$$
$$i=1:3, j=1:n_i,$$

where β represents coefficients of effect and η represents the standard deviation between experiments (i).

Model control was performed with standard techniques. For the APP SAA, the model control revealed that criteria were not met for the response measure, and the tests were subsequently performed as z-tests (continuous fixed effects). All analyses were performed with Splus© software version 6.1 (Insightful Corp. 2002).

## Results

### *Clinical symptoms*

All animals included in the test groups developed mild to moderate clinical signs of FMD, with an increase in body temperature (Figure 1a) and vesicular lesions in the oral cavity being the most prominent findings occurring in all individuals. Less than half of the animals developed vesicular lesions on the feet and only one individual (out of 36) developed clearly visible lameness. Inoculated animals developed clinical signs of FMD at PID 1-2, whilst onset of clinical disease in contact animals was observed from PID 2-5. The animals showed no loss in appetite and all animals recovered from clinical disease within approximately seven days, without further complications, and without the need of supportive medical treatment.

### *Viremia and development of circulating antibodies*

FMDV RNA was detectable in serum from all infected animals for a period of four to seven days (Figure 1b). Inoculated animals showed significant viremia at PID 1, reflecting the very rapid replication and systemic spread of FMDV. Anti-FMDV serotype O antibodies in serum reached the diagnostic cut-off level, defined as a blocking percentage of 50%, at around PID 4 to 5 in inoculated animals and at PID 7 to 9 in the contact group (Figure 1c). Appearance of circulating antibodies was accompanied by a rapid reduction in viremia. There were no detectable differences in the level of viremia (presented as the number of copies of FMDV genome per µl of serum) or the anti-FMDV O antibody titres (data not shown) between directly inoculated and contact infected animals, or between carriers and non-carriers.

### *Prevalence of Carriers*

Carrier status was determined on the basis of detection of FMDV RNA, using qRT-PCR, in probang samples beyond 28 days post infection[50]. An animal was regarded as being a carrier following detection of at least two positive probang samples (Ct value < 40) out of a

minimum of four samples collected beyond PID 28. Detailed information on the extent of virus excretion in probang samples in carriers and non-carriers has been included in a separate report[41]. In summary, animals identified as FMDV carriers had detectable levels of FMDV RNA (Ct<40) in probang samples harvested throughout the entire length of the experimental period whereas FMDV RNA became undetectable in probang samples from the non-carriers by approximately 14 days post infection. One of the experiments (FMD 2010b) was terminated at 14 days post infection, for technical reasons, and so it was therefore not possible to determine carrier-status in this experiment. In the remaining two experiments (FMD 2008 and FMD 2010a) the prevalence of carriers was 6/12 (50%) and 3/12 (25%) respectively (see Table 1), consistent with what has been found previously in experimental infections with FMDV [2,5,35]

***Acute phase proteins***

The serum levels of APPs showed a consistently occurring peak, coinciding with the onset of clinical symptoms (Figure 1e-f). Directly inoculated and contact animals showed similar responses when the areas under the curve (AUC) were compared for the serum concentrations of SAA during the observation period of 14 days after infection. For the HP-response, there was however, a small but statistically significant difference ($p=0.04$) between inoculated and contact animals, with a larger response in the directly inoculated animals. There was a very marked and statistically significant ($p<0.0001$) difference in the AUC values for both SAA and HP when comparing test-groups (FMDV inoculated and contacts) to the uninfected control animals.

It is interesting to note that, there was also a statistically significant difference ($P=0.015$) in the AUC values for the serum concentration of HP between the animals that became carriers and the non-carriers, with lower values in the carrier animals (Figure 2f; table 2 and 3). The difference in the HP-response between carriers and non-carriers was unrelated to, and could therefore not be explained by, the observed difference between inoculated and contact-infected animals for this parameter. There was no difference in the AUC values for serum concentration of SAA between carriers and non-carriers (Table 2).

***Type 1 IFN***

Type 1 IFN bioactivity, as measured by the activation of the Mx promoter in the MDBK-t2 cells, showed a reaction pattern similar to the APPs, with a marked peak at the onset of

clinical symptoms and thus significant differences in response between the infected test-groups and uninfected control animals. Contact animals seemed to show slightly lower peaks in IFN response than directly inoculated animals (Figure 1d), although this difference was not statistically significant (Table 2). There was no difference in the AUC values for the type 1 IFN bioactivity between carriers and non-carriers (Table 2).

**Discussion**

The observed progression of the clinical infection, including the level of viremia and titres of circulating antibodies, was similar to what has been described previously for infection with FMDV O UKG/34 2001 in cattle[1,49].

Measurements of serum concentrations of the APPs, SAA and HP, indicated a rapid acute phase response which coincided with the appearance of clinical signs of disease. It has previously been proposed that the sensitivity of SAA as a marker of infection could be higher than that of HP[30]. In our studies we observed a relatively high basal level and measurable changes in the level of SAA in some of the mock-inoculated control animals, although by no means approaching the significantly up-regulated concentrations observed in animals infected with FMDV. In contrast to this, we recorded very low basal levels in the HP measurements within uninfected animals, whilst both APP markers showed a clear response with significantly increased concentrations within the sera from infected animals. It has been shown that changes in SAA levels are more easily induced by physical stress than for HP[7]. It may be that the procedures used for handling of animals for sampling, including the protocol for sedation used for inoculation, could be responsible for inducing a significant stress response in the animals, which might be the cause of the observed SAA response in some of the mock-inoculated control animals.

The acute phase response to FMD in cattle has previously been investigated by Hofner *et al.*[28] using measurements of HP in sera from cattle that were infected with FMDV O BFS 1860 following aerosol exposure. It was reported that an increase in serum concentrations of HP occurred 8-9 days after virus exposure and 1-3 days after the onset of viremia and appearance of clinical signs. It was concluded that there was no measurable rise in HP during the "pre-viremic" phase of FMD infection. Furthermore, it was proposed that this finding was indicative of little or no tissue damage during initial replication of FMDV in the pharyngeal epithelia.

The clinical course of FMD in our experiments differed slightly from what was described by Hofner *et al.*[28]. We observed a more rapid course of infection, with simultaneous appearance of viremia and clinical signs of disease, including an increase in body temperature, in both directly inoculated and in contact animals. We also detected an increase in serum concentrations of HP as well as SAA, which for the majority of the animals began at the same time as the observed onset of clinical disease and appearance of viremia. Thus, there was no apparent lag phase between the rise in body temperature and the measurable acute phase response in serum.

The differences in clinical observations may partly be a result of the different means of inoculation used in the two studies; direct inoculation in our studies versus aerosol exposure in the study performed by Hofner *et al.*[28]. However, in our studies, we also saw similar timing of reactions in animals infected by contact exposure as in those that had been directly inoculated with simultaneous detection of viremia and clinical parameters. Thus, the observed variations in results are more likely to be derived from differences in sensitivity of the different assays used for quantification of HP and detection of viremia in the two studies. In the earlier study, the HP concentration was quantified through an assay measuring hemoglobin binding capacity while viremia was detected by inoculation of primary cell cultures, whereas we measured HP with a specific and sensitive ELISA assay and viremia with a quantitative RT-PCR.

The measured changes in the bioactivity of type 1 IFN in serum followed a similar pattern to what was recorded for the APPs, with measurable peaks in activity of IFN in the sera at the onset of clinical disease. There seemed to be a slight, although not statistically significant, difference in maximum peak levels of IFN bioactivity in inoculated versus contact animals. Animals in the contact groups acquired the infection through continuous exposure to virus excreted by infected animals, whereas the inoculated animals received a single high level dose of virus in the tongue. The different level and route of virus access to the primary site of replication is likely to cause a variation in the timing and synchronization of the initial cellular immune response responsible for the measured cytokine response.

It is believed that the acute phase response of the liver is induced in response to the presence of inflammatory cytokines in the circulation[10]. Our results indicate near simultaneous timing for the onset of induction of APPs and type 1 IFN in both inoculated and contact-infected animals, with the rate of the initial response in SAA slightly exceeding that of HP. It is possible that the very rapid onset of viremia, with high levels of virus in the blood seen as

early as PID 1 in the inoculated animals, could in some way influence the hepatic APP response in a more direct manner.

Type 1 IFN is produced by virus-infected cells, as well as by inflammatory cells (macrophages and cells of the dendritic cell lineage) present within the circulation and peripheral tissues[21,48]. Previous studies have shown an up-regulation of IFN-α and-β mRNAs within epithelial cells harvested from FMDV lesions of the oral cavity[48]. We have attempted to quantify IFN bioactivity in samples of oropharyngeal scrapings (probang samples) without any success (data not shown), possibly indicating that the activity of IFN is somehow inhibited in this type of sample.

Recent studies in cell culture have demonstrated that the leader protease (Lpro) of FMDV is capable of down-regulation of transcription of IFN-β through interaction with NFκB[20,46]. The Lpro also inhibits host cell protein synthesis[12,32], thus within FMDV-infected cells the expression of IFN mRNA and its translation should be blocked. This may suggest that the IFN present in serum is not produced by cells that are actually infected with FMDV but by populations of immune cells that react upon the presence of virus through activation of pattern recognizing receptors. Plasmacytoid dendritic cells, also referred to as natural interferon producing cells (NIPC), are known to produce high levels of type I IFN in response to virus infection[40]. It is, however, also suggested that it is necessary for non-enveloped viruses such as FMDV to be complexed by bound antibody in order to induce IFN production by cells of this lineage[26]. This putative relationship does not match the timing of events observed in our study as the increase in type 1 IFN activity in serum was detected very early (PID 1-2) which significantly precedes the rise in circulating anti FMDV-antibody. Investigations of the cellular source of the systemically measurable rise in IFN activity might help to further elucidate the interactions between the virus and the immune response of the host. Previous studies have reported an increase in IFN-α mRNA levels in nasal associated lymphoid tissue harvested from the pharyngeal region of calves acutely infected with FMDV serotype O [51]. The same study also reported a difference in the expression of tumor necrosis factor-α (TNF-α) between carriers and non-carriers in similar samples collected from a total of 6 animals at 62 days post infection. We have been unable to find evidence of an up-regulation in IFN mRNA levels in samples of pharyngeal mucosa collected at sequential time points during infection. However, we did corroborate the previous report of a difference in TNF-α mRNA expression in the pharyngeal epithelium of FMDV-carriers and non-carriers during the late phase of infection (>PID 28)[42]

Alsemgeest *et al*.[6] compared serum concentrations of SAA and HP in acute and chronic inflammation in cattle. It was found that the HP/SAA ratio differed during different stages of infection, with the serum concentration of SAA exceeding that of HP in acute inflammation and the opposite occurring during chronic inflammation. In our studies, we saw that concentrations of both SAA and HP declined to baseline-levels by PID 14-21, regardless of whether the animal was persistently infected with FMDV or not. These findings indicate that the carrier status in FMDV infection in cattle is not accompanied by a state of systemically measurable chronic inflammation.

There was no measurable difference in the serum reactions of SAA and type 1 IFN between infected animals which became carriers or non-carriers. For HP, however, there was a statistical difference in the AUC values between animals which were identified subsequently as carriers and non-carriers with a lower HP level in carriers. The observed difference in the HP response is most clearly visible at the latter part of the timescale when viewing the log-transformed data (figure 2f). This data may be an indication of slightly prolonged reaction in HP in the sera of the animals that were successful in clearing the infection completely. When extending the observational period to include analysis of sera collected at PID 21 it was, however, clear that the serum concentration of HP did decline to baseline levels (undetectable serum concentrations) in all animals regardless of carrier-status.

The observed difference in HP-response between directly inoculated and contact infected animals, which only just reached statistical significance ($p=0.04$), could possibly (as previously discussed for the IFN-response), be explained by the differences in the routes and timing of virus exposure.

The relationship between the HP response and the development of FMDV carrier animals should be further investigated in future experiments to elucidate the nature of the apparent difference in the acute host response to FMDV between carriers and non-carriers.

There might also seem to be a slight difference in the time course of SAA and HP responses between animals which became carriers and non-carriers, with a more rapid pattern of reactions in the carrier-group. This apparent difference is, however, most likely caused by a variation in the timing of the reactions between inoculated and contact animals, with a relatively larger number of inoculated animals, compared to contact-infected animals within the group of carriers. However, the route of infection (directly inoculated versus contact-

infected) did not have any effect on whether the animal developed into a persistently infected carrier or not (Table 1).

Observations from our experiments indicate that combined measurements of serum concentrations of SAA and HP can be used as markers of an acute systemic response to FMDV infection in cattle. This could, for example, be of value during vaccine studies or transmission experiments with FMDV isolates of low virulence, where it would be of interest to detect any systemic immune response in animals exposed to the virus. Studies comparing the patterns of the systemic acute phase response in experiments with FMDV isolates of varying virulence have been initiated. Preliminary results indicate a close relationship between the timing and magnitude of the serum APP response with the severity of clinical disease, providing an objective tool for validation of severity of infection.

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**Table 1**

Distribution of FMDV carriers within the directly inoculated and contact infected animals.

| Experiment ID | Number of animals (Inoculated: Contact) | Carriers (Inoculated: Contact) | Non-Carriers (Inoculated: Contact) |
|---|---|---|---|
| **FMD 2008** | 12 (6:6) | 6 (4:2) | 6 (2:4) |
| **FMD 2010 a** | 12 (6:6) | 3 (1:2) | 9 (5:4) |

**Table 2**

Summary of P-values for comparisons of the AUC values of serum concentrations of APPs and IFN during a test period of 14 days after inoculation.

