Efficacy of emergency vaccination against foot-and-mouth disease in pigs

Phaedra Eblé

2006

Omslag: Fred van Welie, Phaedra Eblé
Druk: Ponsen & Looijen B.V.
ISBN-10: 90-393-4314-4
ISBN-13: 978-90-393-4314-2

Efficacy of emergency vaccination against foot-and-mouth disease in pigs

Werkzaamheid van noodvaccinatie tegen
mond-en-klauwzeer bij varkens

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht,
op gezag van de rector magnificus, prof. dr. W.H. Gispen,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op donderdag 14 september 2006
des middags te 2.30 uur

door

Phaedra Lydia Eblé

geboren op 20 februari 1967 te ’s-Gravenhage

Promotoren: Prof. Dr. J.A. Stegeman

Prof. Dr. Ir. M.C.M. de Jong
Wageningen Universiteit

Co-promotoren: Dr. A. Dekker
Centraal Instituut voor DierziekteControle, Lelystad

Dr. A. Bouma

Uitgave van dit proefschrift werd mede mogelijk gemaakt met financiële steun van het Centraal Instituut voor DierziekteControle, Lelystad.

Voor Reinder, Sybren en Tjalle

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Chapter 1

Introduction

Introduction

Aetiology and clinical signs

Foot-and-mouth disease (FMD) was the first animal disease ever for which was discovered that it was caused by a filterable agent [1,2]. The causative agent, foot-and-mouth disease virus (FMDV) is now classified within the Aphtovirus genus of the Picornaviridae [3]. Seven immunologically distinct serotypes of the virus are known: the so-called classical types A, O and C, the Southern African territories types SAT 1, SAT 2 and SAT 3 and type Asia-1. Within each serotype there are a substantial number of strains showing a variable degree of antigenic diversity [4].

FMDV can affect all cloven-hoofed animals, the most important domestic animals being cattle, pigs, sheep, goats and domestic buffalo, but FMD also occurs in many wildlife species. Typical clinical signs of animals infected with FMDV include fever and development of vesicles on epithelia of the mouth and feet and in lactating animals also on the udder. The presence of the painful vesicles leads to depression, salivation, anorexia, decreased milk production and lameness. Young animals may die because of myocardial failure. The clinical severity of FMD can vary between different species and also depends on other factors such as the strain of the virus involved. The disease is clinically most apparent in dairy cattle and intensively reared pigs but in sheep and goats clinical signs are milder and may stay clinically undetected [5,6].

Epidemiology

Despite all efforts to eradicate the disease, foot-and-mouth disease is still present in large parts of the world (Fig. 1) [7]. The disease is endemic in large parts of Africa, Asia and South America, and its distribution is clearly associated with areas with lower levels of development and contributes to severe economic problems of many developing countries [8]. After a successful vaccination program in Western Europe, which resulted in cessation of disease outbreaks after 1989, the European Union adopted a non-prophylactic vaccination policy in 1992 [9]. From 1992 until 2001 there were 20 outbreaks in this area that were all rapidly controlled using either a stamping-out policy only or a stamping-out policy associated with ring vaccination [10]. In North America the last outbreaks were reported in 1952 in Canada and 1953 in Mexico [4]. Parts of South America are free of FMD, but in some areas vaccination is still used in order to remain free of the disease. Australia is also free of FMD, the last case of FMD being recorded in 1872 [11], and New Zealand is historically free of the disease.

The most common route of infection with FMDV is by direct contact between infected and susceptible animals. The infection route can either be respiratory or through abrasions

on the skin or mucosal membranes. Beside direct contact transmission, also infection by intake of infected milk or meat, mechanical transfer of infected se- and excreta and infection by aerosols produced by infected animals can occur [6]. Cattle are very susceptible for infection with FMDV and require as little as 10-20 $TCID_{50}$ of virus by the respiratory route to become infected [12,13]. Because of this high susceptibility and the large respiratory volume, cattle are the most likely to be infected by aerosol virus generated by other infected animals [13,14]. Also sheep are highly susceptible to virus infection via aerosol [14,15]. Because in sheep clinical diagnosis is often difficult, the infection can go unnoticed and therefore they can play a major role in spread of the disease. Pigs can be infected by the same routes as cattle and sheep, but another way to become infected for this species is through (illegal) infected swill feeding, which was reported to be the cause of several outbreaks of FMD [16,17]. Pigs are relatively resistant to aerosol infection [18,19], but can, once infected, produce enormous amounts of aerosol virus and therefore are considered as amplifiers of the virus [14].

Disease outbreaks and control measures

Outbreaks of FMD, especially in unvaccinated populations, can have devastating socio-economic consequences as was unfortunately experienced during some recent epidemics of the disease in countries that had been free of the disease for decades. In 1997, an FMD outbreak was reported in Taiwan. The epidemic that followed resulted in the slaughter of more than 4 million pigs, almost 38% of the entire pig population, at a cost of approximately U.S. $1.6 billion [20,21]. The epidemic was controlled by a combination of slaughter of infected animals and vaccination. In February 2001, an outbreak of FMD was detected in the UK, which was the start of a huge epidemic that lasted until September 2001 and led to virus introductions in The Netherlands, France and Ireland. To control the epidemic in the UK, a stamping out policy was applied in which all the infected and in-contact animals were culled to combat the disease. In total the epidemic resulted in the slaughter of 6.5 million animals [22] and the costs were estimated at U.S. $ 12-14 billion [23]. The epidemic in The Netherlands that started in March 2001 was controlled using a vaccination-to-cull strategy in an area surrounding the outbreaks. The epidemic was controlled within 2 months, and resulted in the slaughter of approximately 260.000 animals, including all vaccinated animals [24,25].

The major epidemics and the massive slaughter of animals that resulted stimulated debate about how the disease should be handled in the EU in the future. In the aftermath of the 2001 epidemic, in the UK a number of inquiries were commissioned to examine the government's handling of the epidemic and to determine how the country should prepare itself for and respond to future infectious disease outbreaks. This resulted in a list of key

recommendations for the preparation for disease outbreaks in the future and it was recommended that emergency vaccination should be considered as part of the control strategy from the start of any FMD outbreak [26]. In addition, public reaction, predominantly within The Netherlands, questioned the need for large-scale slaughter of susceptible animals, particularly the slaughter of vaccinated animals that were healthy [21]. All this resulted finally in the adoption of new regulations by the EU and OIE, which make it more attractive to use a vaccinate-to-live policy in case an outbreak occurs [27,28]. In The Netherlands, the use of emergency vaccination is now implemented in the contingency plan.

However, if emergency vaccination will be applied as a standard emergency tool, there is an urgent need to understand how effective vaccination is as intervention measure during an epidemic and moreover, how fast (herd-)immunity is achieved. The most important issue is then that vaccination should sufficiently reduce transmission of the virus in the population, and preferably quickly after vaccination. To quantify transmission, animal experiments in which transmission of the virus is quantified using a stochastic susceptible-infectious-removed (SIR) model have shown to be very suitable [29,30]. In these studies transmission of infection is quantified using the reproduction ratio R, which is defined as the average number of secondary cases caused by one typical infectious individual, and is a measure for transmission from one generation to the next generation [31]. R has a threshold value of 1 [31,32], and as long as $R > 1$ an infection can spread on a large scale (major and minor outbreaks are possible) but as soon as R becomes < 1 the infection will fade out (only minor outbreaks are possible).

Parameters derived from transmission experiments can also be used in mathematical models, which can for example be used to determine the number of expected outbreaks in the high risk period of an epidemic or to evaluate which control measures can reduce transmission to such a level that the virus will be eradicated. Mathematical models have for example already been used extensively during the 2001 FMD epidemic in the UK [33].

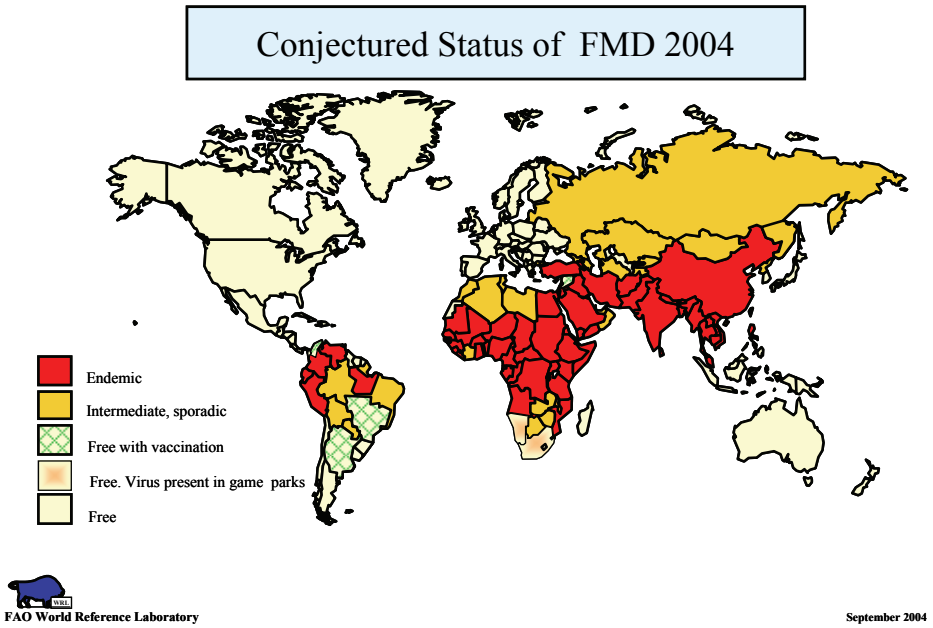
Beside knowledge on the efficacy of vaccines regarding their capacity to reduce transmission of the virus, more knowledge on the induced immune responses after vaccination is desirable. The correlation between induced immune responses and protection can be helpful to determine vaccine efficacy without animal challenge experiments; might facilitate choice-making between vaccines and can be used to develop improved vaccines. Traditionally, the effectiveness of vaccines is determined in PD_{50} experiments, in which the animals are challenged at 21 days post vaccination [34]. For cattle, a correlation between virus neutralizing antibody titres (VN-titres) and protection against challenge at 21 days post vaccination has been established [35]. Due to the early evidence of involvement of antibodies, most studies on vaccine efficacy used humoral immunity responses as indicators

of the overall immunity induced by the vaccines [36], and also for choice-making between available vaccines humoral immune responses are used [37]. However, the correlation between VN-titres and protection is not always precise and also other immune mechanisms play a role in protection, as has been established for cellular immune responses [38,39]. In fact, it is recognized that humoral and cellular immunological response to infection and to vaccination remains poorly described [8,40], and it has been recommended that more studies on the cellular and other types of immunological responses are needed [36].