Levels of significance: ***= p<0.0001; **= p<0.001 *=p<0.05

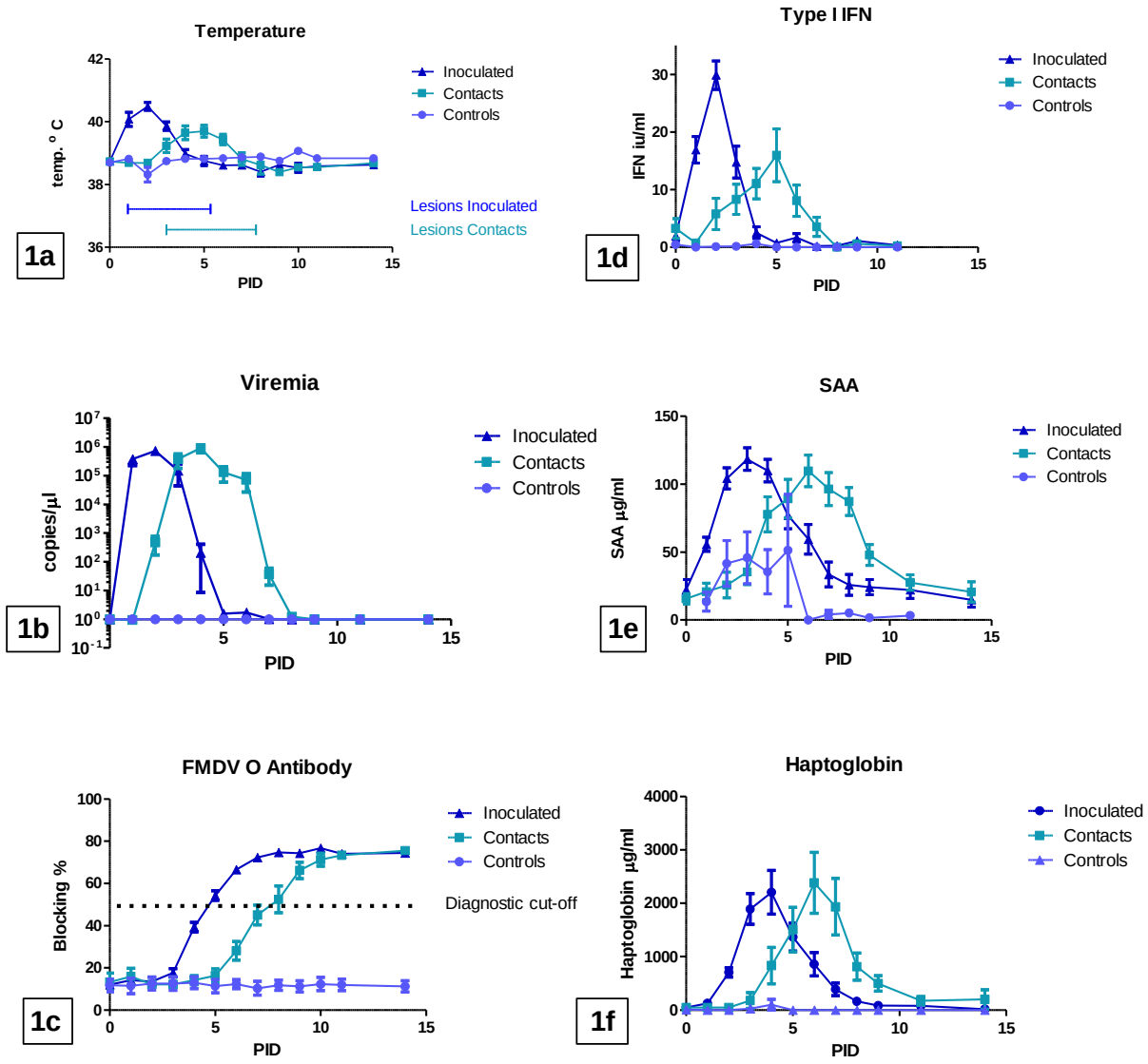
| | Inoculated and Contacts vs Controls | Inoculated vs Contacts | Carriers vs. Non-Carriers |
|---|---|---|---|
| **SAA** | <0.0001*** | 0.61 | 0.49 |
| **IFN** | <0.0001*** | 0.43 | 0.27 |
| **Haptoglobin** | <0.0001*** | 0.04* | 0.015* |

**Table 3**

Coefficients of effect (β) for contribution to AUC values of serum HP of individual animals for statistically significant effects: carrier-status ("Carrier": carrier (1)/ non-carrier (0)), route of infection ("Status": inoculated/ contact-infected (uninfected controls are not included in this analysis as these do not become carriers)). η represents standard deviation between experiments (i)

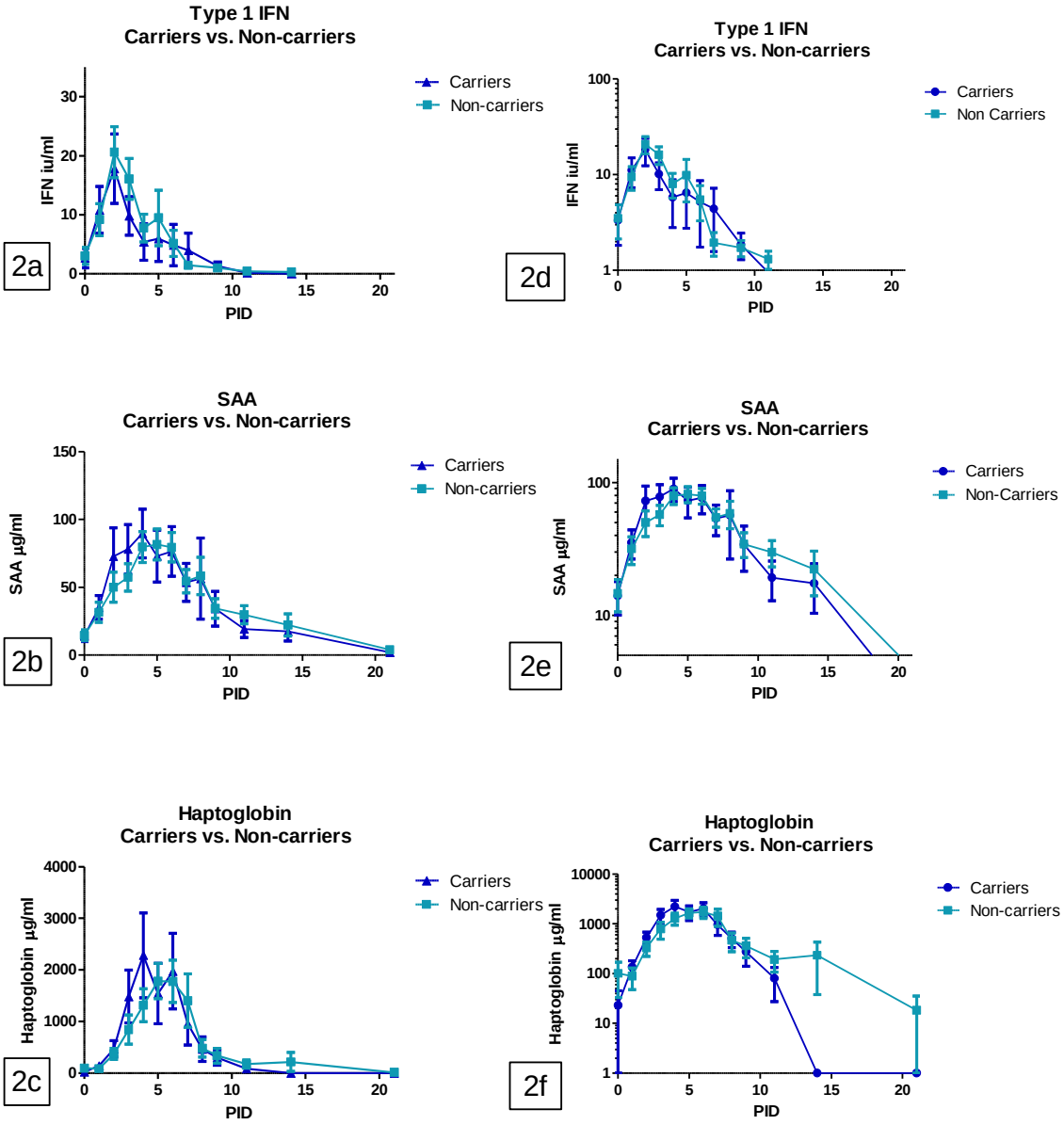
| **Final model** | $\log(Hp_{ij}) = \beta_C Carrier_{ij} + \beta_{Statusij} + \eta Y_{Surveyi} + \varepsilon_{ij}, i=1:3, j=1:n_i,$ | | |
|---|---|---|---|
| | | **$\beta_C$** | **95% confidence interval** |
| **Carrier Status** | Carrier | -1.14 | -1.98<<-0.3 |
| | Non-carrier | 0 | (NA) |
| | | **$\beta_{Status}$** | **95% confidence interval** |
| **Route of infection** | Inoculated | 4.52 | 3.19<<5.85 |
| | Contact | 3.65 | 2.34<<4.96 |

**Figure 1**




**Characterization of clinical signs and acute phase responses in FMDV-infected cattle.** The results shown are the mean values (+/- S.E.M) for the indicated parameters from three separate experiments each including 12 or 15 cattle (6 inoculated with FMDV, 6 in-contact and 3 uninfected controls in 2 of the experiments). a) Temperature and timing of occurrence of characteristic vesicular lesions within the oral cavity. b) Viremia measured by qRT-PCR expressed as copies of FMDV genome/ µl of serum c) anti-FMDV (type O) antibodies in serum expressed as blocking percentage derived from a solid phase blocking ELISA d) Bioactivity of type 1 IFN in serum (iu/ml) E) Haptoglobin in serum (µg/ml) f) Serum Amyloid A in serum (µg/ml).

## Figure 2




**Comparison of acute phase protein and IFN measurements in carrier and non-carrier cattle on linear (a-c) and logarithmic scale (d-f).** a+d) Bioactivity of type 1 IFN in serum (iu/ml) b+e) Serum amyloid A in serum (µg/ml) c+f) Haptoglobin in serum (µg/ml)

# Manuscript II

## Modulation of cytokine mRNA expression in pharyngeal epithelial samples obtained from cattle infected with foot-and-mouth disease virus

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# Modulation of cytokine mRNA expression in pharyngeal epithelial samples obtained from cattle infected with foot-and-mouth disease virus.

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## Abstract

A novel technique of endoscopical collection of small tissue samples from the pharyngeal mucosa of live cattle has been developed. This methodology was used to obtain sequential tissue samples from the dorsal soft palate (DSP) of individual cattle infected with foot-and-mouth disease virus (FMDV) at different phases of the infection. Levels of mRNAs encoding interferon (IFN)-α and-β as well as tumor necrosis factor-α (TNF-α) were measured in these samples using quantitative RT-PCR assays. Expression levels of IFN-β mRNA were significantly down-regulated in the biopsy samples harvested during the acute phase of infection, whereas there was no statistically significant effect on the expression of the IFN-α mRNA compared to baseline levels. In contrast, the TNF-α mRNA was significantly up-regulated in samples collected during both acute and late (>28 dpi) phases of infection. There were also significantly higher levels of TNF-α mRNA expressed in samples derived from animals that were identified subsequently as persistently infected FMDV-carriers. It was concluded that there was a significant difference in the host response in the DSP of calves that were detected as persistently infected, subclinical carriers of FMDV.

## Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral infection of huge financial importance for countries with substantial exports of agricultural products. The disease affects cloven hoofed animal species such as pigs, cattle and sheep with rapid spread amongst susceptible animals. The infection is typically manifested through an increase in body temperature and the development of characteristic vesicular lesions in areas covered by cornified epithelia such as the oral cavity and coronary bands (reviewed by Alexandersen *et al.*[5]). Mortality rates are low amongst adult animals but the occurrence of persistently infected subclinical "carriers" of FMD-virus (FMDV) in ruminant species causes further complications for disease control. FMDV carriers are defined as animals with an intermittent presence of infectious FMDV in oropharyngeal excretions more than 28 days after initial infection[23].

FMDV carriers generally mount an adaptive immune response comparable to that seen in animals which are effective in clearing the infection, with rapid development of high titres of anti-FMDV antibodies in the circulation[4]. A possible difference in the characteristics of early FMDV infection in carriers versus non-carriers, with indications of a difference in the rate of decline in virus excretion in oropharyngeal fluid following the acute clinical infection has been reported[35]. There has also been a report of higher levels of FMDV-specific IgA detectable in the oropharyngeal fluid of cattle that were identified as FMDV-carriers following vaccination and subsequent virus challenge[21]. There is, however, a lack of specific knowledge regarding the innate immune response to FMDV in cattle and its possible role in the development of persistently infected carriers.

FMDV is a positive stranded RNA virus belonging to the *Aphthovirus* genus within the picornavirus family. The viral genome consists of a single copy of RNA encoding a polyprotein from a single large open reading frame. Individual virus proteins are produced by the action of the virus encoded leader- (Lpro) and 3C- proteases giving rise to the structural and non-structural proteins needed for viral RNA replication and assembly of new virus particles[7]. The Lpro of FMDV is also responsible for inhibition of host cell protein synthesis through cleavage of cellular translation initiation factors essential for cap-dependent translation of host cell mRNAs[7,17].

Virus infections of the upper respiratory tract are usually sensed by effector cells of the innate immune system, such as macrophages and other cells of the monocyte-lineage, which are

present in the respiratory mucosa. Activation of pattern recognizing cellular receptors leads to production of an array of pro-inflammatory cytokines, including tumor necrosis factor –α (TNF-α) and the type 1 interferons (IFNs). TNF-α is a "common" pro-inflammatory cytokine with the function of inducing a local inflammatory reaction. The type 1 IFNs, including IFN-α and –β, function by interacting with common cell-surface interferon receptors leading to downstream induction of transcription of a number of genes involved in preventing spread of virus to uninfected cells[24,30]. Induction of type I IFNs has been reported to occur through signaling pathways involving NF-κB as well as interferon regulatory factors 3/7[14] by activation of either one of two complementary receptor systems[27]. The first pathway of activation is through a class of ubiquitously expressed cytosolic receptors which function to detect viral nucleic acids in infected cells and can be found in almost all types of nucleated cells[27]. The second class of receptors comprises the Toll-like receptors (TLRs) which belong to a family of pattern recognizing receptors (PRRs) that detect molecular features shared by entire classes of pathogens[11]. The TLRs involved in the host response to virus infections are mainly located in the membranes within intracellular compartments of phagocytic cells[11], as a result of this, the TLR induced pathway of IFN activation does not require that the cell itself is actively infected by the virus[27].

It has been demonstrated that it is necessary for FMDV to be complexed with immunoglobulins in order to induce transcription of IFN by porcine plasmacytoid dendritic cells in culture[13]. We have previously reported a consistent and clearly detectable systemic acute phase response in cattle experimentally infected with FMDV serotype O that is characterized by an increase in the bioactivity of Type 1 IFN in serum and by up-regulated concentrations of the acute phase proteins (APPs) serum amyloid A and haptoglobin[26]. The increase in APPs and type 1 IFN within serum was coincident with the onset of clinical signs of infection. The rise in IFN occurred at a much earlier time point than the detection of anti-FMDV antibodies in the circulation and has therefore indicated the need for further investigations of the cellular source of the IFN response.

Clinical studies investigating the pathogenesis of FMD in cattle have detected the persistence of FMDV in epithelial cells[36] and germinal centers[15] of the pharynx. The pharyngeal epithelium has also been reported to be involved in FMDV replication during the early phases of infection[3,8,18,20] and this specific anatomical site is therefore of significant interest when investigating FMDV pathogenesis.

The aim of this study has been to investigate the local tissue response, through quantification of mRNA levels encoding IFN-α and -β, as well as TNF-α in tissue samples collected from the pharyngeal mucosa of FMDV infected cattle. The cytokine mRNA expression profiles were analyzed in order to detect potential differences in the local tissue response of the pharyngeal epithelia, during both acute and chronic phases of the infection, between animals that developed into FMDV carriers and those that did not.

## Materials and Methods

### *Animal experiments and samples*

Animal experiments were performed in biosecure research facilities at DTU-Vet, Lindholm Island, Denmark, in accordance with the requirements of the Danish Animal Experiments Inspectorate (License 2003/561-742; 2008/561-1541). Three independently performed experiments, using a similar experimental set up, were included in this study (see Table 1). Each of the experiments included 12 steers (4-5 months old) of mixed-holstein breed. The general experimental protocols have been described in detail elsewhere[26]. In brief, six animals in each experiment were inoculated with FMDV O UKG 34/2001 (original inoculum obtained from IAH-Pirbright, UK, and then passaged once in cattle) using subepidermo-lingual injection, each animal receiving approximately $10^{6.9}$ $TCID_{50}$ in a volume of 0.5 ml. Six other animals were kept in continuous direct contact with the inoculated animals, with two inoculated plus two contact animals in each pen and with the stable facilities also allowing direct contact between animals in separate pens.

All animals were monitored daily, with measurements of rectal temperature and observation of clinical signs. Serum samples were collected daily from 3 days prior to inoculation and throughout the first two weeks of the experiments but then on a weekly basis throughout the remainder of the experiment.

Samples of oropharyngeal fluid (probang samples) were collected, for the quantification of virus shedding, as well as for determining the carrier status of individual animals. Samples were obtained prior to inoculation, once daily during the first week after inoculation, then every other day throughout the second week and on a weekly basis subsequently. Additional samples were collected from post infection day (PID) 28 in order to have probang samples from a minimum of four separate days for determination of carrier status.

Small samples of tissue from the dorsal soft palate was collected using a fiberscope (Vet-vu 2FSb) fitted with cutting biopsy forceps, at three or four time points during the experiments. Animals were sedated through intravenous injection of xylazine (Rompun vet© 1.5 ml/ animal) and positioned in ventral recumbency. The fiberscope was introduced through one nostril and positioned to allow clear visualization of the nasopharynx, with the entrance of the trachea in central view. Tissue samples were collected from the ventral surface (constituting the dorsal soft palate), just to either side of the mid plane, rostral to the epiglottis. The site of sampling is believed to house a high density of diffuse lymphoid tissue, which has been corroborated through standard HE staining of randomly selected biopsy samples (Figure 1). Two pieces of tissue, approximately 1x1x1 mm in size, were harvested from slightly different locations within the sampling area and included in each sample.

The first biopsy sample from each animal was collected one week prior to inoculation (reference sample). The second biopsy was collected during the acute phase of infection, defined as the first day when the animals showed a rectal temperature of above 40$^{o}$C (phase 1), with a third sample being collected 7 days later (phase 2). In the first of the three experiments (FMD 2008), animals were kept under observation for a period of 100 days after inoculation. In this experiment, final collection of tissue samples from the pharyngeal mucosa was carried out through an additional endoscopical examination at PID 28 (phase 3). The other two experiments (FMD 2010 a and c) were terminated at PID 31-35 with post mortem examinations and collection of additional tissue samples from the dorsal soft palate (DSP) at this time point. Tissue samples were stored in RNAlater$^{TM}$ (Qiagen) at 6$^{o}$C overnight, and frozen at -20$^{o}$C until further processing.

***Quantitation of virus excretion in probang samples.***

In order to determine the carrier status of individual animals, the level of FMDV RNA in probang samples was measured using quantitative RT-PCR assays following a protocol described previously[33].

Total RNA was extracted using a Magna Pure LC Total Nucleic Acid Isolation Kit (Roche) with an automated robotic workstation (Roche) using 200µl of sample, according to the manufacturer's instructions. Each RNA sample was eluted in a volume of 50µl and stored at -70$^{o}$C until further processing.

Reverse transcription was carried out using 6µl of extracted RNA in a total volume of 15µl, using a TaqMan RT kit with random hexamer primers (Applied Biosystem) at 48$^{o}$ C for 45

minutes and 95$^{o}$ C for 5 minutes. 7µl of cDNA was mixed with 18µl of 2x TaqMan universal PCR mastermix (Applied Biosystems) containing 10 pmol/µl of FMDV specific forward and reverse primers and 5 pmol/µl of fluorescently labeled probe, targeting the 5'UTR region of the FMDV genome. PCR amplification was carried out for 50 cycles, in a Model Mx 3005P Thermal cycler (Applied Biosystems). Results were analyzed using MxPro software.

***Quantitation of IFN -α/β and TNF-α and mRNAs in tissue samples***

The levels of IFN-α and-β and TNF-α mRNAs were determined using quantitative RT-PCR assays , following a protocol described by Zhang *et al.*[34].

Approximately 20mg of tissue sample was homogenized using a fastprep tissue lyser FP120 (Thermo Electron Corporation BIO 101). RNA extraction from the lysate was performed using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Extracted RNA was eluted in a final volume of 30µl.

Reverse transcription of RNA was carried out as described above with the exception that the cDNA of samples and standards was diluted 1:5 prior to PCR analysis, with a total volume of 5µl of diluted template (hence 1µl of undiluted cDNA) being used for each PCR reaction.

Primers and fluorogenic probes used for the detection of IFN mRNAs have been described previously[28] and were originally designed using multiple sequence alignments of bovine IFN-α and-β available in Genbank (see Table 2). These assays were designed to detect all of the nine known bovine IFN-α gene products or the three known IFN-β gene products. Levels of TNF-α and IFN α- and β mRNA were quantified using a fluorogenic probe-based (TaqMan) detection system with 1µl of cDNA in a final reaction volume of 25µl. Diluted cDNA was mixed with TaqMan Master Mix (Applied Biosystems) containing 22.5 pmol of specific forward and reverse primers plus 5 pmol of fluorescently labeled probes specific for each gene of interest. PCR amplification was carried out for 50 cycles in a Model Mx 3005 Thermal cycler as above. Similar analyses were performed for the mRNA encoding the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each sample.

***Quantitative analysis***

Gene expression was calculated using the comparative Ct method[16]. In this method, ΔCt is determined by calculating the difference in the measured Ct values of the target and the normalizer (GAPDH) within each sample. The pre-inoculation tissue sample was chosen as the reference sample for each individual animal. ΔΔCt is defined as the difference in ΔCt

between the sample of interest, and the reference sample. Finally, values were transformed into absolute values using the formula $2^{-\Delta\Delta Ct}$.

***Statistical analysis***

Prior to analysis, survey data were standardized as relative expression values compared to the normalizer (GAPDH) using the method described above. Deviances in survey data from pre-inoculation values were observed when survey values were different from 1. The data were analyzed with a mixed-effects model where time of observation (phase 1, phase 2 or phase 3) was included as a fixed effect. Also carrier status, inoculated/contact status and the interaction between these were included as fixed effects. Survey number (experiment ID; 2008, 2010a, or 2010c) was included as a random effect. Due to the standardization, individual animal effects were not included in the model.

In order to stabilize the variance, data were transformed using the natural logarithm prior to analysis. The applicability of the models was ensured through standard methods and the Kolmogorov-Smirnov test for normality. Model reduction was performed with the likelihood ratio method[6].

Levels for groups of data points significantly different from 1 were calculated through analytical expressions for means of the inverse transformations. Due to the structure of the Ln-transformations, confidence intervals were calculated by transforming standard confidence interval for transformed data (mean ± 1.96 × standard deviation) with the inverse transformation, and taking the smallest interval symmetric around the level that included this interval.

Pre-inoculation data (from the reference samples) were investigated for any effect of carrier status through standard 1-sided analysis of variance.

For TNF-α, the effect of carrier status and inoculated/contact status were also investigated for each value of time of observation (i.e. phase 1, phase 2 and phase 3).

All analyses were carried out using Splus© version 6.1 (Insightful Corp. 2002).

**Results**

During a series of challenge experiments investigating the host response to infection by FMDV O UKG 34/2001 in cattle, we have developed a technique for collecting small tissue samples consisting of pharyngeal epithelia with subjacent submucosa and diffuse lymphoid tissue (see Figure 1) from live animals, using an endoscope fitted with cutting biopsy forceps.

In contrast to previous study designs, this novel approach allows for collection of samples of pharyngeal mucosa at sequential time points during the course of infection by FMDV.

***Progression of clinical disease***

Characteristics of the observed progression of the clinical infection of cattle by FMDV, together with results from measurement of viremia and the development of anti-FMDV antibodies has been presented in a separate report[26].

In brief, all animals included in the experiments developed mild to moderate clinical signs of FMD. Inoculated animals developed clinical signs of FMD at PID 1-2, whilst onset of clinical disease in contact animals was observed from PID 2-5. FMDV RNA was detectable in serum from all infected animals for a period of three to five days. Anti-FMDV serotype O antibodies in serum reached the diagnostic cut-off level at around PID 4 to 5 in inoculated animals and at PID 7 to 10 in the contact group (data not shown). Appearance of circulating antibodies was accompanied by a rapid reduction in viremia. There were no detectable differences in the appearance of the clinical infection, levels of viremia, or the anti-FMDV O antibody titres (data not shown), between directly inoculated and contact infected animals, or between carriers and non-carriers.

***Prevalence of carriers***

Carrier status was determined on the basis of detection of FMDV RNA genome, using qRT-PCR, in probang samples at or beyond PID 28[33]. An animal was regarded as being a carrier following detection of at least two positive probang samples (Ct value <40), out of a minimum of four analyzed samples collected on or after PID 28.

The prevalence of carriers detected in the three separate experiments was 6/12 (50%), 3/12 (25%) and 7/12 (58%) respectively (Table 1), with an overall average of 44% (16/36) which is consistent with what has been found previously in experimental infections with FMDV [2,5,19]. A summary of the presence of FMDV RNA in probang samples at different times post-infection in carriers and non-carriers is shown in Figure 2. The initial profile of virus excretion in these samples is very similar but in non-carrier animals complete clearance of FMDV RNA occurs by about 20 days post infection (DPI) whereas it is maintained in carriers beyond 30 DPI. A more detailed study on the level and duration of virus excretion in probang samples has been presented elsewhere[25].

***Cytokine mRNA expression in tissue samples from the dorsal soft palate***

Sequential biopsy samples were collected from each animal prior to virus infection, then on the first day of acute disease (phase 1) plus 7 days later (phase 2) and finally between 28 and 35 DPI (phase 3). Relative levels of mRNA expression in these samples encoding IFN-α-and-β, and TNF-α were determined using qRT-PCR assays and are presented in Figure 3. A significant down-regulation of the expression of IFN-β mRNA was observed in tissue samples collected during the acute phase of infection (see Figure 3b, phase 1: relative expression of IFN-β = 0.65 +/- 0.25, compared to a baseline-expression (1), level of significance: p= 0.02). There was no statistically significant up-or down regulation of the levels of IFN-α mRNA observed at any of the measured time points of infection, although the observed expression levels for this cytokine mRNA during infection were also below the baseline expression measured in the pre-inoculation samples (Figure 3a).

In contrast to the down-regulation of IFN-β mRNA, the expression of TNF-α mRNA was significantly increased during phase 1 (p=0.005) and 3 (p=0.009), although with levels not significantly different from baseline values observed at phase 2 (Figure 3c).

To determine whether the expression of TNF-α mRNA was different between animals which were subsequently identified as carriers or not, the expression levels were also compared between these two separate groups of animals. The levels of TNF-α mRNA in the biopsy samples harvested from animals that were subsequently identified as FMDV-carriers were found to be significantly higher when compared to the non-carriers (see Figure 4, relative expression levels are presented in Table 3, level of significance: p=0.048). The calculated p-values did not reach significance when results from these two groups from the three different phases of sampling were analyzed separately. The plotted data (Figure 4) does, however, indicate a clear trend with higher values in carriers during both acute (phase 1) and persistent phases (phase 3) of infection consistent with the overall data presented in Figure 3c.

## Discussion

The characteristics of the clinical infection observed in the experiments included in this study were consistent with what has previously been reported for infection with FMDV O UKG 34/2001 in cattle[1,32].

Sequential biopsy samples from the pharyngeal mucosa have been harvested from FMDV infected calves at different times after infection and the expression of specific genes related to

the host response to infection have been analyzed. This system has significant advantages compared to post-mortem analysis since the expression levels of each gene are related to the baseline level of each animal individually. Furthermore it was possible to analyze tissue samples collected during the early phases of infection, whilst still being able to determine whether each FMDV infected animal went on to become a carrier or not.

A key observation was that the IFN-β mRNA levels in biopsy samples harvested from the DSP during acute infection (phase 1) were significantly down-regulated when compared to baseline expression (Figure 3b). This observation is consistent with the results from De Los Santos *et al*.[9] who have previously demonstrated that FMDV is capable of inhibiting the induction of IFN-β in cultured porcine kidney cells. By comparing the IFN response induced by either a wild type FMDV or the corresponding mutant lacking the leader protease (Lpro), it was concluded that the Lpro was responsible for a specific inhibition of IFN-β induction in addition to a previously demonstrated general inhibition of host cell protein synthesis[9,12,17]. In a subsequent publication, the same group concluded that the observed inhibition of IFN-β induction was caused, at least in part, by the degradation of NFκB induced by the FMDV Lpro[10]. In addition, it has recently been reported that the Lpro is also responsible for the degradation of interferon regulatory factor 3/7 (IRF 3/7) which, in addition to NFκB, is also an important factor in the induction of transcription of type 1 IFNs[29]. Observations from the current study indicated that the IFN-β mRNA levels in samples harvested during the acute phase of infection were significantly below the levels found in the reference samples collected prior to inoculation. This could indicate that in addition to inhibition of the IFN response that is normally induced by virus infections, FMDV also affects the baseline expression of this cytokine. The levels of the measured responses in the biopsy samples used for analysis in the current investigation, could, however, be influenced by the fact that these samples consist of a combination of different types of cells (see Figure 1). This mixture of cell types should not be expected to respond as homogenously to infection with FMDV as cultured cell-lines with known susceptibility to the infection.

No statistically significant changes in the expression of IFN-α were detected in the DSP at any stage of FMDV infection in the current studies, although the observed expression levels were, for this cytokine, below baseline levels throughout the study period (Figure 3a). It has been reported that mRNA levels encoding IFN-α and-β were up-regulated in nasal associated lymphoid tissue (NALT) collected from the DSP of animals acutely infected with FMDV O

UKG 34/2001, when compared to uninfected control animals[34]. It should, however, be noted that, in a subsequent publication based on a similar experimental set-up it was concluded that there was no measurable up-regulation of IFN-α/β or TNF-α mRNA in microdissected epithelium from the DSP during the acute phase of infection[31]. These results were based on tissue samples obtained from a relatively small number of animals, with 2 and 4 animals euthanized during acute infection in the two experiments respectively. The levels of the mRNAs in harvested tissue samples were compared to a baseline derived from expression levels in one or two un-infected control animals. Our results are based on measurements from 36 animals, with mRNA levels expressed in relation to the base-line levels determined for each animal individually. By basing the statistical analysis on results from analysis of samples obtained from a larger number of animals, it should be possible to reduce the variance and thereby achieve a more robust model. The system of comparing the expression levels to a baseline derived from analysis of pre-inoculation samples from each individual animal should also minimize possible effects of individual animal variations in the analysis.

We have previously reported a consistent peak in the bioactivity of type 1 IFN in sera from cattle infected with FMDV serotype O[26] which occurred at 1 to 5 DPI, coincident with the onset of acute disease (phase 1). This finding has been corroborated recently by Reid *et al.*[22] using a similar experimental system. In relation to the findings of an induction in systemic type 1 IFN, it is interesting to note that we observed a down-regulation in the expression of IFN-β within the biopsy samples obtained from the DSP during the acute infection, while the IFN-α mRNA levels were not significantly altered from base-line levels at any of the time-points targeted for sampling (Figure 3a-b). As mentioned above, an IFN response can be induced both by cells that are directly infected by the virus, as well as by cells of the innate immune system which detect common pathogenic features through activation of pattern recognizing receptors. The results from our studies indicate that although a clearly measurable systemic IFN response was observed, these increased levels of type 1 IFN are not derived from cells of the pharyngeal region and hence must be the result of activation of cells located elsewhere. As indicated above, porcine plasmacytoid dendritic cells isolated from peripheral blood mononuclear cells, can be induced to express IFN by FMDV when it is within complexes with IgG but this does not occur with the virus alone[13]. Analogous results for bovine large low-density cells from blood and lymph nodes have recently been published by Reid *et al.*[22]. However, it should be noted that the systemic IFN response in cattle, that we

have previously reported, occurred very early post-infection and significantly in advance of the induction of measurable anti-FMDV antibodies[26].

There was an overall increase in mRNA levels of TNF-α during acute (phase 1) and persistent (phase 3) stages of infection (Figure 3c). TNF-α is known to be involved in the induction of local inflammatory reactions in response to tissue injury or infection. TNF-α activation causes an increase in vascular permeability, facilitating the exit of activated cells of the immune system from the blood vessels at the site of tissue damage. It is therefore reasonable to believe that the presence of any form of infectious agent could result in increased levels of TNF-α in the affected tissue.