Scope of this thesis

The research that is reported in this thesis started in 1999, in the aftermath of the huge Classical Swine Fever epidemic of 1997-1998 in The Netherlands [41]. In the same year, an epidemic caused by a pig-adapted FMDV strain occurred in Taiwan. These epidemics raised the awareness of the consequences in case an FMDV introduction in the densely populated, highly susceptible (pig) population in The Netherlands should occur. The research in this thesis, therefore, focused on the efficacy of emergency vaccination against FMDV in pigs. Two important aspects with regard to emergency vaccination were studied. First, the efficacy of emergency vaccination was studied. In Chapter 2, it is described whether and at what time interval homologous vaccination could reduce transmission of FMDV among pigs. In Chapter 3, the effects of homologous and (inta-typic) heterologous vaccination were compared. In Chapter 4, the influence of (4-fold) increase of vaccine dose was studied. The results of all experimental studies were combined and analysed to estimate transmission parameters for both non-vaccinated and vaccinated pigs that might be used in quantitative modelling. In Chapter 5, the within- and between-pen transmission in non-vaccinated pigs was quantified and in Chapter 6, the transmission rate β, the infectious period T and the reproduction ratio R after the vaccination strategies that were used in the previously described experiments were quantified. Secondly, the correlation between the immunological responses after vaccination and protection was studied. In Chapter 3, humoral and cellular immune responses after homologous and heterologous vaccination were compared and in Chapter 4, the correlation between several humoral immune responses shortly after vaccination and protection against FMDV infection, and the serological and mucosal antibody responses after successive vaccination and infection were studied.

Fig 1. FMD occurrence in the world (2004)



www.iah.bbsrc.ac.uk/virus/Picornaviridae/Aphthovirus/index.html [7]

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Chapter 2

Vaccination of pigs two weeks before infection significantly reduces transmission of foot-and-mouth disease virus

P.L. Eblé[a], A. Bouma[b], M.G.M. de Bruin[c], F. van Hemert-Kluitenberg[a], J.T. van Oirschot[c], A. Dekker[a]

[a]Central Institute for Animal Disease Control (CIDC-Lelystad), P.O. Box 2004, 8203 AA Lelystad, The Netherlands
[b]Department of Farm Animal Health, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 7, 3584 CL Utrecht, The Netherlands
[c]Animal Sciences Group Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands

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Abstract

The objective of this study was to investigate whether and at what time interval could vaccination reduce transmission of foot-and-mouth disease virus (FMDV) among pigs. Reduction of virus transmission by vaccination was determined experimentally. Transmission of FMDV was studied in three groups of ten pigs: one non-vaccinated group and two groups that were vaccinated 7 days (-7 dpi) and 14 days before inoculation (-14 dpi), respectively. Five randomly selected pigs from each group were inoculated with FMDV type O Taiwan, while the other five pigs left in the groups were exposed to the inoculated pigs by direct contact. Clinical signs were recorded, virus isolation and RT-PCR were carried out on oropharyngeal fluid, and the neutralizing antibody titres and the antibody response against non-structural proteins of FMDV were determined. No virus transmission was observed in the –14 dpi group, whereas virus transmission was observed in all contact pigs affecting both the non-vaccinated and the –7 dpi group. The reproduction ratio R in the –14 dpi vaccinated group was significantly lower than that of the non-vaccinated group. This study confirms the potential of vaccination as an important tool to reduce transmission of FMDV.

Introduction

Foot-and-mouth disease (FMD) is a contagious viral disease of cloven-hoofed animals. In Europe, the livestock population is highly susceptible to FMD as a result of the non-prophylactic-vaccination policy for FMD, which has been in force in the European Union since 1991. Furthermore, the direct costs of control and indirect costs of trade embargoes owing to outbreaks of FMD in Europe have serious economic consequences as the recent epidemics in the UK and the Netherlands have shown. Outbreaks of FMD in FMD-free countries are usually controlled by ‘stamping-out’ of infected and suspected herds, supplemented by zoo-sanitary measures, but these control measures are often inadequate to prevent the spread of FMD in densely populated livestock areas, necessitating the pre-emptive culling of contact (contiguous) herds. During the FMD epidemic in the Netherlands in 2001 - when the destruction capacity for pre-emptively culled herds proved to be insufficient - emergency vaccination was carried out as an additional measure to reduce the transmission of FMDV. It is unknown however, how soon after vaccination transmission of FMDV is reduced and when adequate herd immunity will be achieved. Clearly discerning the effect of vaccination from among all other measures taken during an outbreak is not possible. Therefore, experimental studies are necessary to quantify reduction of virus transmission by vaccination. Transmission experiments in which a

stochastic susceptible-infectious-removed (SIR) model is used to quantify reduction of transmission have been shown to be very suitable [1, 2]. In these experiments, transmission is quantified by the reproduction ratio R, which is the average number of secondary cases per infectious individual during its entire infectious period [3]. This implies that an infection will fade out in a population when $R < 1$, but can spread on a large scale when $R > 1$. In our study, this approach was followed using pigs that we vaccinated and subsequently challenged with FMDV strain O Taiwan. We chose pigs because they can play an important role in the epidemiology of FMD as they can function as amplifiers of the virus [4]. The O Taiwan strain was used because during the outbreak in Taiwan in 1997, transmission of this virus within pens as well as between pens was extremely high leading to enormous economic damage [5, 6]. Our aim was to quantify the reduction of transmission of FMDV by vaccination at different intervals between vaccination and infection.

Materials and methods

Animals and experimental design

We used thirty-two conventional 6-week-old weaner pigs. Thirty pigs were randomly allocated to one of three pens (12 m^2 per pen) in a high containment unit (groups 1-3). Two pigs were used as a vaccine-control group (group 4). After a 5-day acclimatisation period, the pigs in groups 2, 3 and 4 were vaccinated with a double-oil-in-water emulsion [DOE] vaccine that contained inactivated O Taiwan virus as antigen. The pigs in group 1 were not vaccinated. The pigs in groups 2 and 3 were vaccinated 7 and 14 days before inoculation respectively. The vaccine-control group was vaccinated at the same moment in time as group 3. On day 0, five randomly selected pigs in each pen of groups 1-3 were challenged with FMDV type O Taiwan. The contact pigs were removed before challenge and were put back with their groups again 24h later. Group 4 pigs were not challenged. Rectal temperatures and clinical signs (vesicles, lameness) of the pigs were recorded daily. A body temperature of >40°C for a minimum of two successive days was considered as fever. Care was taken by the animal technicians to avoid mechanical transfer of virus during feeding and watering of the animals and during cleaning out of the pens. In each pen the contact pigs were first sampled and then the inoculated pigs. After challenge, oropharyngeal fluid (OPF) was collected daily using cotton mouth swabs and serum samples were taken at days -14, -11, -7, -4, 0, 3, 7, 10 and 14 dpi. We gave the pigs Buprenorfine to reduce pain caused by generalised FMD, if necessary. Pigs that suffered severely were euthanized.

Vaccine and challenge virus inoculation

The vaccine used for application was prepared in a double-oil-in-water emulsion [DOE], and contained 3µg of O Taiwan 146S antigen per 2 ml dose. The pigs were vaccinated intramuscularly with 2 ml vaccine, 2 cm behind the ear. Intra-dermal inoculation was done in the bulb of the heel of the left hind-foot with 0.1 ml of challenge virus containing 10^5 $TCID_{50}$/ml FMDV O Taiwan (O TAW 3/97). The inoculum we used was a second pig passage of material that originally came from the World Reference Laboratory in Pirbright, UK (O Taiwan 3/97, RS_1 26/3/97).

Sampling procedures

Oropharyngeal fluid (OPF) was collected daily from 0-14 dpi in groups 1-3 with cotton mouth swabs. The swabs were incubated in the laboratory for 30 minutes in 4 ml EMEM containing 5% FBS and 10% antibiotics and then centrifuged, weighed and stored at -70°C for virus isolation. Serum samples were collected at -14, -11, -7, -4, 0, 3, 7, 10 and 14 dpi by puncture of the vena cava anterior. Samples were centrifuged and serum was stored at -20°C.

Laboratory tests

Virus isolation

OPF samples were assayed for the presence of virus by plaque titration on monolayers of secondary pig kidney cells (PK-2 cells) [7]. Ten-fold dilution series (10^0 to 10^{-2}) of the OPF samples (200 µl, tested in duplicate) were allowed to adsorb for 1h on monolayers of PK-2 cells in a six-well tissue-culture plate (Greiner) after which maintenance medium containing 1% methylcellulose was added. After 2 days of incubation the plates were washed in tap water with citric acid and the monolayers were rinsed with tap water and stained with amido-black (0.1% amidoblack in 1M acetic acid, 0.09M sodium acetate, and 10% glycerol). The plaques were counted macroscopically. All incubations were done at 37°C in a humidified atmosphere containing 5% CO_2. Virus titres were expressed as ^{10}log plaque forming units (pfu) per ml.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR):

RNA from the OPF samples was isolated using the MagNA Pure® LC kits for total nucleic acid isolation and the MagNA Pure® system (Roche). The isolated nucleic acid was tested in a LightCycler based RT-PCR, as described elsewhere [8], using primers in the conserved 3D region of the FMDV genome.