The expression levels of TNF-α were significantly higher in animals that were subsequently identified as FMDV-carriers compared to the non-carriers. The difference between the two groups was most clearly apparent in samples harvested during the persistent phase of infection (phase 3) (Figure 4 and Table 3). These results are consistent with and extend what has previously been reported by Zhang *et al.*[34] since the results from this previous study were based on samples from only 6 animals (2 carriers and 4 non-carriers) whereas our results are based on measurements in samples collected from 36 animals (16 carriers and 20 non-carriers).

It could be argued that the procedure of harvesting tissue biopsies from live animals could possibly results in a provoked inflammatory response within the tissue targeted for sampling. It should, however, be noted, that the animals were allowed a period of rest of at least 21 days between the samplings performed during phase 2 and phase 3. Minor lesions on mucosal surfaces are known to heal within a very short time, and it is therefore unlikely that the increased levels of TNF-α that were observed in samples harvested during phase 3 (>28 days post infection) within the group of FMDV-carriers would have been caused by the sampling procedure in itself.

We have previously reported that the carrier state in FMDV infection is not associated with a sustained systemic inflammatory reaction, based on measurements of the acute phase proteins serum amyloid A and haptoglobin combined with the bioactive type 1 IFN in sera of infected cattle[26]. The results from measurements of TNF-α mRNA in this study could, however, indicate that there could still be a prolonged local inflammatory reaction in the pharyngeal region during FMDV persistence.

There is still a lack of consensus regarding the nature of pathogenesis during persistent FMDV-infection in cattle. Some researchers have reported that persistent FMDV is localized

in the basal layers of the pharyngeal epithelium[32,36], whereas others have reported virus persistence within germinal centers of lymphoid tissue[15]. Results from the current study, with significantly up-regulated levels of TNF-α in the DSP of carrier-animals, could possibly support the theory of an active involvement of the pharyngeal region in the persistence of FMDV in cattle. We have, however, with the current techniques, only been able to detect low levels of FMDV RNA in tissue biopsies from the DSP during acute and persistent infection[25]. It is possible that the cytokine response measured in the harvested biopsy samples could be influenced by signals from neighbouring cells and may thus react to the local infection without being actively infected. Further investigations will be necessary to elucidate the relationship between the innate host response to FMDV and the mechanisms involved in the pathogenesis of the infection, including the anatomical sites involved in virus replication during early and persistent infection. We believe that the newly developed experimental procedure, with the possibility of collection of tissue biopsies from live animals during different stages of infection, could be useful for further investigations of the host-response to FMDV infection in cattle.

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**Table 1**

General features of the three animal experiments included in the study.

| Experiment ID | Number of animals (Inoculated: Contact) | Carriers / Non-Carriers | Duration (PID) |
|---|---|---|---|
| **FMD 2008** | 12 (6:6) | 6/6 | 98 |
| **FMD 2010 a** | 12 (6:6) | 3/9 | 31 |
| **FMD 2010 c** | 12 (6:6) | 7/5 | 35 |

**Table 2**

Primers and probes for bovine IFNs and GAPDH. Primers and probes used for detection of the bovine IFNs and GAPDH. Primers and probes used for amplification of IFN-α and-β were designed using alignments of the bovine IFN-α and IFN-β sequences available in GenBank (for IFN-α, A00145, A00146, A00147, A00148, X93087, X93088, X93089, E00133, E00134, E00135, M11001 and Z46508; for IFN-β, M15477, M15478, and M15479) as previously described by Valarcher et al. (2003).

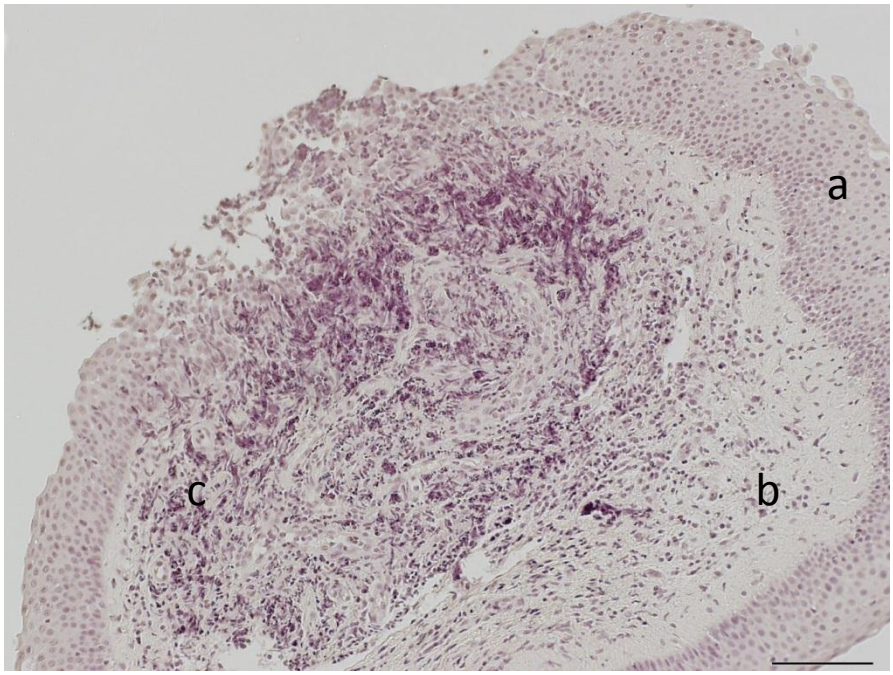
| Target | Primer/probe | Primer/probe sequence (5'-3') |
|---|---|---|
| **IFN-α** | FP | GTG AGG AAA TAC TTC CAC AGA CTC ACT |
| | RP | TGA RGA AGA GAA GGC TCT CAT GA |
| | Probe | TGC TCT GAC AAC CTC CCA GGC ACA |
| **IFN-β** | FP | CCT GTG CCT GWT TTC ATC ATG A |
| | RP | GCA AGC TGT AGC TCC TGG AAA G |
| | Probe | ATG GTT CTC CTG CTG TGT TTC TCC ACC AC |
| **GAPDH** | FP | GCA TCG TGG AGG GAC TTA TGA |
| | RP | GGG CCA TCC ACA GTC TTC TG |
| | Probe | CAC TGT CCA CGC CAT CAC TGC CA |
| **TNF-α** | FP | CCG GTG GTG GGA CTC GTA T |
| | RP | GCT GGT TGT CTT CCA GCT TCA |
| | Probe | CCA ATG CCC TCA TGG CCA ACG |

**Table 3**

Expression levels of TNF-α mRNA in the biopsies harvested from the DSP during acute (phase 1), sub-acute (phase 2) and persistent (phase 3) stages of FMDV infection. Relative mRNA levels are expressed as fold-increase compared to a baseline value of 1.

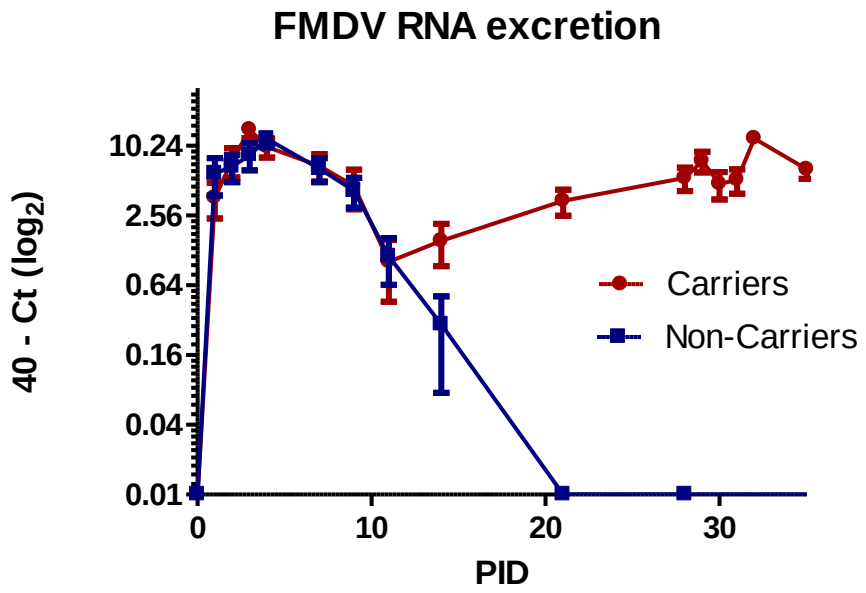
| | **Carrier** | **Non-Carrier** |
|---|---|---|
| **Phase 1** | 1.89 (+/- 0.41) | 1.47 (+/- 0.26) |
| **Phase 2** | 1.28 (+/- 0.20) | 1 |
| **Phase 3** | 1.81 (+/- 0.38) | 1.41 (+/- 0.25) |

**Figure 1**



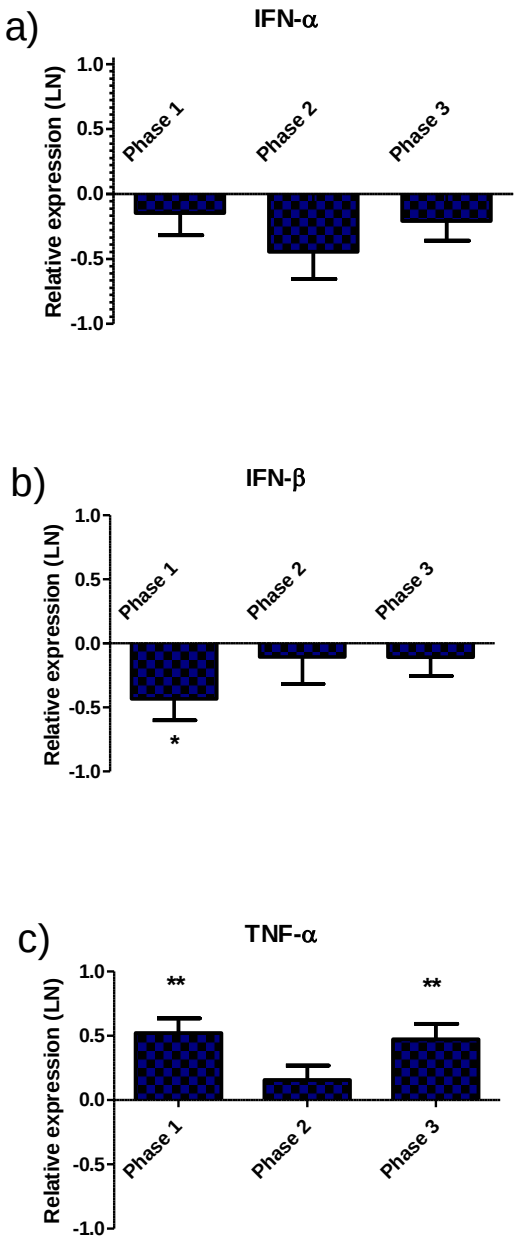

Section from formalin fixed and paraffin wax-embedded biopsy sample from the DSP harvested prior to inoculation. This section includes intact non-cornified stratified squamous epithelium (a), underlying submucosa (b) and lymphoid tissue (c). bar, 100µm

**Figure 2**



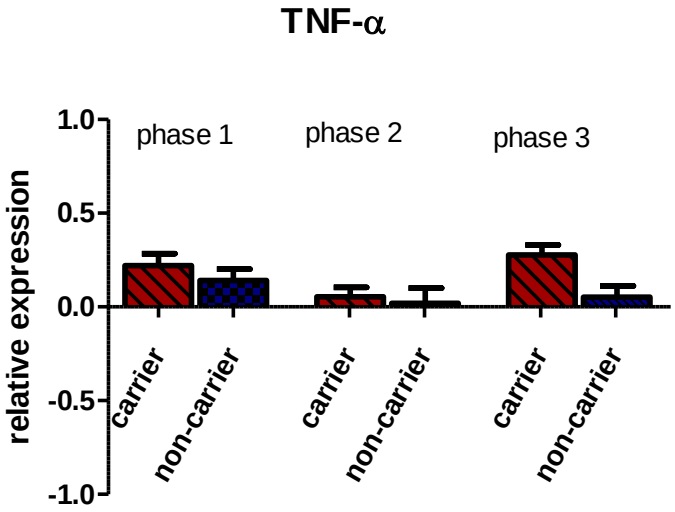

FMDV RNA levels detected by qRT-PCR in probang samples harvested from a total number of 36 calves during an experimental period of 35 days. In total, 16 animals were detected as FMDV carriers, with FMDV RNA detected in a minimum of two samples collected on or later than PID 28. In animals that were not detected as carriers of FMDV, virus excretion became undetectable by approximately PID 20.

**Figure 3**




mRNA expression levels of a) IFN-α, b) IFN-β and c) TNF-α, in biopsies harvested from the DSP of FMDV infected calves (n=36). Samples were collected on the first day of appearance of clinical signs of infection (phase 1), seven days after the first sampling (phase 2) and at 28-35 days post infection (phase 3). Relative expression levels of target mRNAs were calculated by comparison to the expression of the housekeeping gene GAPDH within each sample. Expression levels for each phase of sampling were related to the baseline expression measured in pre-inoculation samples harvested from each individual animal. In order to stabilize the variance for the statistical analysis of data, the values have been transformed using the natural logarithm so that the baseline-expression levels equal "0" on the y-axis.

**Figure 4**




Expression levels of TNF-α mRNA in sequentially harvested samples of DSP from FMDV-carriers and non-carriers at the onset of clinical signs (phase 1), 7 days later (phase 2) and 28-35 DPI (phase 3). There was a significant difference in the overall expression levels of TNF-α mRNA, with higher levels in animals that were eventually identified as FMDV-carriers ($p=0.048$).

# Manuscript III

## Detection of foot-and-mouth disease virus RNA in pharyngeal epithelium biopsy samples obtained from infected cattle; investigation of possible sites of virus replication and persistence

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# Detection of foot-and-mouth disease virus RNA in pharyngeal epithelium biopsy samples obtained from infected cattle; investigation of possible sites of virus replication and persistence.

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## Abstract

Foot-and-mouth disease (FMD) is a highly contagious viral infection of significant financial importance to the export and trade of agricultural products. The occurrence of persistently infected "carriers" of FMD-virus (FMDV) in ruminant species adds further complications to disease control. There have been significant discrepancies in reports regarding the pathogenesis of FMDV infection in cattle with specific emphasis on the anatomical sites involved in early and persistent virus replication. In this study, collection of small biopsy samples from the dorsal soft palate (DSP) of live animals was used to investigate the level of FMDV RNA present at this site at sequential time points during the infection. Results were compared to measurements of virus excretion in samples of oropharyngeal fluid collected at corresponding time points. Possible sites of virus persistence were investigated through measurements of the levels of FMDV RNA in the DSP as well as mandibular and retropharyngeal lymph nodes beyond 28 days after infection.

Results indicated only low levels of FMDV RNA present in samples of pharyngeal epithelia during both early and persistent phases of infection with significantly higher levels of virus detected in pharyngeal excretions. It is concluded that the targeted area for sampling within the DSP does not harbour significant levels of virus replication during acute or persistent FMDV infection in cattle. Furthermore, the DSP and the mandibular and retropharyngeal lymphnodes cannot be concluded to be principal sites for persistence of FMDV.

## Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral infection which affects cloven-hoofed animals including cattle, pigs and sheep[1]. The disease spreads rapidly amongst susceptible animals, with devastating consequences for farmers and agricultural industries in affected areas.