Antibody detection

Neutralizing antibody titres (VN-titres) to FMDV in serum samples were measured using the neutralization assay (VNT) described by De Leeuw et al. [7]. End-point titres were calculated as the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of homologous FMDV in 50% of the wells. Serum samples were also tested in a commercially available ELISA (UBI® FMDV NS EIA Swine) for detection of antibodies against non-structural proteins (NS-proteins) of FMDV (NSP-ELISA). This ELISA detects antibodies against non-structural protein 3B and thus differentiates infected pigs from vaccinated ones.

Statistical Methods

We calculated the mean daily virus excretion in OPF (MDV: average per day for the days virus was excreted); the duration (in days) of virus excretion in OPF; the first day that virus was excreted in OPF; and the area under the curve (AUC) of the virus excretion for each pig. We also calculated the virus excretion per day for each dpi for each group. For samples in which no virus was detected in OPF, the detection limit was calculated which we used in the analysis of the AUC and to compare the virus excretion per day of the groups. The detection limit of each individual OPF-sample was calculated by multiplying the dilution factor of the OPF sample with the detection limit of the VI assay (0.4 ^{10}log pfu/ml). The mean detection limit was 1.72, ranging from 0.99-1.84 ^{10}log pfu/ml. For calculation of the AUC, the missing values of pigs that were euthanised before the end of the experiment, were replaced by the average value of the remaining pigs of the same group.

Differences between the three groups for all these parameters were statistically analysed using the non-parametric Kruskal Wallis test (StatXact©) [9]. A non-parametric permutation test was used (StatXact©) [9] for pair wise comparison between groups if the Kruskal-Wallis test produced a significant result. In the case of multiple comparison, Bonferroni correction was applied.

VN-titres of each individual pig were compared to the mean titre of the vaccine-control group (group 4) on the comparable day after vaccination. A titre that was at least 4 times higher than the mean titre of the vaccine-control group was considered as a titre with a significant rise.

We used a stochastic susceptible-infectious-removed (SIR) model [10] to calculate the reproduction ratio R by means of a maximum likelihood estimator [11]. The estimation of R was based on the final size of the outbreak observed in the experiments. Clinical signs of FMD or detection of virus in OPF or a significant rise in VN-titre or response in the NSP-ELISA were considered as evidence of infection with FMD. To test whether transmission

differed significantly between groups, the null hypothesis was that there was no difference in transmission between the non-vaccinated and the vaccinated group.

Results

Clinical signs

In the non-vaccinated group, all inoculated and all contact pigs developed generalized FMD; vesicles and lameness appeared 2-3 dpi. In the –7 dpi vaccinated group, all inoculated pigs developed generalized FMD; vesicles and lameness appeared 2-3 dpi. Three of the contact pigs also developed generalized FMD; vesicles and lameness appeared 2-4 dpi. All the pigs that developed generalized FMD had fever. Two of the inoculated pigs in the –14 dpi vaccinated group developed vesicles on the foot in which they were inoculated, but no generalization of FMD was detected. No clinical signs were detected in the remaining pigs of this group, both inoculated and contacts (Table 1).

Virus isolation of OPF

All the pigs in the non-vaccinated group, both inoculated and contact pigs shed virus, as did all the pigs of the –7 dpi vaccinated group. In contrast, no virus was isolated from OPF in the –14 dpi vaccinated group (Table 1). This resulted in a significant difference between the –14 dpi vaccinated group and the other two groups with regard to MDV ($p<0.05$), duration of virus excretion ($p<0.05$), onset of virus excretion ($p<0.05$) and AUC ($p<0.05$). In contrast, no differences were detected between the non-vaccinated and the –7 dpi vaccinated group for these parameters.

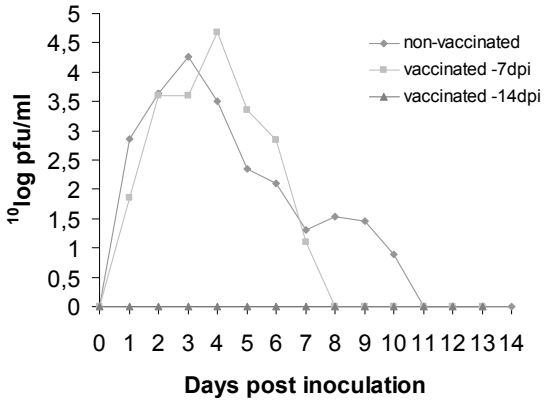

Fig. 1. Virus isolation OPF: mean virus excretions per day per group

Table 1: Virus isolation OPF (^{10}log pfu/ml), RT-PCR, clinical signs, NSP-ELISA and VNT results

	Days post infection																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	clinical	NSP-ELISA		rise[f] in
Non-vaccinated																**signs**	**dpi -**	**dpi +**	**VNT**
inoculated	-	3.4[a,b]	3.0	3.4	3.7	3.3	2.6	2.8	2.2	1.8	-	-	-	-	-	yes	0-9	10-14	+
inoculated	-	4.2	3.4	3.3	3.0	2.9	2.8	-	3.1	2.0	-	-	-	-	-	yes	0-6	7-14	+
inoculated	-	2.9	4.8	3.4	2.9	1.1	3.0	2.3	2.1	2.8	-	-	†			yes	0-9	10-11†	+
inoculated	-	2.9	4.4	4.4	†[d]											yes	0-3†		-
inoculated	-	4.3	3.5	5.0	3.6	2.2	2.6	2.9	†							yes	0-6	7†	+
contact	-	-	2.7	4.1	4.8	3.0	2.4	2.2	1.8	2.1	3.0	-	-	-	-	yes	0-9	10-14	+
contact	-	2.5	2.1	4.9	3.2	2.7	-	-	†							yes	0-7†		+
contact	-	2.9	3.8	4.0	3.2	2.0	1.7	-	-	-	-	-	-	-	-	yes	0-9	10-14	+
contact	-	1.8	3.8	5.1	3.0	1.8	1.8	1.7	-	-	2.4	-	†			yes	0-9	10-11†	+
contact	-	3.6	4.8	5.0	4.1	2.1	1.8	-	†							yes	0-7†		+
Vaccinated -7 dpi																			
inoculated	-	-	3.3	3.7	4.1	2.8	2.2	1.7	†							yes	0-7†		+
inoculated	-	3.6	4.2	4.3	†											yes	0-3†		-
inoculated	-	3.7	4.3	4.7	5.5	3.5	2.2	-	-	-	-	-	-	-	-	yes	0-9	10-14	+
inoculated	-	2.7	3.5	-	4.1	2.8	2.4	-	-	-	-	-	-	-	-	yes	0-9	10-14	+
inoculated	-	3.1	5.2	4.7	4.6	3.7	1.6	-	-	-	-	-	-	-	-	yes	0-9	10-14	+
contact	-	-	3.4	3.4	4.6	4.5	4.9	3.4	-	-	-	-	-	-	-	no	0-14		+
contact	-	-	3.1	4.8	5.4	3.5	1.8	-	-	-	-	-	-	-	-	yes	0-9	10-14	+
contact	-	3.2	3.5	4.3	5.1	4.0	5.0	2.5	-	-	-	-	-	-	-	no	0-14		+
contact	-	2.3	2.6	2.8	4.0	2.1	2.3	-	-	-	-	-	-	-	-	yes	0-9	10-14	+
contact	-	-	2.9	3.3	4.6	3.2	3.2	2.2	-	-	-	-	-	-	-	yes	0-6	7-14	+
Vaccinated -14 dpi																			
inoculated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	no	0-14		-
inoculated	-	-	tox[c]	tox	-	-	-	-	-	-	-	-	-	-	-	yes[e]	0-14		+
inoculated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	no	0-14		+
inoculated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	yes[e]	0-14		+
inoculated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	no	0-14		+
contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	no	0-14		-
contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	no	0-14		-
contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	no	0-14		-
contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	no	0-14		-
contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	no	0-14		-

[a] log titre VI; -: no virus was detected; [b] in grey RT-PCR positive samples are indicated; [c] toxic sample; [d] euthanasia pig, if not indicated pigs were euthanized at 14 dpi

[e] only inoculated foot; [f] titre at least 4 times higher than the mean titre of the vaccine-control group

The mean virus excretion at 1 to 6 dpi of the –14 dpi vaccinated group was significantly lower than the mean virus excretion of both the –7 dpi vaccinated group and the non-vaccinated group ($p<0.05$). At 4 and 5 dpi, however, the mean virus excretion of the –7 dpi vaccinated group was significantly higher than the mean virus excretion of the non-vaccinated group ($p<0.05$) (Figure 1).

RT-PCR of OPF

Although the results of the RT-PCR largely confirmed the VI data, the RT-PCR of 24 samples that were negative in the VI assay were found to be positive. It usually concerned samples collected at the end of the shedding period. Two samples were positive in the VI assay, but negative in the RT-PCR (Table 1).

VNT

All the vaccinated pigs developed a neutralizing antibody titre as well as all the pigs of the non-vaccinated group that lived beyond 3 dpi. The VN-titres of all the individual pigs of the –7 dpi and the –14 dpi vaccinated group were compared with the concordant mean titre of the vaccine-control group. Four of the five inoculated pigs in the –7 dpi vaccinated group showed a significant rise in VN-titre after challenge, apart from the pig that was euthanized 3 days after challenge which showed no significant rise in VN-titre. The contact pigs of the –7 dpi vaccinated group all demonstrated a significant rise in VN-titre. Four of the inoculated pigs in the –14 dpi vaccinated group, showed a significant rise in VN-titre after challenge but none of the contact pigs of this group did (Table 1). Mean neutralizing antibody titres of each group are presented in Figure 2.