Foot-and-mouth disease virus (FMDV) is a non-enveloped, positive stranded RNA virus belonging to the *Aphthovirus* genus of the picornavirus family[9]. The clinical disease is manifested by development of vesicular lesions in areas covered by cornified stratified squamous epithelia such as the oral cavity and coronary bands, causing excessive salivation, inappetence and lameness. Infected animals develop transient viremia lasting for about 2-4 days, which is effectively counteracted by the development of circulating anti-FMDV antibodies. Disease severity varies greatly between different virus isolates, as well as between different host species, with pigs developing the most severe clinical signs, followed by cattle with mild to moderate clinical disease, and sheep in which the infection may sometimes go unnoticed[1]. Mortality rates are generally very low in adult animals, whereas juvenile animals may develop fatal myocarditis as a consequence of the infection. The clinical course of the disease is usually over within 14 days after infection, but the occurrence of persistently infected, so called "carrier-animals" within ruminant species causes further complications for disease control. FMDV carriers are defined as animals with infectious FMDV in oropharyngeal fluid more than 28 days post infection[28]. In practice, the level of FMDV in the oropharyngeal fluid is normally low and detection is intermittent. It has previously been demonstrated that analysis of samples of oropharyngeal fluid using specific qRT-PCR, is suitable for detection of FMDV-carriers in experimentally infected animals[26]. Development of the carrier state is unaffected by the presence of neutralizing antibodies in the circulation following vaccination or previous exposure to the virus, and thus vaccinated animals may become carriers of FMDV without the pre-occurrence of clinical disease[12,15,23].

FMD pathogenesis has been studied in detail by several research groups. The initial site of FMDV replication in cattle has been suggested to be located within epithelia and lymphoid associated tissues of the pharyngeal area[2,5,13,18,20] or alternatively, within the lungs [6,10,11].

An experimental study of the early pathogenesis of FMDV in cattle performed by Pacheco *et al*[20] showed that FMDV RNA could be isolated from tissue samples from both the upper and lower respiratory tracts at 24 hours post aerosol exposure to serotype O FMDV. It was

proposed that FMDV could reach the lower respiratory tract following aerosol exposure but that the pulmonary tissues were only poorly permissive for FMDV replication whilst primary virus replication was found within respiratory-associated lymphoid tissues of the upper airways[20]. Subsequently the same group defined the initial site of viral replication as being located within the epithelial cells overlying the lymphoid crypts of the pharyngeal mucosa[7]. Thus, it was proposed that primary replication of FMDV in cattle occurs in pharyngeal epithelia with subsequent widespread FMDV replication within pulmonary pneumocysts accompanied by a significant decrease in viral load within pharyngeal tissues. In contrast to these recent findings, previous work of other authors have failed to find evidence of significant FMDV replication within the lungs[2,5,13]. Thus, there is still no clear consensus regarding the early pathogenesis of FMD and comparison of published results is confounded by variations in experimental design with the use of different virus strains plus varying routes of administration of the virus.

With regard to the localization of persistent FMDV in carrier animals the published results are somewhat more consistent; generally suggesting that the virus shed in oropharyngeal fluid originates from within tissues of the pharyngeal region[4,5,12,17,21,27,30]. There are, however, some differences regarding what is believed to be the exact anatomical localization of persistent virus. Some have suggested that FMDV persists at a low level of replication within pharyngeal epithelial cells[12,26,30], whilst others have reported that persistent FMDV is found as intact, but largely quiescent, viral particles within lymphoid tissues of the pharynx[17].

In this study, endoscopical collection of small tissue samples from the pharyngeal mucosa from live animals was used to investigate the sites of virus replication in cattle with FMD. Biopsy samples from this specific anatomical region were collected from the same animals at sequential time points; prior to inoculation, during the acute phase of infection with serotype O FMDV and subsequently. The presence of FMDV RNA in these biopsy samples and concurrent viral shedding in oropharyngeal fluid were determined. Other possible sites of viral persistence were investigated through analysis of samples collected post mortem.

## Materials and Methods

### *Animal experiments and samples*

Animal experiments were performed in biosecure research facilities at DTU-Vet, Lindholm Island, Denmark, in accordance with the requirements of the Danish Animal Experiments Inspectorate (License 2003/561-742; 2008/561-1541). Three independently performed experiments, using a similar experimental set up, were included in this study (see Table 1). Each of the experiments included 12 steers (4-5 months old) of mixed-holstein breed. The general experimental protocols have been described in detail elsewhere[25]. In brief, six animals in each experiment were inoculated with FMD O UKG 34/2001 (original inoculum obtained from IAH-Pirbright (UK), and then passaged once in cattle) using subepidermo-lingual injection, each animal receiving approximately $10^{6.9}$ $TCID_{50}$ in a volume of 0.5 ml. Six other animals were kept in continuous direct contact with the inoculated animals, with two inoculated plus two contact animals in each pen and with the stable facilities also allowing direct contact between animals in separate pens.

All animals were monitored daily, with measurements of rectal temperature and observation of clinical signs. Serum samples were collected daily from 3 days prior to inoculation and throughout the first two weeks of the experiments but then on a weekly basis throughout the remaining part of the experiment.

Samples of oropharyngeal fluid (probang samples) were collected, for the quantification of viral shedding, as well as determining the carrier status of individual animals. These samples were obtained prior to inoculation, once daily during the first week after inoculation, then every other day throughout the second week and on a weekly basis subsequently. Additional samples were collected after post infection day (PID) 28 in order to have probang samples from a minimum of four separate days for determination of carrier status.

A recently developed technique for endoscopical collection of biopsy samples from the pharyngeal region of live animals was used in order to obtain sequential tissue samples from the dorsal soft palate from the infected animals. The method used for sampling has been described in detail elsewhere[24].

In brief, small samples of tissue from the dorsal soft palate was collected using a fiberscope (Vet-vu 2FSb) fitted with cutting biopsy forceps, from sedated animals at three time points during the experiments. Tissue samples were collected from the caudal end of the dorsal soft palate, at a site which has previously been proven to house a high density of diffuse lymphoid

tissue[24]. Two pieces of tissue, approximately 1x1x1 mm in size, were harvested from slightly different locations within the sampling area and included in each sample.

The first biopsy sample from each animal was collected one week prior to inoculation. The second biopsy was collected during the acute phase of infection, defined as the first day when the animals showed a rectal temperature of above 40$^{o}$C, with a third sample being collected 7 days later. In the first of the three experiments (FMD 2008), animals were kept for observation for a period of 100 days after inoculation. In this experiment, final collection of tissue samples from the pharyngeal mucosa was carried out through an additional endoscopical examination at PID 28. Lymphnodes from post mortem examinations of the 12 animals included in this experiment were not included in the analysis. The other two experiments (FMD 2010 a and c) were terminated at PID 31-35 with post mortem examinations and collection of mandibular and retropharyngeal lymphnodes A section of approximately 2-3 mm thickness was cut perpendicular to the longitudinal axis of the harvested lymph nodes and several small tissue samples of approximately 1x1x1 mm were collected from the lymphoid cortex of each lymph node. Post mortem samples of pharyngeal mucosa were harvested from the dorsal soft palate from the anatomical region that had previously been targeted during the endoscopical sampling procedure. All tissue samples were stored in RNAlater$^{TM}$ (Qiagen) at 6$^{o}$C overnight, and frozen at -20$^{o}$C until further processing.

***Quantitation of viremia and virus excretion in probang samples***

The level of FMDV RNA in serum and probang samples was determined using quantitative RT-PCR assays following a protocol described previously[26].

Total RNA was extracted using a Magna Pure LC Total Nucleic Acid Isolation Kit (Roche) with an automated robotic workstation (Roche) using 200µl of sample, according to the manufacturer's instructions. Each RNA sample was eluted in a volume of 50µl and stored at -70$^{o}$C until further processing.

Reverse transcription of FMDV RNA was carried out using 6µl of extracted RNA in a total volume of 15µl, using a TaqMan RT kit with random hexamer primers (Applied Biosystem) at 48$^{o}$ C for 45 minutes and 95$^{o}$ C for 5 minutes. 7µl of cDNA was mixed with 18µl of 2x TaqMan universal PCR mastermix (Applied Biosystems) containing 10 pmol/µl of forward and reverse primers and 5 pmol/µl of fluorescently labeled probe, targeting the 5'UTR region

of the FMDV genome. PCR amplification was carried out for 50 cycles, in a Model Mx 3005P Thermal cycler. Results were analyzed using MxPro software.

***Quantification of FMDV in tissue samples***

Approximately 20mg of tissue sample was homogenized using a Fastprep tissue lyser FP120 (Thermo Electron Corporation BIO 101). RNA extraction from the lysate was performed using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Extracted RNA was eluted in a final volume of 30µl.

Reverse transcription of RNA and qRT-PCR was carried out following the protocol described above with the exception that the cDNA of samples and standards was diluted 1:5 prior to PCR analysis, with a total volume of 5µl of diluted template (hence 1µl of undiluted cDNA) being used for each PCR reaction.

Each sample was analyzed for the presence of FMDV RNA using two different sets of primers and fluorogenic probes. One assay targeted the 5'UTR region[22] whilst the other targeted the 3D protein coding region[14]. Both assays used were previously validated and accredited for FMDV diagnostics according to the quality assurance system of DTU-Vet. In addition, quantification of the mRNA corresponding to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed for each sample[28] in order to ensure a consistent amount of template in the samples.

***Quantification of anti-FMDV Antibodies using a solid phase blocking ELISA***

Serum concentrations of FMD specific antibodies were measured using a serotype-specific solid phase blocking ELISA[8]. In brief, microtitre plates were coated using guinea-pig immune sera raised against FMDV O Manissa before addition of inactivated FMDV antigen. Samples of the bovine sera were added, incubated overnight and then rabbit anti-FMDV serotype O serum was added and the bound antibodies were detected using horseradish-peroxidase conjugated porcine anti-rabbit IgG. All serum samples were initially screened at a dilution of 1:5 and positive samples (blocking percentage >50%) were analyzed in a two-fold titration starting at a dilution of 1:10 to allow determination of antibody titers.

## Results

### *Progression of clinical disease*

All animals included in the three experiments developed mild to moderate clinical signs of FMD, with an increase in body temperature (Figure 1a) and vesicular lesions in the oral cavity being the most prominent findings occurring in all individuals. Less than half of the animals developed vesicular lesions on the feet and only two individuals (out of 36) developed clearly visible lameness. Inoculated animals developed clinical signs of FMD at PID 1-2, whilst onset of clinical disease in contact animals was observed from PID 2-5. The animals showed no apparent loss in appetite and all animals recovered from clinical disease within approximately seven days, without further complications, and without the need of supportive medical treatment.

### *Viremia and development of circulating antibodies*

FMDV RNA was detectable in serum from all infected animals for a period of four to seven days (Figure 1b). Inoculated animals showed significant viremia at PID 1, reflecting the very rapid replication and systemic spread of FMDV. Anti-FMDV serotype O antibodies in serum reached the diagnostic cut-off level at around PID 4 to 5 in inoculated animals and at PID 7 to 10 in the contact group (Figure 1c). Appearance of circulating antibodies was accompanied by a rapid reduction in viremia. There were no detectable differences in the level of viremia, (presented as the number of copies of FMDV genome per µl of serum), or the anti-FMDV O antibody titres (data not shown), between directly inoculated and contact infected animals, or between carriers and non-carriers.

### *Prevalence of Carriers*

Carrier status was determined on the basis of detection of FMDV RNA genome, using qRT-PCR, in probang samples beyond PID 28. An animal was regarded as being a carrier following detection of at least two positive probang samples (Ct value < 40), out of a minimum of four analyzed samples collected on or after PID 28[27].

The prevalence of carriers detected in the three separate experiments was 6/12 (50%), 3/12 (25%) and 7/12 (58%) respectively in the three different experiments (Table 1), with an overall average of 44% (16/36) which is consistent with what has been found previously in experimental infections with FMDV[1,5,19].

***Detection of FMDV genome using qRT-PCR analysis of tissue and probang samples***

The presence of FMDV RNA in probang and tissue samples, as determined from qRT-PCR analysis, is presented for each separate experiment in Tables 2A-C. Due to technical problems with the equipment used for sampling, there are a few samples missing at different time points in two of the experiments (indicated as "NA" in Table 2 A and B).

In biopsy samples of pharyngeal mucosa, collected through endoscopical examination during the acute phase of infection, FMDV RNA was only detected in 13 out of a total of 34 samples analyzed (38%). At the subsequent sampling, performed 7 days after initial detection of clinical disease, 8 out of a total of 28 samples (29%) were found positive with either, or both, of the two assays used for analysis. In tissue samples derived from the final sampling at post mortem examinations (experiments FMD 2010 a + c; at PID 31-35), or endoscopical examination (experiment FMD 2008; PID 28) the prevalence of positive samples was 5 out of 36 (14%). For animals detected as FMDV-carriers, the prevalence of FMDV-RNA positive samples of pharyngeal mucosa at PID 28-35 was 31% (5 out of 16 animals).

In tissue samples of the mandibular and retropharyngeal lymph nodes collected at PID 32-35, the prevalence of FMDV-positive samples was 25% (6 out of 24) and 17% (4 out of 24) respectively. For carrier animals, these tissues were positive in 4 out of 10 (40%) animals for both of the lymph nodes.

The Ct values in FMDV-positive samples were generally very high for both assays, indicative of low levels of FMDV RNA, with some samples having Ct-values above 40 (presented in brackets in Table 2 A-C). Positive controls included in the assays were found within the normal range accepted for the diagnostic assays. The level of mRNA derived from the housekeeping gene GAPDH was at a rather consistent detection level in all samples, with Ct-values within the range of 22 to 25 cycles.

For probang samples collected at similar times as the samples of pharyngeal mucosa, the prevalence of FMDV RNA positive samples was 100% during the acute phase of infection, whilst only 38% of the corresponding tissue samples were found positive. At the second phase of biopsy collection, which was performed 7 days after onset of clinical disease, 50% of probang samples, and 29% of tissue samples were found positive for FMDV. At the final stage of sampling (PID 28-35), 44% of probang samples were positive whilst FMDV could only be detected in 14% of the collected tissue samples (Table 2 A-C, Figure 2A). A summary of the level of FMDV RNA in probang and DSP biopsy samples that were found positive at the different stages of infection is shown in Figure 2B. This indicates that even in

the small proportion of DSP samples (see Figure 2A) which did contain detectable FMDV RNA at the final sampling stage, the levels were low. It is apparent that throughout the time course of infection, the proportion of animals containing FMDV RNA in the biopsy samples from the DSP (or from lymph nodes) was much lower than the proportion of animals which were excreting FMDV in probang samples (Figure 2A).

In animals which did not become carriers, it was found that the presence of FMDV RNA in probang samples was lost by PID 14 or earlier (Tables 3 A-C, Figure 3). However, at the earlier stages of infection no difference in the level of FMDV RNA was apparent in the probang samples from the animals which became carriers and those which did not (Figure 3).

**Discussion**

The pharyngeal mucosa is believed to be an important site for FMDV replication during both acute[2,13,18] and persistent stages of infection[30]. In the current study, sequential collection of biopsy samples from pharyngeal epithelia and subjacent mucosa within the dorsal soft palate (DSP) of live animals was used to investigate possible sites of virus replication and persistence following infection of cattle with FMDV serotype O. The study-design allowed for comparison of possible differences between animals that would subsequently develop into FMDV carriers and those that would not. The original idea was to harvest biopsy samples, which would enable monitoring of the progression of infection within individual animals, with minimal interference to the natural course of infection

The average prevalence of animals detected as FMDV carriers was 44% in the three experiments. Results of the FMDV qRT-PCR analysis of the probang samples showed a consistent pattern of positive samples in carrier animals, whilst the samples from non-carriers became negative at around PID 14 (Tables 3 A-C, Figure 3) and stayed negative throughout the remaining parts of the experimental periods.