NSP-ELISA

Seven of the pigs in the non-vaccinated group developed antibodies against NS-proteins which were detected for the first time either 7 or 10 dpi (Table 1). Three pigs whose serological response against NS-proteins remained negative were euthanized at 3 or 7 dpi. In the –7 dpi vaccinated group, three of the inoculated pigs developed antibodies against NS-proteins. The other two pigs were euthanized at 3 and 7 dpi respectively. Three of the contact pigs of this group developed antibodies against NS-proteins, the other two remained negative until 14 dpi. None of the pigs in the -14 dpi vaccinated group developed antibodies against NS-proteins until 14 dpi (Table 1).

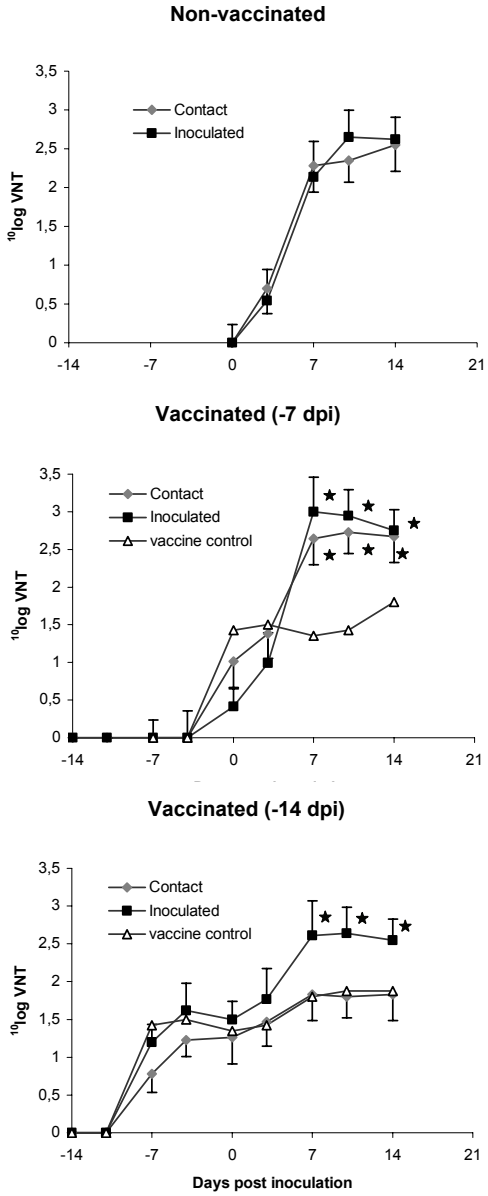

Figure 2. Serological response. * titre at least 4 times higher than the mean titre of the vaccine-control group

Transmission of FMDV

All the contact pigs in both the non-vaccinated group and the –7 dpi vaccinated group became infected. For both groups, the R was estimated to be ∞ (0.67-∞, 95% confidence interval), which is not significantly > 1 (p=0.083). None of the contact pigs in the –14 dpi vaccinated group became infected. The R was estimated to be 0 (0-2.18), which was significantly lower than the R of the non-vaccinated group (p=0.013), however, the R in the –14 dpi vaccinated group was not significantly < 1 (p=0.13).

Discussion

Our results demonstrate that vaccination successfully prevented transmission of FMDV among a group of pigs vaccinated 14 days before infection, but not among those vaccinated 7 days before infection. Vaccination did induce clinical protection in all pigs of the –14 dpi vaccinated group while only in 2 of the 5 contact pigs in the –7 dpi vaccinated group. However, these 2 clinically protected contact pigs did shed virus at titres comparable to those of pigs that showed signs of FMD. That pigs that were clinically protected against challenge with FMDV can still shed virus has been previously shown [12]. In our experiment, protection against clinical signs after challenge in the –7 dpi vaccinated group was barely observed, whereas in other experiments [12, 16], protection against clinical signs was seen as early as 4 days after vaccination. This difference is probably the result of a more severe challenge in our study. Salt et al. [16] and Barnett et al. [12] exposed their vaccinated pigs for 1 or 4 hours to infected donor pigs, whereas the pigs in our experiment were exposed continuously.

There were no significant differences observed in the mean daily virus excretion in OPF, the duration of virus excretion and the AUC of the mean daily virus excretion between the non-vaccinated and the –7 dpi vaccinated group. Remarkably, at 4 and 5 dpi, the virus excretion of the –7 dpi vaccinated group was significantly higher than that of the non-vaccinated group. This was probably caused by a one day delay in rise of virus in OPF in the –7 dpi vaccinated group. The delay resulted in the decline in virus titre observed in the non-vaccinated group coinciding with the peak in virus titre in the –7 dpi vaccinated group (Figure 1). Although this delay could have been a chance happening, a delay in mucosal immune response could also be a likely explanation for this finding. Experiments with cattle have demonstrated that serum IgG antibodies in vaccinated animals can delay the mucosal immune responses after infection [13, 14].

As opposed to VI, we detected more samples that were positive for FMDV using RT-PCR, especially beyond 6 dpi (Table 1), which might have been due to a difference in sensitivity of the methods, however, the sensitivity of the RT-PCR we used was almost

equal to the sensitivity of VI for FMDV strain O Taiwan [8]. Probably, neutralizing antibodies that are present interfere in the VI but not in RT-PCR. Treatment of OPF samples with 1,1,2-trifchloro-1,2,2-trifluorethaan could be applied in future studies to see whether this would result in more positive samples in the VI, as is seen in OPF samples of cattle [15].

In our experiment, we detected a four-fold rise in antibody titre in all the pigs in the non-vaccinated group and the –7 dpi vaccinated group, confirming that virus replication had taken place. However, such a secondary antibody response after challenge was also detected in 4 of the 5 challenged pigs in the –14 dpi vaccinated group from which no virus was isolated. Similarly, in the experiment of Salt et al. [16] pigs that were protected against challenge at 4 days after vaccination showed a secondary antibody response after challenge, indicating that probably limited virus replication had taken place in these pigs.

No antibodies to NS-proteins were detected in most of the samples taken from the pigs that were euthanized at 3 or 7 dpi. This is not remarkable because samples started to be positive in the NSP-ELISA from 7-10 dpi onwards. However, samples from two pigs in the –7 dpi vaccinated group and samples from all the pigs in the –14 dpi vaccinated group remained negative in the NSP-ELISA up to 14 dpi, whereas some of these pigs demonstrated a positive VI of OPF and/or a four-fold rise in antibody response compared to the control pigs. Probably 14 days was too short after infection for the NSP-antibodies to appear or virus replication in these pigs was too limited to induce a response against NS-proteins. New EU legislation allows vaccination in case of an outbreak, together with the use of NSP-ELISAs to distinguish between infected and vaccinated animals. However, the results of this study show that the use of NSP-ELISAs might be useful, but more extensive validation is necessary. In our study, VI and RT-PCR were better single indicators of infection in the contact pigs than the NSP-ELISA, but this will probably differ later after infection.

This is the first experiment for FMDV using a SIR model to determine transmission of the virus. Transmission of FMDV has been studied before by placing susceptible seronegative sentinel pigs together with vaccinated pigs that had been challenged [12, 16]. However, the effectiveness of a vaccine cannot be quantified properly if a group of pigs consists of vaccinated and unvaccinated animals [17]. In our experimental set-up we could determine the reduction of transmission, at different moments after vaccination. The R in the –14 dpi vaccinated group was significantly lower than the R of the non-vaccinated group confirming the potential of vaccination as an important tool to reduce transmission of FMDV. Another aspect of evaluating the effectiveness of vaccination in transmission experiments is to test whether R of a vaccine is significantly < 1. In the –14 dpi

vaccinated group, R was not significantly < 1 (p=0.13), therefore we cannot exclude the possibility that major outbreaks of FMD could occur in groups of pigs vaccinated at -14 dpi. To demonstrate that R in the –14 dpi vaccinated group is statistically significantly < 1, more transmission experiments are needed. In a recent similar experimental set-up, we demonstrated for the second time that transmission of FMDV was absent in a group of pigs vaccinated at –14 dpi (Eblé et al, paper in preparation). If the results of the two studies are combined, R for the –14 dpi vaccinated group would become significantly < 1 (p= 0.017) implying that only minor outbreaks will occur and that the infection will disappear.

Our results suggest that effective within-pen protection is attained between 7 and 14 days after vaccination. However, the onset of an infectious disease in a neighbouring pen can be delayed compared to the onset within a pen. In another study we found a delay in the onset of FMD in pigs in a neighbouring pen compared to the onset of FMD in pigs within a pen (Eblé, unpublished result). For Classical Swine Fever, Klinkenberg et al. [18] found the R value for between-pen transmission to be lower than that of within-pen transmission. Therefore, if vaccination is carried out during an outbreak of FMDV, transmission between farms will probably be effectively reduced well before 14 days after vaccination.

Acknowledgements

This work was funded by the European Union (FAIR5-PL97-3665) and the Netherlands Ministry of Agriculture, Nature and Food Quality.

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Chapter 3

Comparison of immune responses after intra-typic heterologous and homologous vaccination against foot-and-mouth disease virus infection in pigs

P.L. Eblé[a], M.G.M. de Bruin[b], A. Bouma[c], F. van Hemert-Kluitenberg[a], A. Dekker[a]

[a]Central Institute for Animal Disease Control (CIDC-Lelystad), P.O. Box 2004, 8203 AA Lelystad, The Netherlands
[b]Division of Infectious Diseases, Animal Sciences Group Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands
[c]Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands

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Abstract

This study compares the immune responses and protection induced by intra-typic heterologous vaccination with that induced by homologous vaccination against challenge with foot-and-mouth disease virus (FMDV). Humoral and cell-mediated immune responses and protection against challenge with FMDV O Taiwan were examined in a non-vaccinated group, a group vaccinated with O Taiwan FMD vaccine and a group vaccinated with O Manisa FMD vaccine. Five pigs from each group were challenged with FMDV type O Taiwan 14 days after vaccination and five other pigs were contact-exposed to the inoculated pigs. Both homologous and heterologous vaccination protected against challenge with FMDV O Taiwan at two weeks after vaccination. In the heterologous vaccinated group, cross-neutralising antibody titres against O Taiwan could be detected although the ratio 'r_1' was 0.4, which was significantly smaller than the critical r-value. Cell-mediated immune responses were detected after both homologous and heterologous vaccination. Virus-induced *in vitro* lymphocyte (cross-) proliferation and production of both a Th1-type (IFN-γ) and a Th2-type (IL-10) cytokine response were demonstrated in cultures of peripheral blood mononuclear cells (PBMC).

The findings show that heterologous (emergency) vaccination can prevent clinical disease and shedding of virus. The induction of the cell-mediated immune responses after (heterologous) vaccination needs more research but data on these responses might provide additional tools for both vaccine choice and vaccine development.

Introduction

Foot-and mouth disease (FMD) is a highly contagious and economically devastating disease of susceptible cloven-hoofed animals. The European Union (EU) has had a non-prophylactic-vaccination policy for FMD since 1991 and the control policy for outbreaks of FMD used to be primarily based on the implementation of hygienic measures, movement restrictions and culling of infected animals [1,2]. Additionally, culling of susceptible in-contact animals and susceptible animals in neighbouring herds has been applied. During the last outbreaks in the EU, millions of animals were culled and destroyed. This led to an intense debate on the ethical aspects of the non-vaccination policy, culminating in the adoption of a new directive of the FMD legislation by the EU (2003/85/EC), in which the emphasis on various control measures has been shifted in favour of emergency vaccination. However, if we are to implement (emergency) vaccination as the preferred control measure, we must broaden our understanding of its effectiveness.

Vaccines to be used during an outbreak will probably be provided by vaccine banks that store a selection of concentrated, inactivated FMD antigen for this purpose. The efficacy of possible vaccine candidates is usually examined by carrying out serological tests in which the field isolate and a panel of sera against the vaccine strains are used to determine the serological relationship between the field isolate and a vaccine strain. This serological relationship can be quantified by the ratio 'r_1' [3]. The value of 'r_1' reflects whether or not a (reasonable) level of cross-protection between the vaccine candidates and the field strain could be expected. Because the selection of stored antigen is usually limited, vaccines provided during an emergency are usually (intra-typic) heterologous, necessitating careful examination of the immune responses and protection induced by heterologous vaccination.

Although protection against FMDV is often associated with the induction of high levels of circulating neutralizing antibodies in serum, this response does not ensure clinical protection and animals with low VN-titres may nevertheless be protected [4]. It is known that cell-mediated immunity is required and may be crucial for protection against FMDV [5] and cytokine profiles induced by vaccination can be considered as determinants for the efficacy and characteristics of the developing immune defences. However, the cell-mediated immune responses are usually not examined during the selection of vaccine strains. Cell-mediated immune responses, including virus-induced cytokine production after (repeated) homologous vaccination have been described previously [6], but it is not yet known whether these responses are comparable after heterologous vaccination, which is more representative of a field situation.

While vaccination may protect against clinical disease and reduce or even prevent virus excretion, an important feature of an (emergency) vaccine is that it should be able to stop transmission of the field virus [7]. We recently demonstrated that homologous vaccination of pigs with vaccine-strain O Taiwan two weeks before infection prevented transmission of FMDV O Taiwan [8], but it cannot be concluded from that study that vaccination with a different subtype would have the same effect.

The current study compares the protection induced by (intra-typic) heterologous vaccination with an O Manisa vaccine with that of homologous vaccination with an O Taiwan vaccine against subsequent challenge with FMDV O Taiwan. In an experimental set-up designed to quantify reduction of transmission, we examined the humoral immune response and determined the serological relationship between FMDV O Taiwan and FMDV O Manisa by calculating the ratio 'r_1' with the sera obtained in this study. To examine how homologous and heterologous vaccinations influence the cell-mediated immune response, we monitored the virus-induced *in vitro* lymphocyte proliferation after re-stimulation with FMDV O Taiwan. The Th1-type (IFN-γ) and Th2-type (IL-10) cytokine responses were

determined by assessing the concentrations of IFN-γ and IL-10 in supernatants of cultured PBMC.

Materials and methods

Animals and experimental design

We used 34 conventional 6-week-old piglets that were randomly allocated to five groups. Three groups (groups 1, 3 and 5) consisted of ten pigs each; two groups of 2 pigs each (groups 2 and 4) were used as vaccine-control groups. The pigs were housed in our high-containment unit, each group in a separate room except for groups 2 and 4, which were housed together. Groups 1 and 2 were vaccinated at day 0 with a double-oil-in-water emulsion [DOE] vaccine that contained inactivated O Taiwan virus as antigen. Groups 3 and 4 were vaccinated at day 0 with DOE vaccine that contained inactivated O Manisa virus as antigen. Group 5 (challenge control group) remained non-vaccinated. At 14 days post vaccination (dpv), five randomly selected pigs in groups 1, 3 and 5 were challenged with FMDV type O Taiwan. The contact pigs were removed before challenge and were added to their groups again 24h later. Groups 2 and 4 were not challenged. Buprenorfine was administered to reduce pain caused by generalized FMDV, and pigs that suffered severely were euthanized. Approval of the Ethics Committee for Animal Experiments of CIDC-Lelystad was obtained before the start of the experiment.

Vaccine and challenge virus inoculation

The vaccine used for application was prepared in a double-oil-in-water emulsion and contained 3μg of O Taiwan (O Taiwan 3/97) or O Manisa (O Manisa/Turkey/69) 146S antigen per 2 ml dose. The pigs were challenged by intra-dermal inoculation in the bulb of the heel of the left hind-foot with 0.1 ml of challenge virus containing 10^5 $TCID_{50}$/ml FMDV O Taiwan (O Taiwan 3/97), which was a second pig passage of material originally derived from the World Reference Laboratory in Pirbright, UK (O Taiwan 3/97, RS_1 26/3/97). The inoculum was titrated before and after inoculation of the pigs.

Sampling procedures

Clinical signs (fever, vesicles, lameness) of the pigs were recorded daily. Oropharyngeal fluid (OPF) was collected daily after challenge from 14-28 dpv in groups 1, 3 and 5 using cotton mouth swabs. The swabs were incubated in the laboratory for 30 minutes in 4 ml EMEM containing 5% FBS and 10% antibiotics and then centrifuged, weighed and stored at -70°C until virus isolation was performed. Heparinized blood samples and serum samples were collected at days 0, 3, 7, 10, 14, 17, 21, 24 and 28 dpv by puncture of the

vena cava anterior. Plasma samples for virus isolation were obtained by centrifugation of the heparinized blood samples; the plasma was stored at –70°C. Peripheral blood mononuclear monocytes (PBMC) were obtained from heparinized blood samples by density gradient at 0, 14, 21 and 28 dpv. Serum samples were centrifuged and serum was stored at –20°C.

Laboratory tests

Virus isolation

Challenge virus, OPF samples and plasma samples were assayed for the presence of virus by plaque titration on monolayers of secondary pig-kidney cells [9]. Ten-fold dilution series of the samples (200 µl, tested in duplicate) were allowed to adsorb for 1h on monolayers of PK-2 cells in a six-well tissue-culture plate (Greiner), and then maintenance medium containing 1% methylcellulose was added. After 2 days of incubation at 37°C in a humidified atmosphere containing 5% CO_2, the plates were washed in tap water with citric acid; monolayers were then rinsed with tap water and stained with amido-black (0.1% amido-black in 1M acetic acid, 0.09M sodium acetate, and 10% glycerol). Plaques were counted macroscopically. Virus titres expressed as ^{10}log plaque forming units (pfu) per ml were corrected for the dilution by the medium that was added before storage.

Antibody detection

Neutralizing antibody titres (VN-titres) against FMDV O Taiwan and VN-titres against FMDV O Manisa in serum samples were measured using the neutralization assay as described previously [9]. End-point titres were calculated as the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of FMDV (O Taiwan or O Manisa) in 50% of the wells.

Antibodies against non-structural proteins of FMDV in serum samples were determined using a commercially available ELISA (UBI® FMDV NS EIA Swine) (NSP-ELISA). This ELISA detects antibodies against non-structural protein 3B allowing differentiation between infected and vaccinated pigs [10].

We determined the antigenic relationship of O Taiwan and O Manisa based on their neutralization by antibodies by using the ratio 'r_1', which is the neutralization antibody titre against the heterologous virus divided by the neutralization antibody titre against the homologous virus [3,10,11]. The titres of the sera collected 14 days after vaccination were used for this calculation. We divided the VN-titres against O Taiwan by the VN-titres against O Manisa of the O Manisa vaccinated pigs and next calculated the mean 'r_1' value. The calculated ratio 'r_1' was subsequently compared to the critical r-value. This critical r-value is defined as the highest value of r, which is distinguishable from 1 for a given

number of replicates and a probability P [11,12]. For 10 replicates (which is the number of sera we used) the critical r-value is 0.459 (p=0.01) [12]. We obtained the ratio 'r_2' by dividing the VN-titres of the O Taiwan vaccinated pigs against O Manisa by the VN-titres of the O Taiwan vaccinated pigs against O Taiwan.