The prevalence of FMDV RNA in the DSP during both acute (38%) and sub-acute (29%) phases of infection (Tables 2 A,B,C) was much lower than the presence of FMDV in the probang samples collected at the same time points which reached 100% in the acute phase of infection, and then declined to 50% by 7 days later (Figure 2). Low quantities and prevalence of FMDV RNA were also found in samples of the DSP (14%), as well as mandibular (25%) and retropharyngeal (17%) lymph nodes collected at PID 28-35.

Quantification of the mRNA levels from the housekeeping gene GAPDH using qRT-PCR analysis indicated that the biopsy samples could be used to provide RNA in the consistent yield and quality needed for these analyses. Immunohistochemical staining of randomly selected biopsy samples showed that they contained intact epithelia with subjacent mucosa, as well as diffuse lymphoid tissue as expected[24].

The low level and prevalence of FMDV RNA that was detected in the biopsy samples suggested that the principal site of FMDV replication is not located within the area of the DSP targeted during sampling. With the limited size of the collected tissue samples, it could be argued that an uneven distribution of FMDV replication within the DSP could easily be missed, and hence, interpreted as "false negative" results. However, taking into account the number of samples analyzed in this study it would, even assuming a patchy distribution of viral replication, be expected to find some samples with a very high content of virus whilst other samples could be negative. The levels of FMDV RNA detected within positive tissue samples were always very low (Ct values of 35-45), whilst the assays showed significantly higher levels and prevalence of FMDV RNA within the probang samples.

Even though the DSP has been identified previously as the site of virus persistence in FMDV carriers[30], other studies have failed in finding persistent virus in pharyngeal epithelia[17]. Furthermore, in the recent study by Juleff et al.[17], laser capture microdissection, in combination with qRT-PCR, determined the site of persistent FMDV to be within germinal centers of lymphoid tissue within the pharyngeal region, with the highest amounts of virus RNA, obtained at PID 29-34, found in the mandibular lymph node. Indeed, FMDV RNA was found to persist within the germinal centers of this specific lymph node even in animals that did not excrete FMDV in oropharyngeal scrapings. It was thus concluded that FMDV may persist to some degree in all animals following infection, regardless of whether they can be detected as FMDV carriers[17]. In the current study, we found relatively low numbers of biopsy samples which were positive for FMDV RNA which were derived from the dorsal soft palate or in tissue samples from mandibular and retropharyngeal lymph nodes at PID 28-35. This was the case even though a substantial proportion of the cattle (44%) were excreting clearly detectable amounts of FMDV in oropharyngeal scrapings at the time of biopsy sampling. It is noteworthy that it was not possible to detect convincing amounts of FMDV RNA in any of the tissue samples derived from animals that did not excrete FMDV in probang samples.

Animals included in this study were infected either through direct inoculation in the tongue, or through continuous direct contact with inoculated animals. This method has previously

been used in numerous experimental studies of FMD in cattle[3,26,29]. There were no observed differences in severity or nature of the clinical infection between animals infected through direct inoculation or contact exposure. It is however, reasonable to believe that the route of initial exposure to the virus could have a significant influence on the site of primary replication. In this study, we have found that there was no effect of the route of virus exposure on the prevalence of FMDV-RNA positive samples in the DSP or lymph nodes.

Arzt et al.[7] used qRT-PCR, in combination with immunohistochemistry and virus isolation in cell culture, to investigate the early pathogenesis of FMDV in cattle following aerosol exposure. It was concluded that widespread replication of FMDV took place within pulmonary tissue following an initial phase of replication within the pharyngeal epithelia. It has previously been concluded that the size of particles generated during aerosol exposure will influence the site of deposition within the respiratory tract, and hence the possible sites of viral entry[5]. Other studies have concluded that aerosol exposure, with deposition of artificially produced small droplets of virus inoculum in the lungs, will lead to virus uptake and entry through the pulmonary circulation, with subsequent virus replication at distant predilection sites[16].

It would be relevant to investigate further the involvement of the lungs as a site of uptake and primary replication of FMDV in cattle infected by other routes of exposure, most importantly in animals infected through direct contact with infected animals. Furthermore, it can be discussed whether it is appropriate to conclude that virus replication occurs within a specific tissue following just detection of FMDV RNA or isolation of infectious virus since high levels of viremia occur. Specific detection of negative strand FMDV RNA, which is only present within cells in which virus replication is taking place, should make it possible to determine if the virus is actively replicating within the sampled tissues.

The exact micro-anatomical compartments included in the tissue samples analyzed in this study have not been subjected to stringent control. It would however, be expected that germinal centers would be included in the majority of samples derived from the mandibular and retropharyngeal lymph nodes, as well as in a large proportion of samples derived from the dorsal soft palate (as supported by immunohistochemical staining[24]). For future investigations, the technique of endoscopical collection of tissue samples from live animals could be further developed in order to target different anatomical regions such as pharyngeal and palatine tonsils as well as epithelia from the lower respiratory tract.

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**Table 1.**

General features of the three animal experiments included in the study.

| Experiment ID | Number of animals (inoculated: contact) | Carriers/non-carriers | Duration (days) |
|---|---|---|---|
| **FMD 2008** | 12 (6:6) | 6/6 | 98 |
| **FMD 2010a** | 12 (6:6) | 3/9 | 31 |
| **FMD 2010c** | 12 (6:6) | 7/5 | 35 |

**Table 2 (A, B, C)**

Results of qRT-PCR analysis for FMDV RNA in tissue samples from the dorsal soft palate (DSP), lymph nodes and probang samples from cattle infected with FMDV O UKG 34/2001.

12 animals were included in each experiment, of which 6 were directly inoculated (nos. 1, 2, 5, 6, 9, 10) and 6 were contact- infected (nos. 3, 4, 7, 8, 11, 12). Times of sampling: (1) the acute phase of infection defined as the first day where animals showed a temperature > 40$^{o}$C; (2) seven days after the first sampling (3) PID 28-35. Ct values: +++ <20; ++ 20<30; + 30<40; (+)>40, Ct values for the mRNA expressed from the housekeeping gene GAPDH were within the range of 22-25 Ct for all analyzed tissue samples.

| **A) FMD 2008** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | | | | | | | | | | | | | |
| **Acute phase** | | **Animal ID** | | | | | | | | | | | |
| **sample** | **assay** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| probang | 5'UTR | ++ | ++ | ++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| DSP | 5'UTR | - | - | - | - | - | *NA* | - | - | *NA* | - | - | - |
| | 3D | - | - | - | - | - | *NA* | - | - | *NA* | - | - | - |
| **2** | | | | | | | | | | | | | |
| **Acute phase +7** | | **Animal ID** | | | | | | | | | | | |
| **sample** | **assay** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| probang | 5'UTR | - | - | + | + | - | - | - | - | - | - | + | + |
| DSP | 5'UTR | - | - | (+) | + | - | *NA* | - | - | *NA* | - | (+) | - |
| | 3D | - | - | + | + | - | *NA* | - | - | *NA* | + | - | - |
| **3** | | | | | | | | | | | | | |
| **PID28** | | **Animal ID (FMDV carriers marked with*)** | | | | | | | | | | | |
| **sample** | **assay** | **1*** | **2** | **3** | **4*** | **5*** | **6*** | **7** | **8** | **9*** | **10** | **11** | **12*** |
| probang | 5'UTR | + | - | - | + | + | + | - | - | + | - | - | + |
| DSP | 5'UTR | - | - | - | - | + | - | - | - | - | - | - | + |
| | 3D | - | - | - | - | + | - | - | - | - | - | - | + |

| **B) FMDV 2010a** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | | | | | | | | | | | | | |
| **Acute phase** | | **Animal ID** | | | | | | | | | | | |
| **sample** | **assay** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| probang | 5'UTR | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | +++ | ++ | ++ | +++ |
| DSP | 5'UTR | (+) | (+) | - | (+) | - | - | - | - | (+) | - | - | - |
| | 3D | + | - | - | - | - | - | - | - | - | - | - | - |
| **2** | | | | | | | | | | | | | |
| **Acute phase + 7** | | **Animal ID** | | | | | | | | | | | |
| **sample** | **assay** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| probang | 5'UTR | - | - | + | (+) | + | + | - | - | + | + | + | + |
| DSP | 5'UTR | - | - | *NA* | *NA* | - | - | *NA* | *NA* | - | - | *NA* | *NA* |
| | 3D | - | - | *NA* | *NA* | - | - | *NA* | *NA* | - | - | *NA* | *NA* |
| **3** | | | | | | | | | | | | | |
| **PID 31** | | **Animal ID (FMDV carriers marked with *)** | | | | | | | | | | | |
| **sample** | **assay** | **1** | **2*** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11*** | **12*** |
| probang | 5'UTR | - | + | - | - | - | - | - | - | - | - | + | ++ |
| DSP | 5'UTR | - | - | - | - | - | - | - | - | - | - | - | - |
| | 3D | - | + | - | - | - | - | - | - | - | - | - | - |
| Mandibular ln. | 5'UTR | - | - | - | - | + | - | - | - | - | - | - | + |
| | 3D | - | - | - | - | - | - | - | - | - | - | - | - |
| Retropharyngeal ln. | 5'UTR | - | - | - | - | - | - | - | - | - | - | - | - |
| | 3D | - | - | - | - | - | - | - | - | - | - | - | - |

| **C) FMD 2010 c** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1.** | | | | | | | | | | | | | |
| **Acute phase** | | **Animal** | | | | | | | | | | | |
| **sample** | **assay** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| probang | 5'UTR | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| DSP | 5'UTR | (+) | - | + | (+) | - | - | + | - | - | - | - | + |
| | 3D | - | + | - | - | - | - | + | - | - | - | - | - |
| **2.** | | | | | | | | | | | | | |
| **Acute phase +7** | | **Animal** | | | | | | | | | | | |
| **sample** | **assay** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| probang | 5'UTR | - | + | + | + | - | - | - | + | - | + | + | - |
| DSP | 5'UTR | (+) | - | - | (+) | - | - | - | - | - | - | (+) | - |
| | 3D | - | - | - | - | - | - | - | - | - | - | - | + |
| **3.** | | | | | | | | | | | | | |
| **PID 35** | | **Animal** | | | | | | | | | | | |
| **sample** | **assay** | **1*** | **2*** | **3*** | **4*** | **5** | **6** | **7** | **8** | **9*** | **10** | **11*** | **12*** |
| probang | 5'UTR | ++ | + | ++ | ++ | - | - | - | - | + | - | + | + |
| DSP | 5'UTR | + | - | (+) | - | - | - | - | - | - | - | - | - |
| | 3D | + | - | - | - | - | - | - | - | - | - | - | - |
| Mandibular ln | 5'UTR | (+) | - | - | (+) | - | (+) | - | - | - | - | - | - |
| | 3D | - | - | + | + | - | - | - | - | - | - | - | - |
| Retropharyngeal ln. | 5'UTR | - | - | (+) | + | - | - | - | - | - | - | - | (+) |
| | 3D | + | - | + | + | - | - | - | - | - | - | - | - |

**Table 3 (A, B, C)**

Ct-values from qRT-PCR analysis for FMDV RNA in probang samples collected from three separate experiments.

FMDV carriers were defined as animals with positive detection (Ct<40) in probang samples collected on at least two separate days from PID 28 and after (Ct-values included in determination of carrier-status are marked in separate boxes in tables).

**A) FMD 2008**

| | Animal ID (FMDV carriers marked with *) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PID** | **1**** | **2** | **3** | **4*** | **5*** | **6*** | **7** | **8** | **9*** | **10** | **11** | **12*** |
| **0** | - | - | - | - | - | - | - | - | - | - | - | - |
| **1** | 24.5 | 40.4 | - | - | 35.2 | 37.3 | - | 19.4 | 35.0 | 27.8 | 23.9 | 36.8 |
| **2** | 37.5 | 37.6 | - | 18.9 | 31.8 | 31.7 | 22.9 | 22.2 | 35.3 | 34.9 | 35.5 | 22.1 |
| **3** | 20.2 | 31.5 | 28.6 | 26.6 | 19.2 | 27.2 | 35.0 | 24.7 | 33.2 | - | 29.0 | 29.3 |
| **4** | 22.7 | 25.5 | 18.9 | 24.3 | 34.0 | 30.3 | 28.7 | 26.0 | 32.6 | 32.0 | 23.8 | 24.4 |
| **5** | 37.7 | 36.4 | 25.5 | 28.4 | 40.3 | 34.7 | 24.1 | 26.4 | 37.6 | 36.3 | 21.8 | 26.3 |
| **7** | 32.9 | 33.9 | 26.7 | 30.7 | 39.8 | 37.0 | 31.1 | 30.8 | - | - | - | - |
| **11** | - | - | 38.5 | 42.3 | - | - | - | - | - | - | 44.6 | 42.7 |
| **14** | - | - | - | - | 43.3 | - | - | - | - | - | - | 49.9 |
| **21** | - | - | - | 36.2 | 34.2 | 37.7 | - | - | 37.2 | - | - | - |
| **28** | 34.8 | - | - | 33.7 | 44.3 | 38.4 | - | - | 34.2 | - | - | 37.5 |
| **35** | *NA* | - | - | 33.0 | 34.5 | 37.1 | - | - | 41.4 | - | - | 37.2 |
| **42** | *NA* | - | - | 34.2 | 42.3 | 40.1 | - | - | 36.4 | - | - | 38.6 |
| **49** | *NA* | - | - | - | 44.0 | 42.5 | - | - | 38.6 | - | - | 33.3 |
| **56** | *NA* | - | - | 42.2 | - | - | - | - | 38.4 | - | - | - |
| **63** | *NA* | - | - | - | 42.5 | 38.1 | - | - | 37.6 | - | - | 40.6 |
| **70** | *NA* | - | - | 33.7 | 39.4 | 38.6 | - | - | 37.2 | - | - | 38.1 |
| **72** | *NA* | - | - | 39.9 | 38.6 | - | - | - | - | - | - | 42.8 |
| **78** | *NA* | - | - | 37.1 | 38.8 | 38.4 | - | - | 42.8 | - | - | 41.5 |
| **91** | *NA* | - | - | 34.7 | 43.5 | 38.2 | - | - | 38.3 | - | - | 34.8 |
| **98** | *NA* | - | - | 34.6 | - | 40.9 | - | - | 39.5 | - | - | 39.5 |

**Animal number 1 in experiment FMD 2008 (Table 4A) was euthanized at PID 28 due to reasons unrelated to the experiment. The definition of this animal as an FMDV carrier was confirmed by virus isolation in primary bovine thyroid cell culture from the probang sample collected at PID 28.