Lymphocyte proliferation assay

To determine the extent of the cell-mediated immune response, we determined the FMDV-induced *in vitro* lymphocyte proliferation response. Initially, only the inoculated pigs were assayed because for these pigs the exact time of infection with FMDV was known. The proliferation assay was essentially performed as described for pseudorabies virus [13]. Briefly, PBMC were isolated from heparinized blood samples by centrifugation onto Lymphoprep (Nycomed Pharma) and were washed twice with PBS. Viable cells were counted using trypan blue and adjusted to a concentration of 5.10^6 cells per ml in RPMI complete medium (RPMI containing 10% porcine serum, 2 mM L-glutamine, 50 μM β-mercapto-ethanol, 200U/ml penicillin, 200 μg/ml streptomycin and 100 U/ml of mycostatin).

Lymphocyte proliferation assays were done in quadruplicate in 96-well flat-bottom plates (Greiner). To each well, 100 μl of the PBMC and 100 μl of the antigen or control suspension were added. Viral antigen (FMDV O Taiwan) for the lymphocyte proliferation assay was prepared in a suspension of a BHK cell culture in a large-scale fermentor. The suspension was frozen (-70°C), thawed and clarified after collection. Material from an uninfected BHK cell culture was treated likewise and used as mock-control. The PBMC were cultured for five days at 37°C in a humified atmosphere containing 5% CO_2 with medium alone, with medium containing FMDV O Taiwan (10^6 $TCID_{50}$ per 100 μl), with mock-control or with human IL-2. The cultures were consequently pulsed with 0.4 μCi [^{3}H]-Thymidine (Amersham, The Netherlands) and after 4 hours of incubation, cells were harvested and the radioactivity was measured in a Betaplate scintillation counter (Wallac, EG&G Instruments, The Netherlands). Proliferation was expressed as delta counts, which is the number of counts (mean of quadruplicate) of FMDV-stimulated cultures minus number of counts of the mock-control stimulated PBMC.

Cytokine ELISAs

Concentrations of IFN-γ and IL-10 were determined in supernatant of cultured PBMC. Supernatants of PBMC cultured with FMDV or control suspension were collected 4 days after the start of the culture. Initial experiments had shown that IFN-γ and IL-10 concentrations in supernatant were optimal at this time point. Samples were stored at -70°C

until analysis and IFN-γ and IL-10 concentrations were determined using commercially available ELISA-kits (Biosource). Assays were performed according to the supplier's instructions. In each assay, a control recombinant porcine cytokine was diluted over the recommended detection range from which a standard curve was generated. Concentrations in the samples were calculated using this standard curve. For IFN-γ concentrations the values obtained in the virus stimulated wells were corrected by subtracting the value obtained in the mock control.

Statistical methods

The mean daily virus excretion in OPF (the average virus titre found in OPF for the days on which virus was excreted) and the duration (in days) of virus excretion were calculated for each pig. Statistical analysis of the differences between the groups was done using the non-parametric Kruskal-Wallis test (StatXact) [14]. If the Kruskal-Wallis test gave a significant result, for pair wise comparison between groups a non-parametric permutation test was used (StatXact). In case of multiple comparisons, Bonferroni's correction was applied.

A linear mixed effects model (S-Plus 6.2) using maximum likelihood optimisation was used to determine the difference between the day of vaccination (day 0) and the other days when samples were collected and differences between the homologous and heterologous vaccinated-challenged groups (groups 1 and 3) of the VN-assay, the lymphocyte proliferation and the cytokine concentration data. Vaccination group and number of days after vaccination were included as explanatory variables in the model and, additionally, animal was included as a random effect variable, to take into account that observations originating from the same animal are not independent. Moreover, also the interactions between vaccination group and number of days after vaccination were tested and the best-fitted model was selected based on the lowest AIC (Akaike's Information Criterium) [15]. For the VN-assay data a Gaussian distribution of the data was assumed. We replaced the VN-titres with a value of < 0.3 by 0 for the calculations. For analysis of the cytokine concentration data, we replaced all negative values by 1 and transformed the data by taking the natural logarithm to enable the use of a Gaussian error distribution.

Contact infections

Contact pigs were considered infected if they had clinical signs of FMD; or virus was isolated in OPF or plasma samples; or if a boost (rise ≥ 0.6 at 28dpv as compared to the mean titre of the vaccine-control group) in VN-titre; or a response in the NSP-ELISA was detected.

Results

Clinical signs

All the inoculated pigs and all the contact pigs in the non-vaccinated group developed generalized FMD. Clinical signs of FMD appeared 2-4 days after challenge. No clinical signs of FMD were detected in either the O Manisa or the O Taiwan vaccinated groups.

Virus isolation

The titre of the challenge virus was $10^{5.2}$ $TCID_{50}$/ml. Virus was isolated from OPF of all pigs of the non-vaccinated group (Table 1). Virus was also detected in all plasma samples taken from this group at 3 days post challenge. No virus was detected in any of the OPF and plasma samples collected from vaccinated pigs. This resulted in a significant difference between the non-vaccinated and the vaccinated groups with regard to mean daily virus excretion ($p<0.01$) and duration of virus excretion ($p<0.01$).

Table 1. Virus isolation OPF and NSP-ELISA results of the non-vaccinated group

	dpi[c]													NSP-ELISA + at dpi
	0	1	2	3	4	5	6	7	8	9	10	11	12-14	
Inoculated	-	-	4.3[a]	5.4	4.8	†[b]								
Inoculated	-	-	3.2	6.5	6.6	†								
Inoculated	-	-	5.4	3.4	3.4	†								
Inoculated	-	-	5.6	4.4	4.4	†								
Inoculated	-	2.5	5.5	3.4	2.8	†								
Contact	-	-	3.1	5.0	3.5	†								
Contact	-	-	2.7	5.5	4.6	4.5	2.8	2.3	1.9	-	-	1.7	-	10-14
Contact	-	-	2.8	5.3	4.0	4.3	3.3	1.3	†					7
Contact	-	-	2.1	3.9	5.3	†								
Contact	-	-	4.0	3.3	3.1	†								

[a] ^{10}log pfu/ml; -: no virus was detected

[b] euthanasia pig

[c] days post inoculation

Antibody responses

Virus neutralization assays (VN-assays)

All vaccinated pigs developed neutralizing antibodies against both FMDV O Taiwan and O Manisa. The O Taiwan vaccinated pigs showed a higher neutralizing antibody response against O Taiwan than against O Manisa. The reverse was the case in the O Manisa vaccinated pigs (Figure 1). The responses of the vaccine-control groups were comparable to the responses of the vaccinated-challenged groups (results not shown).

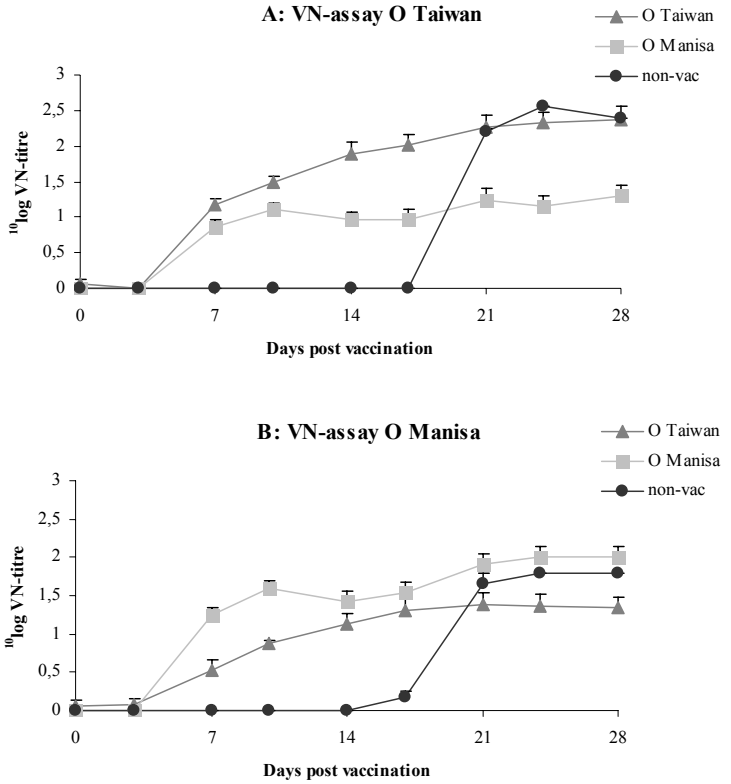

Fig. 1. Neutralizing antibody responses

^{10}log VN-titres, error bars represent standard error of mean (SEM)

O Taiwan = mean response of all the pigs of the O Taiwan vaccinated-challenged group (group 1);

O Manisa= mean response of all the pigs of the O Manisa vaccinated-challenged group (group 3);

non-vac= mean response of all the pigs of the non-vaccinated challenge control group (group 5);

VN-titres < 0.3 are represented as 0.

After vaccination for both the O Manisa and the O Taiwan VN-assay, a significant difference in titre compared to day 0 was observed at days 7, 10, 14, 17, 21 and 28 ($p<0.01$). Also, a significant difference ($p<0.01$) was detected between the titres of the homologous and heterologous vaccinated groups. One challenged pig from the O Manisa group had a rise of ≥ 0.6 at 28dpv compared to the vaccine-control group, however, this difference in titre had already been observed in this pig on the day of challenge.

In the non-vaccinated group, an increase in VN-titre was detected at 3 or at 7 days after challenge. Using the sera of the vaccinated pigs collected at 14 dpv an average 'r_1' of 0.4 was calculated, which was significantly distinguishable from 1 as compared to the critical r-value for 10 replicates ($p<0.01$). The value 'r_2' was calculated to be 0.2.

ELISA against non-structural proteins

The two pigs that lived beyond 4 dpi of the non-vaccinated group were the only animals that developed antibodies against non-structural proteins. No antibodies against non-structural proteins were detected in the serum samples of the pigs of the vaccinated groups after vaccination or after challenge (Table 1).

In vitro lymphocyte proliferation response and cytokine production of PBMC

Figure 2 shows the lymphocyte proliferation responses and the IFN-γ and the IL-10 concentrations as detected in cultures of PBMC of the inoculated pigs of the O Taiwan vaccinated group (group 1) and the O Manisa vaccinated group (group 3). The results of the vaccinated control groups were comparable with the results of the vaccinated-challenged groups (data not shown).

Unfortunately, we were unable to establish the responses of the inoculated non-vaccinated control pigs (group 5) because we had to euthanize most of them for welfare reasons shortly after challenge. We sampled the remaining contact infected pigs of this group instead (for numbers see Table 1), but the (few) samples we obtained failed to show a clear response in the lymphocyte proliferation assay or the cytokine ELISAs (Figure 2).

In vitro lymphocyte proliferation response

A lymphocyte proliferation response in re-stimulated PBMC was detected in both the O Taiwan and O Manisa vaccinated groups. The mean proliferation response of the groups increased at 14 dpv and peaked at 21 dpv. A significant difference was found in both groups between day 0 and days 14 ($p<0.01$) and 21 ($p=0.02$) after vaccination, but no significant difference was detected between the groups ($p=0.4$). Peak responses varied between the individual pigs and were detected at 14, 21 or 28 dpv.

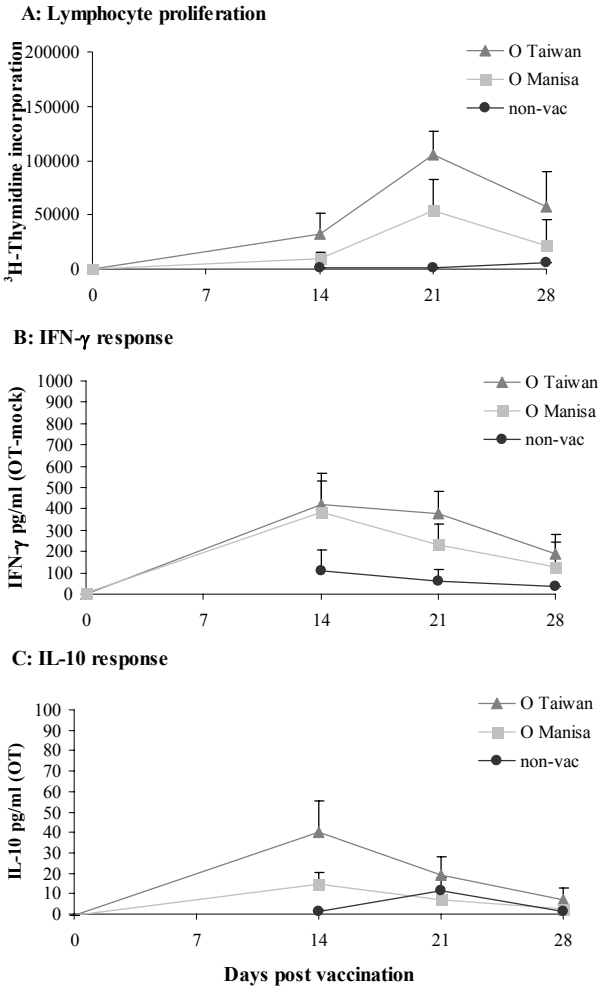

Fig. 2. Lymphocyte proliferation and cytokine responses

2A: Lymphocyte proliferation: ^{3}H-Thymidine incorporation (delta counts), error bars represent SEM;

2B: IFN-γ response: IFN-γ pg/ml (O Taiwan minus mock stimulated), error bars represent SEM;

2C: IL-10 response: IL-10 pg/ml, error bars represent SEM;

O Taiwan = mean response of the inoculated pigs of the O Taiwan vaccinated-challenged group (group 1); O Manisa = mean response of the inoculated pigs of the O Manisa vaccinated-challenged group (group 3); non-vac = mean response of the inoculated or remaining contact infected pigs of the non-vaccinated challenge control group (group 5);

Cytokine concentrations < 0 are represented as 0.

Two pigs in the O Manisa vaccinated group did not show a clear response in the lymphocyte proliferation assay.

Cytokine production of PBMC

We found IFN-γ as well as IL-10 in supernatant of virus-stimulated PBMC in both O Taiwan and O Manisa vaccinated groups. The mean IFN-γ concentration of the groups increased at 14 dpv and decreased in the period thereafter. Both groups showed a significant difference between day 0 and days 14 and 21 ($p<0.01$) after vaccination, but no significant difference was detected between the groups ($p=0.74$).

Also, in both vaccinated groups an increase of IL-10 was observed at 14 dpv with a decrease thereafter. For both groups, a significant difference was found between day 0 and days 14, 21 and 28 ($p<0.01$) after vaccination. No significant difference between the groups was detected ($p=0.25$). Between individual pigs the peaks in the cytokine responses for both IFN-γ and IL-10 varied and were seen at 14 or 21 days after vaccination, and occasionally a low- or even a non-responder was detected.

In the mock-stimulated cultures of PBMC production of IL-10 could be detected. However, this was present at a constant level from day 0 until day 28 and had no time or group effect.

Contact infections

None of the contact pigs in both the O Taiwan and the O Manisa vaccinated group became infected, whereas in the non-vaccinated group all contact pigs became infected.

Discussion

The aim of this study was to compare the efficacy of a heterologous and a homologous vaccine with respect to clinical protection, prevention of virus transmission, sero-response and T-cell responses at 14 days after vaccination. Both vaccines worked so well that inoculated pigs did not became infectious after challenge with FMDV. The induced immunity appeared to be complete, because no clinical signs, no virus excretion and no significant rise in antibody titres were detected in the vaccinated-challenged pigs. No response was detected in the NSP-ELISA either, which might be explained by the low level of infection in the vaccinated animals [16]. Because the inoculated pigs did not become infectious, there were no seeder pigs in these experiments, and consequently the effect of vaccination on virus transmission could not be determined. These findings suggest that in a field situation, emergency vaccination with both a homologous and a heterologous vaccine would be effective in controlling an outbreak of FMDV in a pig population. Similar results

were shown after emergency vaccination with a heterologous O Manisa vaccine in cattle, when clinical protection against (contact) challenge with FMDV O UKG 2001 was observed [17]. However, in contrast to our results, in cattle local virus replication in the oropharynx could be detected after challenge, although it was reduced by the vaccination. The more severe (contact) challenge that was used in the cattle experiment might explain this discrepancy.

The usual way to select an emergency vaccine is to calculate the antigenic relationship of the vaccines with a field isolate using the ratio 'r_1'. From our calculations using the sera obtained in our study, 'r_1' was found to be 0.4, which was lower than the 'r_1' of 0.85 calculated in a previous study by Barnett et al. [18]. In their study 'r_1' was determined using a bovine polyclonal antiserum, whereas we used the mean value of several pig antisera, which could explain the difference in 'r_1' values. Our relatively low 'r_1' value corresponds with the results of Chen et al. [19], who evaluated the immune response in pigs after vaccination with several imported FMDV vaccines during the outbreak in Taiwan in 1997. Based on VN-titres against FMDV O Taiwan of sera of vaccinated pigs, they concluded that the O Manisa vaccines that they studied were not optimal for vaccination against FMDV O Taiwan. Our study shows, however, that the pigs were protected against challenge with O Taiwan after vaccination with O Manisa, despite the relatively low 'r_1' value. This might be explained by the height of the VN-titres against O Manisa obtained in our study, because it is known that heterologous vaccines with a high potency, such as we used, might provide sufficient protection against a heterologous field strain [20].

Although antibodies are considered to be the most important element in the protective immune response against FMDV, cell-mediated responses including production of cytokines are also induced by FMD vaccination. We demonstrated cell-mediated responses after vaccination using the lymphocyte proliferation assay, in which responses are probably mainly attributable to CD4+ T cells [21]. In our study we found lymphocyte proliferation of cultured PBMC after re-stimulation with FMDV O Taiwan in the O Taiwan vaccinated pigs, as well as cross-proliferation in the O Manisa vaccinated group. Cross-reactive lymphocyte proliferation after FMD vaccination has been described before in cattle [22] and pigs [21] and is probably caused by recognition of conserved epitopes within or even between serotypes [21,22]. More studies are needed to determine to what extent these heterologous responses contribute to protection.

Moreover, although cell-mediated immune responses have been extensively investigated during recent years [23] little is known about the functional role and balance of induced cytokines and how they contribute to protective responses. In our study, we observed production of the Th1-cytokine IFN-γ and the Th-2 cytokine IL-10 in cultures of

FMDV O Taiwan stimulated PBMC of the O Taiwan as well as the heterologous O Manisa vaccinated pigs. It has previously been shown that systemic levels of cytokines related to monocytic cell activity increased within days following vaccination in pigs [24] and also a prolonged systemic Th-2 response could be detected after vaccination [25]. However, a systemic Th-1 response has not been described. Since cytokines are generally produced locally and at low levels, they might be difficult to detect systemically, therefore, *in vitro* studies of cultured PBMC can be useful to investigate virus-induced cytokine production. Previous analysis of cytokine mRNA profiles of homologous re-stimulated PBMC identified both a Th-1 and Th-2 profile following repeated FMD vaccination, and it was demonstrated that the Th-1 cytokine proteins IFN-γ and IL-2 and the Th-2 cytokine proteins IL-6 and IL-10 were inducible by FMDV [6]. Our findings correspond with these previous results. However, we demonstrated that the production of a Th-1 and Th-2 cytokine could also be induced by a single vaccination and moreover, that a comparable production was detected after heterologous vaccination, which will often be practised during emergency vaccination. In keeping with the results of Barnard et al. [6], we also detected production of IL-10 in mock-stimulated cultures, but this was at a constant level and did not influence the analysis of the virus-stimulated production.