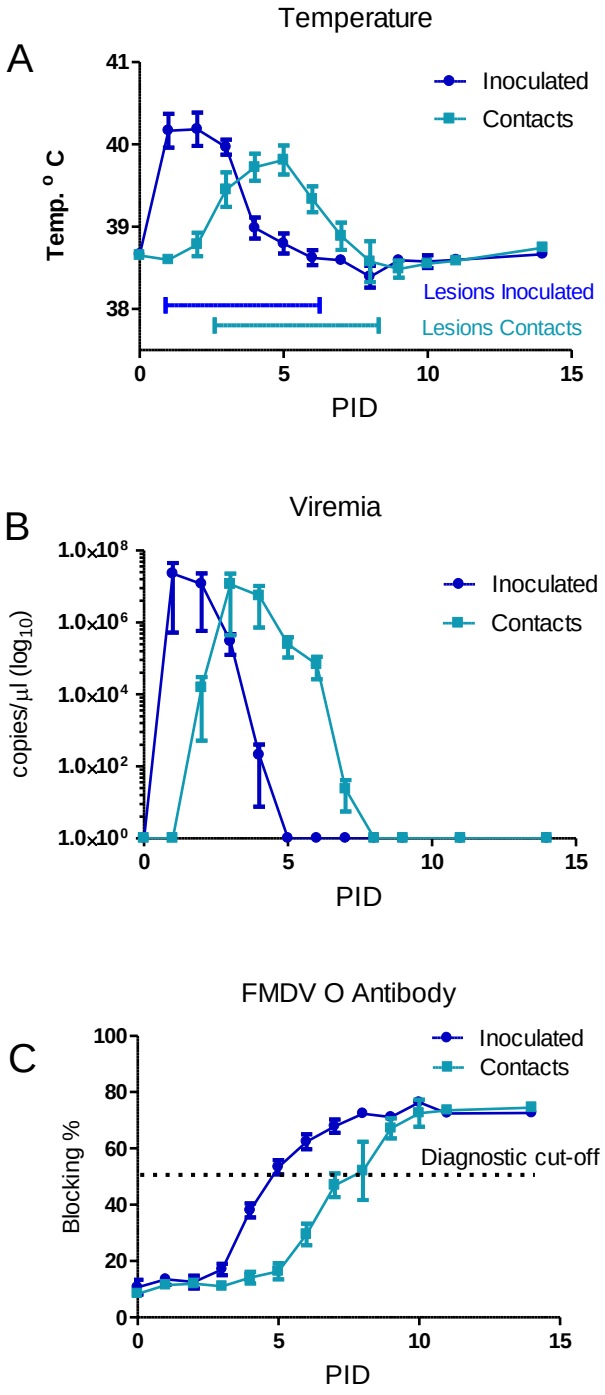
**B) FMD 2010 a**

| | Animal ID (FMDV carriers marked with *) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PID** | **1** | **2*** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11*** | **12*** |
| **0** | - | - | - | - | - | - | - | - | - | - | - | - |
| **1** | 18.4 | 27.7 | - | - | - | 46.8 | - | - | 14.4 | 44.6 | - | - |
| **2** | 37.9 | 28.5 | 44.3 | - | 22.1 | 20.9 | - | - | 37.65 | 38.2 | - | - |
| **4** | 34.9 | - | 21.5 | 20.1 | 37.1 | - | - | - | 38.1 | 25.9 | 41.5 | - |
| **7** | - | - | 41.2 | 42.0 | 40.3 | 33.3 | 24.4 | 26.0 | - | - | 23.8 | 17.8 |
| **11** | 38.6 | - | 36.8 | - | - | - | 32.3 | - | - | 34.0 | - | - |
| **14** | - | 37.6 | - | 43.3 | - | - | - | - | - | 39.7 | 33.0 | 38.7 |
| **21** | - | 36.7 | - | - | - | - | 40.0 | - | - | - | 31.6 | 30.3 |
| **28** | - | 39.3 | - | - | - | - | - | - | - | - | 37.8 | - |
| **29** | - | 41.0 | - | - | - | - | - | - | - | 40.4 | - | 35.5 |
| **30** | - | - | - | - | - | - | - | - | - | - | 38.7 | - |
| **31** | - | 39.1 | - | - | - | - | - | - | - | - | - | 29.2 |

**C) FMD 2010 c**

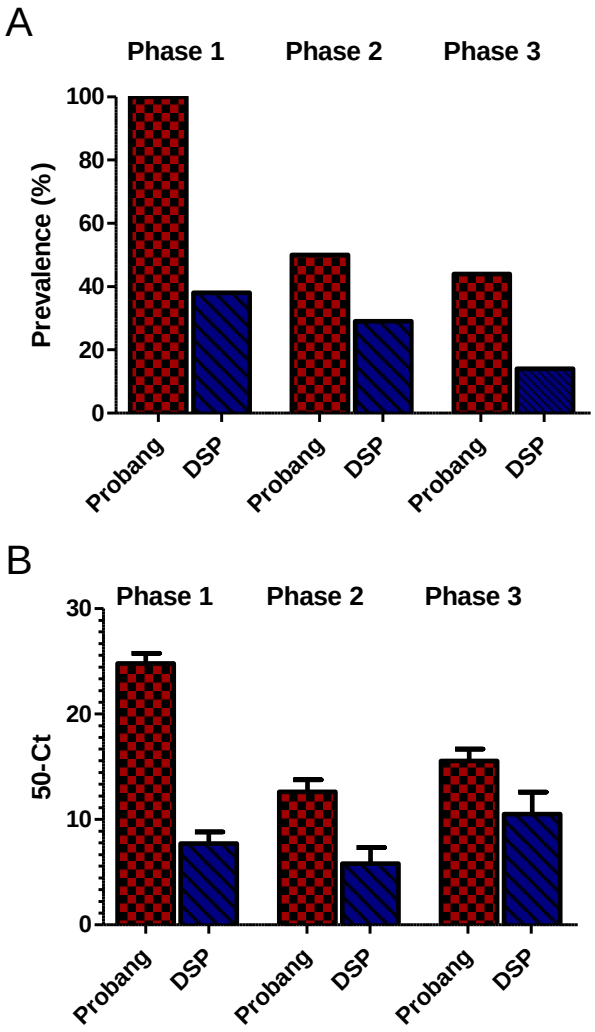
| | Animal ID (FMDV carriers marked with *) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PID** | **1*** | **2*** | **3*** | **4*** | **5** | **6** | **7** | **8** | **9*** | **10** | **11*** | **12*** |
| **0** | - | - | - | - | - | - | - | - | - | - | - | - |
| **1** | 26.4 | 31.1 | - | - | 37.7 | 43.7 | - | - | 39.5 | 20.9 | - | - |
| **2** | 36.8 | 19.0 | - | 26.7 | 28.4 | 18.3 | 44.1 | - | 16.9 | 26.2 | 43.2 | - |
| **4** | - | 25.5 | 23.5 | 23.7 | 23.2 | 26.7 | 16.6 | 14.0 | 30.5 | 33.3 | 16.7 | 22.8 |
| **7** | - | 30.6 | 30.5 | 27.8 | 37.2 | - | 30.4 | 29.1 | - | 40.0 | 32.4 | 24.0 |
| **11** | 38.0 | 37.9 | - | - | - | - | - | 37.3 | 36.2 | - | 31.7 | - |
| **14** | 38.3 | 31.7 | - | - | - | - | 35.7 | 38.9 | 33.3 | - | 40.2 | 35.4 |
| **21** | - | 29.3 | - | 29.5 | - | - | - | - | 30.3 | - | 34.9 | 37.0 |
| **28** | - | 28.9 | 25.1 | 27.8 | - | - | - | - | 27.3 | - | 35.9 | 33.1 |
| **29** | 34.3 | 28.7 | 30.0 | 28.1 | - | - | - | - | 25.9 | - | 32.1 | 30.0 |
| **30** | 33.8 | 32.8 | 40.1 | 32.2 | - | - | - | - | 31.9 | - | 33.4 | 29.3 |
| **31** | 34.3 | 34.6 | 33.6 | - | - | - | - | - | 34.2 | - | 34.0 | 29.3 |
| **32** | 25.8 | 29.9 | 28.9 | 28.1 | - | - | - | - | 28.7 | - | 28.0 | 27.9 |
| **35** | 28.6 | 37.2 | 28.4 | 29.4 | - | - | - | - | 32.8 | - | 31.4 | 33.7 |

**Figure 1**



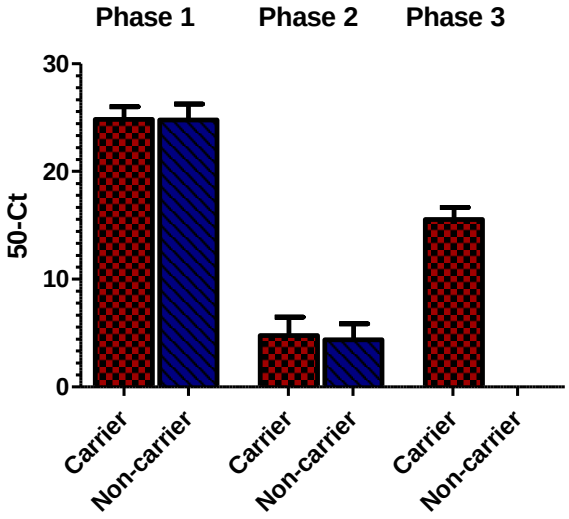

Characterization of clinical signs in FMDV-infected cattle. Infection parameters. The results shown are the mean values (+/- S.E.M) for the indicated parameters from three separate experiments each including 12 cattle (6 inoculated with FMDV and 6 in-contact). A) Temperature and timing of occurrence of clinical lesions B) Viremia measured by qRT-PCR and expressed as copies of FMDV genome/ µl of serum C) anti-FMDV (type O) antibodies in serum expressed as blocking percentage derived from a solid phase blocking ELISA.

**Figure 2**




(A) Prevalence of probang samples and tissues samples harvested from the DSP containing detectable levels of FMDV RNA at three time points during infection. Number of animals included: 36 (probang samples, in each phase), 34 (DSP phase 1), 28 (DSP phase 2) and 36 (DSP phase 3).
(B) Levels of FMDV RNA in probang samples and DSP tissue samples that were found positive (mean +/- SEM). The presented values are calculated as the 50-Ct value from the qRT-PCR assay targeting the 5UTR region of the FMDV genome. Phase 1= the first day the animal showed clinical signs of infection, Phase 2= 7 days later, Phase 3 = PID 28-35.

**Figure 3**




Levels of FMDV RNA in probang samples at three time points during infection in animals detected as FMDV carriers versus non-carriers (mean +/- SEM of 50-Ct for the FMDV 5'UTR qRT-PCR assay). Number of animals: 16 (carriers) and 20 (non-carriers).

# 4. General discussion and conclusions.

The overall aim of the project included in this thesis has been to investigate the innate host response to FMDV infection in cattle. An additional focus has been to elucidate possible variations in host response between animals that developed into persistently infected carriers of the virus and those that did not.

The project has involved the performance of seven animal experiments, which have all been performed within the Biosafety Level (BSL) 3+Ag stable facilities at DTU-Vet, Lindholm. A novel technique of endoscopical collection of small tissue biopsies from the pharyngeal region of live animals has been developed and implemented in order to investigate the local tissue response to FMDV infection in this specific anatomical region. Manuscripts for publication have been based on results from investigations of both the systemic (manuscript 1) and local (manuscript 2) host response to infection, as well as investigations of FMDV-pathogenesis through analyses of sites of virus replication and persistence (manuscript 3).

The first manuscript was based on results from three separate cattle experiments (FMD 3C 2008, FMD 5C 2010 and FMS 6C 2010). As the latter of these three experiments was terminated after 14 days due to technical circumstances, the carrier-status of individual animals was only known for the two earlier experiments (24 out of 36 infected animals included in the study).

The investigation included measurements of the acute phase proteins SAA and HP, as well as measurements of the bioactivity of type 1 IFNs in sera of FMDV-infected calves, compared to un-infected age-matched controls. Results indicated a clear and consistent acute phase response in all measured parameters, which was closely correlated in timing to the onset of clinical disease and the development of viremia. Statistical analysis of data indicated a significantly different HP-response in animals that did not develop into FMDV- carriers compared to those that did. The HP-response was determined through calculations of the area under the curve (AUC) of serum HP-concentration during a 21 days test period. The plotted data indicated that the larger values of the AUC in the non-carriers could be the consequence of a slightly slower rate of decline in serum HP concentrations in this group of animals, which were effective in clearing the infection. This possible finding will, however, need to be confirmed through further investigation with measurements of the HP response in sera from a larger number of FMD infected animals with known carrier-status.

In this first manuscript, it was further concluded that there was no measurable systemic inflammatory reaction related to the carrier state of FMD. The measured acute phase response, for all of the included parameters, declined to base-line levels well in advance of PID 28, which is the generally accepted time post infection from which carrier status can be determined[62].

The second manuscript dealt with the local immune response, through measurements of mRNA expression of inflammatory cytokines IFN-α and-β as well as TNF-α in sequentially collected biopsy samples from the pharyngeal mucosa of FMD infected cattle. This study was based on a slightly different combination of experiments compared to the first manuscript (FMD 3C 2008, FMD 5C 2010 and FMD 7C 2010). The carrier-status of individual animals had been determined in all of the experiments included.
Biopsy samples were collected during acute, sub-acute and late phases of infection, with relative expression levels of individual targets compared to levels measured in pre-inoculation samples harvested from each individual animal. Results indicated significantly down regulated expression of IFN-β mRNA during acute infection, which is consistent with previously published findings from cell-culture based experiments[27,28,73]. There was also a significant difference in the expression levels of TNF-α, with higher levels in carriers compared to non-carriers. These findings are also in line with an earlier report[77] which was based on only a small number of animals. The study design used to investigate variations in the relative gene expression levels has been substantially improved during the current project when compared to previous investigations. The possibility of analyzing sequentially harvested tissue samples from a relatively large number of animals provides further strength for this kind of investigation.

The third manuscript was based on an investigation of FMDV-pathogenesis through measurements of FMDV RNA in sequentially collected biopsies of pharyngeal mucosa. The experiments included in this analysis were similar to those included in the previous manuscript.

The pharyngeal epithelia has been reported to be an important site for FMDV replication during both early[6,21,59,75] and persistent[75,80] phases of infection. Despite this, the levels of FMDV RNA found in biopsy samples collected during the early infection, and in samples of the dorsal soft palate and associated lymph nodes harvested *post mortem* during the late phase of infection, were unexpectedly low. The investigation was based on results from two qRT-

PCR assays, targeting different regions of the FMDV genome. Samples of oropharyngeal fluid (probang samples) were analyzed using a similar assay and were found to contain much larger amounts of FMDV RNA, than the tissue samples harvested at the same stage of infection.

The low prevalence of FMDV RNA detected in biopsies and tissue samples could be interpreted to indicate that the predominant sites of FMDV replication and persistence are not located within the region targeted for sampling. Further experiments will be needed in order to fully elucidate the pathogenesis of early and persistent phases of FMDV infection in cattle.

Analysis of virus excretion in probang samples from the three full-term experiments indicated that the overall prevalence of FMDV-carriers amongst infected animals was 44%, which is consistent with previous reports[2,6,52]. An interesting finding from the analysis of probang samples was that virus excretion in non-carriers became undetectable at approximately PID 14, whereas FMDV RNA remained detectable at consistent levels throughout the experiments (up to 100 days) in the carriers.

Previous publications investigating sites of FMDV replication have all been based on the analysis of tissue samples collected from euthanized animals. The suitability of using biopsy samples of the DSP for this type of investigation could be evaluated through comparisons of the levels of FMDV detectable in biopsy samples and larger tissue samples collected *post mortem*. For instance, biopsy samples could be collected from individual animals which would subsequently be euthanized for collection of larger tissue samples from the corresponding region.

The apparent advantage of being able to collect biopsy samples from live animals is that it is possible to collect sequential samples from individual animals, and also to be able to evaluate the early events of infection in animals which could then be followed to determine their eventual carrier-status.

It should be possible to develop the technique of enodscopical sampling of live animals further by harvesting biopsies from different anatomical regions. Relevant targets could, for example, include epithelia from the lower regions of the respiratory tract.

In relation to this, it could also be relevant to compare the characteristics of FMDV infection in animals which have been exposed to the virus through different routes. There have been recent publications investigating the early events of FMDV pathogenesis in animals infected through controlled aerosol exposure[8,59]. It would be reasonable to believe, that there could be

some significant variations in the characteristics of the early infection in animals that had been infected via more natural routes of exposure, such as direct contact with infected animals.

The carrier status of the animals included in this project has been determined through detection of FMDV RNA in probang samples using qRT-PCR. The definition of an FMDV-carrier is based on the detection of infectious FMDV in oropharyngeal excretions beyond 28 days post infection. When using qRT-PCR for this analysis, it is not possible to differentiate between infectious and non-infectious virus, as can be done through virus isolation in cell culture. Virus isolation in cell culture was attempted in the earlier experiments performed within the project, but due to technical problems, with an apparent need of optimization of the protocol in use, this method was abolished for the current purpose. As the results from qRT-PCR analysis of probang samples were very consistent, it was concluded that this method was suitable for detection of FMDV-carriers in experimental infections, which has also been supported by previous reports[76]. It would, however, add further strength to the investigations if the proposed carrier status of the included animals could be corroborated through isolation of infectious virus from probang samples.

***Conclusions and perspectives.***

Through measurements of the acute phase proteins SAA and HP, as well as bioactive type 1 IFN in sera of FMDV infected cattle, it was concluded that there was no measurable systemic inflammatory reaction related to the carrier status of FMD. The three markers could, however, be used for detection of a systemic reaction during the early stages of FMDV infection in cattle. Preliminary studies also indicated that the magnitude of the acute phase response in SAA and HP could be used in combination with observations of clinical signs of infection in order to achieve a more objective evaluation of the severity of the clinical infection.

There was an indication of a possible difference in the HP-response in sera from animals that developed into FMDV-carriers and those that did not. This finding should be confirmed through further investigations.

There was a measurable down-regulation in expression of IFN-β mRNA in biopsies from the DSP harvested during acute infection, which corroborates previous reports from other groups[27,28,73].

TNF-α mRNA levels in DSP biopsies was significantly up-regulated during acute and late phases of infection, with significantly higher levels in carriers compared to non-carriers. This finding could indicate a prolonged inflammatory reaction in the DSP of FMDV-carriers despite the lack of a continued APP response.

The pathogenesis of FMDV infection in cattle needs further investigations in order for consistent conclusions to be made. There is a general lack of consensus between researchers regarding the exact events involved in both the early and persistent stages of the infection. The results from the investigations included in this project add further uncertainties in relation to some of the commonly cited reports within the area. Collaborations between groups interested in research within this area, with some harmonization of the methods used for investigations would be highly beneficial for future investigations.

The developed technique of endoscopical collection of tissue samples from live animals can be further optimized, and should be highly relevant for future studies of the pathogenesis of FMDV-infection. Results of the performed gene expression analysis in biopsy samples harvested from the DSP indicate that further development of this technique would be relevant for future experiments with aims of investigating the detailed mechanisms involved in the host response to FMDV in cattle.

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**Appendix I**

# Detection of FMDV non-structural protein 3A in frozen tissue sections by indirect immunoflourescence.

### *Introduction and methods*

Detection of FMDV antigens in frozen tissue sections by indirect immunofluorescence was performed using a protocol obtained from IAH-Pirbright [1].

Biopsy samples of pharyngeal mucosa (see manuscript number 2), as well as samples from the dorsal soft palate and mandibular and retropharyngeal lymphnodes, collected *post mortem*, were analyzed for the presence of both structural and non-structural FMDV proteins.

Murine Mab 2C2[2], raised against nonstructural protein 3A was kindly provided by Emiliana Brocchi, Instituto Zooprofilactico Sperimentale della Lombardia, Italy. Mab IB11[1] raised against 146 S FMDV type O antigen was supplied by Nick Juleff, IAH-Pirbright as cellculture supernatant, which was subsequently purified using HiTrap protein G columns (GE Healthcare, Hillerød, Denmark).

Tissue samples were frozen in OCT medium (Tissue-Tek) on dry-ice directly after harvesting, and were subsequently stored at -70$^{o}$C until further processing. Cryo-embedded tissue samples were sectioned and mounted on microscope slides (Superfrost*, Thermo Scientific) and left to dry at room-temperature prior to fixation in acetone. Hydrophobic and electrostatic forces were blocked through incubation with 5% heat inactivated normal goat serum in PBS. Sections were incubated with primary Mabs (diluted 1:1000 in 5% goat serum/PBS) for 30 minutes, before washing and incubation with secondary antibodies (Alexa fluor range, Invitrogen) for another 30 minutes. Nuclear staining was performed with DAPI (Invitrogen, 1:20000 dilution), and slides were mounted using Prolong Gold fluorescence mounting medium (Invitrogen). Following overnight incubation, sections were analyzed using an Olympus BX 51 Fluorescence microscope in combination with Acell© imaging software.

### *Results*

Initial results from staining with the Mab 2C2, which recognizes non-structural protein 3A, showed a consistent and specific pattern of intra-cellular staining in sections of the mandibular lymph nodes from infected animals (see Figures I-1 and I-2) This distinct staining pattern was not found in samples harvested from other organs, nor in corresponding samples from un-infected animals or isotype-control sections (incubated with non-specific murine IgG, Dako). There was, however, a significant amount of unspecific background staining, and due to lack of time, further optimization of this method was not achieved.

Staining with the anti-structural protein Mab IB11 showed non-specific binding, in both test- and control sections. Similar results were observed through staining with anti DC-cell marker CNA.42 (Dako) and anti-CD8 T cell marker HB 264-15 (kindly provided by Jens Nielsen,

DTU-Vet, Lindholm). Due to practical circumstances, further optimization of these methods was not attempted.

***Conclusion***

Initial results indicated a distinct intra-cellular staining pattern with the anti-FMDV 3A Mab 2C2 in the mandibular lympnodes harvested from cattle previously infected with FMDV, during both the early (results not shown) and late (PID< 28) stages of infection. The specific pattern of staining was found both in animals that were identified as FMDV carriers and in those that did not continue to excrete FMDV in oropharyngeal fluid. These findings will require further investigations, including optimization of the protocol used in order to reduce non-specific background staining, in order for any conclusions to be made.

Further optimization should also be performed in order to achieve reliable results from staining with the Mab IB11 (which recognizes structural proteins), as well as additional cellular markers (DC, T-cell sub-populations). Staining with the 2C2 should be combined with staining for cellular markers in order to determine the exact localization of binding sites within the lymphoid tissue. Furthermore, the procedure for nuclear staining with DAPI should be optimized in order to obtain a clearer view of the intracellular localization of the specific staining.

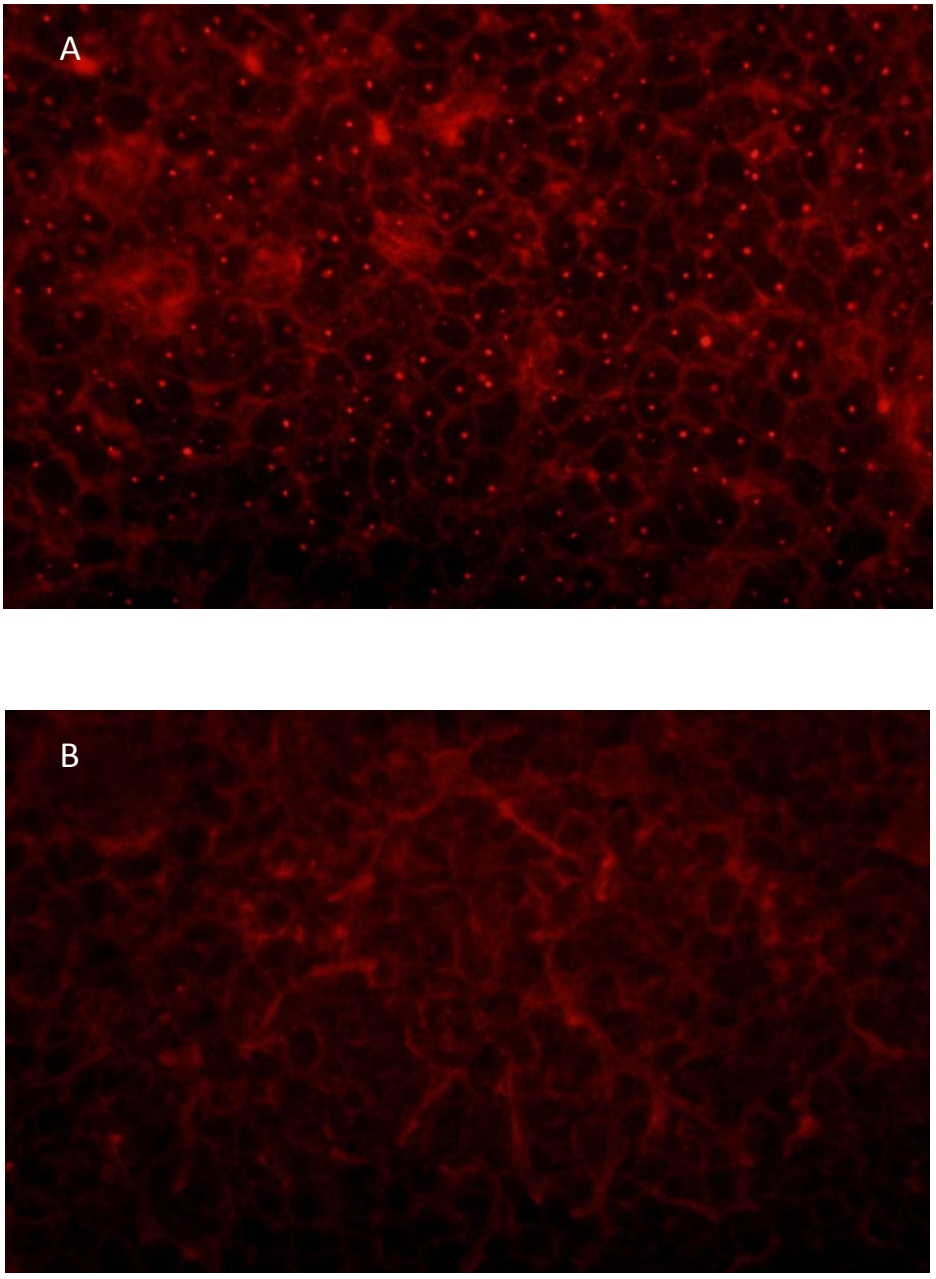


*Figure I-1. Immunofluorescence for FMDV non-structural protein 3A. Mandibular lymph node harvested at PID 35 from calf identified as FMDV-carrier. A) staining with Mab 2C2 shows distinct intracellular pattern. B) parallel control-section incubated with non-specific isotype control (murine IgG, DAKO) without the specific pattern of staining.*

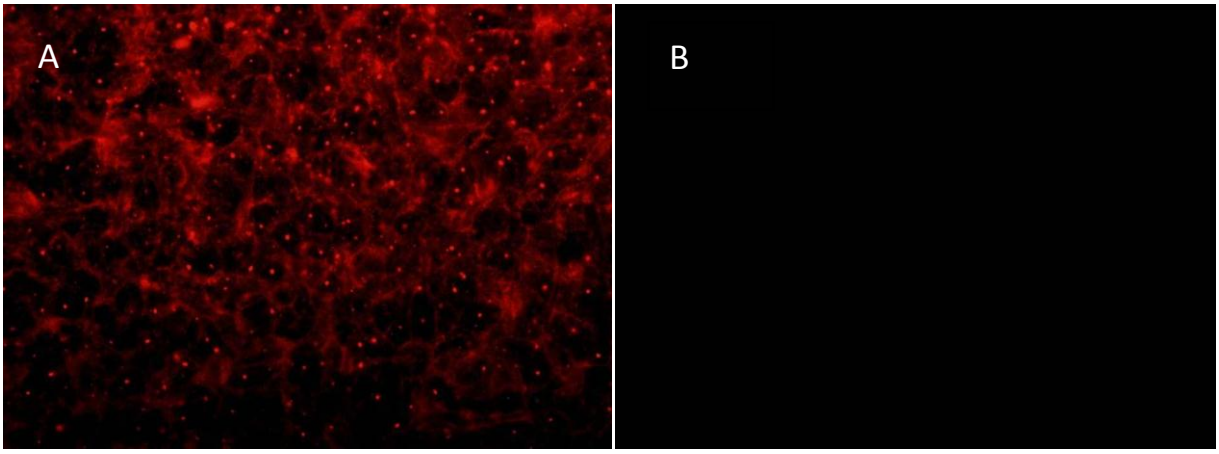


*Figure I-2. Immunofluorescence for FMDV non-structural protein 3A. Mandibular lymph node harvested at PID 35 from calf identified as non-carrier. A) staining with Mab 2C2 shows distinct intracellular pattern. B) parallel control-section incubated with non-specific isotype control (murine IgG, DAKO) without the specific pattern of staining.*

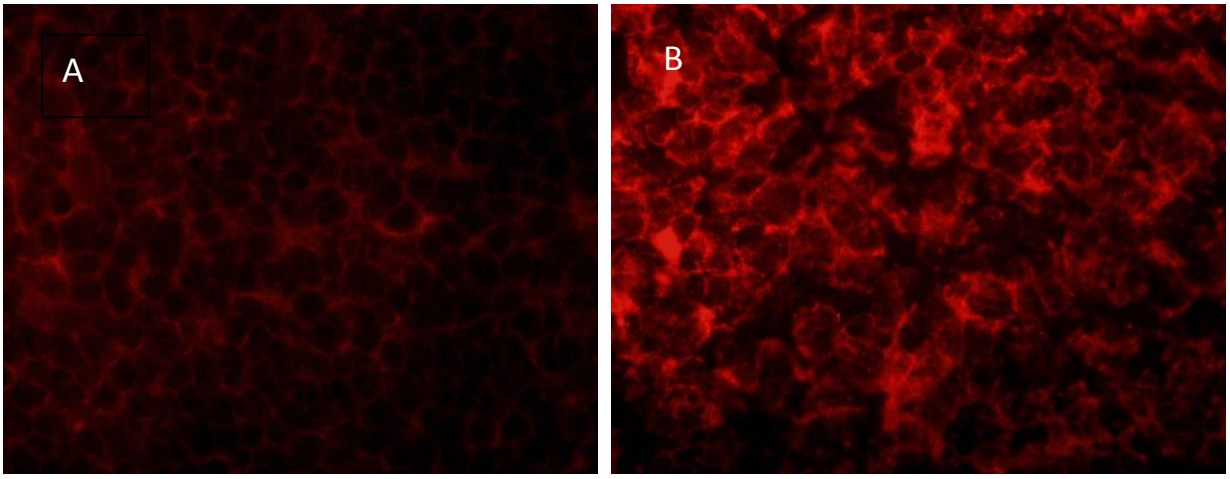


*Figure I-3. Immunofluorescence for FMDV non-structural protein 3A. Retropharyngeal lymphnode harvested at PID 35 from 2 different calves detected as FMDV-carrier (A) and non-carrier (B) respectively. The sections from the retropharyngeal lymphnodes do not show similar staining as seen in the Mandibular lymphnodes in the figures above.*

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