Unfortunately, because we had to euthanize all inoculated and most of the contact pigs of the challenge-control group shortly after challenge, we were unable to compare the cell-mediated immune responses of the vaccinated-challenged pigs with that of the non-vaccinated challenged pigs. Also, because both vaccinated groups were protected against challenge, we were unable to compare differences in cell-mediated immune responses after protective versus non-protective vaccination. What remains to be investigated, therefore, is whether or not and how the demonstrated lymfoproliferation and induced cytokine production contribute to protection against infection with FMDV.

The O Manisa strain that we used for our heterologous vaccination is a frequently used immunodominant vaccine strain [26], which can be illustrated by the calculated 'r_2' value of 0.2, which is lower than the 'r_1' value. This indicates that vaccination with O Manisa vaccine probably better protects against challenge with O Taiwan than vice versa. Whether heterologous vaccination with a less immunodominant vaccine strain also would result in a similar cell-mediated immune response as homologous vaccination, remains to be examined.

We demonstrated that protective single homologous and heterologous vaccination induced a humoral and a cell-mediated immune response, with the induction of both a Th1-like and a Th2-like activity and that after both homologous and heterologous vaccination no polarism towards a Th1-like or Th2-like response could be found. However, it is still largely unknown how and what cell-mediated immune responses are induced and

maintained after vaccination against FMDV. More studies are needed to improve our knowledge of the induced cytokine production and cytokine profiles, which might help to elucidate the contribution of different T-lymphocyte subsets in protective immunity and might prove worthwhile for determining both the effectiveness of existing vaccines and the development of new vaccines.

Acknowledgements

We gratefully acknowledge the financial support we received from the European Union (FAIR5-PL97-3665) and the Netherlands Ministry of Agriculture, Nature and Food Quality.

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Chapter 4

Serological and mucosal immune responses after vaccination and infection with FMDV in pigs

P.L. Eblé[a], A. Bouma[b], K. Weerdmeester[c], J.A. Stegeman[b], A. Dekker[a]

[a]Central Institute for Animal Disease Control (CIDC-Lelystad), P.O. Box 2004, 8203 AA Lelystad, The Netherlands
[b]Department of Farm Animal Health, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 7, 3584 CL Utrecht, The Netherlands
[c]Animal Sciences Group Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands

Vaccine (accepted for publication)

Abstract

The aim of this study was to determine a possible correlation between humoral immune responses shortly after vaccination and protection against foot-and-mouth disease virus (FMDV) infection and to study the serological and mucosal antibody responses after vaccination and infection.

We used three groups of ten pigs, one non-vaccinated group, one group vaccinated with a single dose vaccine and one group vaccinated with a 4-fold dose vaccine. At 7 days post vaccination, five pigs per group were challenged intra-dermally with FMDV O TAW 3/97 and the remaining pigs of each group were contact-exposed to the inoculated pigs. In each group, virus excretion and number of contact infections were quantified. The serological and mucosal antibody responses were evaluated until 116 days post infection.

Vaccination resulted in a significant decrease of virus excretion. Stepwise linear regression analysis of variables from individual vaccinated pigs revealed the virus excretion after challenge to be correlated with neutralising antibody titres at the day of challenge ($p< 0.01$). In serum and OPF samples comparable isotype specific antibody responses (IgM, IgG and IgA), could be detected after vaccination as well as after infection. Remarkably, the pigs with the highest IgA responses after vaccination were protected against contact exposure. After infection, a long lasting (up to 116 dpi) IgA response was seen in the non-vaccinated and to a lesser extent in de single dose vaccinated pigs. The induction of NSP antibodies in the vaccinated pigs after infection were lower and of shorter duration as compared to the non-vaccinated infected pigs.

This experiment shows that vaccination can reduce virus excretion in pigs, which will contribute to reduced transmission of FMDV in the field, even if the pigs are not fully protected. Moreover, vaccines that induce local IgA responses may be more effective, which merits further investigation.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that can cause huge economic losses. Since 1991, the European Union has had a non-prophylactic vaccination strategy for FMD and the control policy for outbreaks in the European countries used to be primarily based on ‘stamping out’ in combination with movement restrictions and hygienic measures. Preventive vaccination during an outbreak was usually not applied because of the adverse economic consequences of vaccination as compared to slaughter of infected animals. The large number of animals slaughtered during the outbreaks in the UK and The Netherlands in 2001 led to an intense debate about this

policy. As a consequence, the OIE and the EU [1,2] have adopted new regulations, which favour a vaccinate-to-live policy during an outbreak of FMD, provided that for regaining of the FMDV-free status a serological surveillance is undertaken to support the absence of circulating virus [1].

However, if emergency vaccination during an outbreak of FMD is going to be applied, there is an urgent need to understand how effective vaccination is as intervention tool. Since the interval between vaccination and new virus introduction might be short during an epidemic, we wanted to study the correlation of immunological parameters shortly after vaccination and protection against infection with FMDV. In cattle, it is well established that protection against challenge at 3 weeks after vaccination is correlated with induced VN-antibody response caused by vaccination [3]. For pigs [4] and shorter vaccination-challenge intervals however, this correlation is less clear. It has been shown that pigs can be protected against clinical disease after vaccination against FMD, as soon as 4 days post vaccination [5,6]. Recently, we demonstrated that after vaccination with a single dose vaccine at 7 days post vaccination, some contact pigs were protected against clinical disease although all contact pigs became infected [7]. In the presented study, we examined the correlation of vaccine-induced humoral immune responses and protection against challenge with FMDV. For this purpose, we repeated the experiment in which we challenged at 7 days post vaccination and we added a group that we vaccinated with a higher vaccine dose with the aim to induce more (partly) protected pigs. Because mucosal immunity is considered as a crucial factor in protection against FMDV infection, we also studied the mucosal antibody responses.

Vaccine-induced immunity might also influence the induction and duration of antibodies against non-structural proteins (NSP) of FMDV, which are used for post-outbreak surveillance. Therefore we monitored the induced NSP responses after infection until 116 days after infection (dpi) and also evaluated whether the isotype specific immune responses after challenge in serum and OPF might give additional information on the differentiation of infected and vaccinated pigs.

Materials and methods

Animals and experimental design

We used 34 conventional 6-week-old piglets that were randomly allocated to five groups. Three groups (groups 1, 2 and 3) consisted of ten pigs each; two groups (groups 4 and 5) of 2 pigs each were used as vaccine-control groups. The pigs were housed in stables of our high-containment unit, groups 1-3 in separate stables and groups 4 and 5 housed together. The pigs in group 1 (challenge control group) remained non-vaccinated. Pigs in groups 2 and 4 were vaccinated intra-muscularly at one week before inoculation (-7 dpi) with a

single dose double-oil-in-water emulsion vaccine that contained 3μg of O Taiwan (O TAW 3/97) 146S antigen per 2 ml dose. Pigs in groups 3 and 5 were vaccinated at – 7 dpi four times with a 2ml dose and thus received a 4-fold dose (4FD) vaccine. To exclude differences in responses due to difference in vaccine composition, we used one vaccine batch (instead of two batches with a different antigen-payload) and increased the vaccine dose.

At 7 days post vaccination (0 dpi), five randomly selected pigs in groups 1, 2 and 3 were challenged by intra-dermal inoculation in the bulb of the heel of the left hind-foot with 0.1 ml of challenge virus containing $10^{5.4}$ $TCID_{50}$/ml FMDV O Taiwan (O TAW 3/97). The contact pigs were removed before challenge and were added to their groups again 24h later. Groups 4 and 5 were not challenged.

To reduce pain caused by generalized FMDV buprenorfine was administered, and pigs that suffered severely were euthanized. During the experiment in groups 2 and 3 some pigs were removed from the experiment and euthanised for other research purposes at 28 dpi (1 pigs) and 54 dpi (2 pigs). Approval of the Ethics Committee for Animal Experiments of CIDC-Lelystad was obtained before the start of the experiment.

Data collection and sample preparation

Clinical signs (fever, vesicles, lameness) of the pigs were recorded daily. Oropharyngeal fluid (OPF) was collected daily after challenge from 0-14 dpi in groups 1, 2 and 3 using cotton mouth swabs. From 14-116 dpi swabs were taken twice a week. Heparinized blood samples were collected daily after challenge from 0-14 dpi in groups 1, 2 and 3. Serum samples were collected daily after challenge from 0-14 dpi and twice a week from 14-116 dpi in all groups. Reduction of transmission was tested by inclusion of contact pigs in the experiment. These contact pigs were considered infected if they showed clinical signs of FMD, if virus or viral genome was detected in OPF or plasma samples, if a boost (rise ≥ 0.6 as compared to the titre at 0 dpi) in VN-titre or if a response in the NSP-ELISA was detected.

In the laboratory, the swabs were incubated in the laboratory for 30 minutes in 4 ml EMEM and then centrifuged and weighed. Half of each sample was stored at –20°C until ELISAs were performed. To the other part of each sample 5% FBS and 10% antibiotics was added and stored at -70°C for virus isolation and RT-PCR. Plasma samples for virus isolation and RT-PCR were obtained by centrifugation of the heparinized blood samples; the plasma was stored at –70°C. Serum samples were centrifuged and serum was stored at –20°C.

Laboratory tests

Virus isolation assay

Challenge virus, OPF samples and plasma samples were assayed for the presence of virus by plaque titration on monolayers of secondary pig-kidney cells [8]. Ten-fold dilution series of the samples (200 µl, tested in duplicate) were allowed to adsorb for 1h on monolayers of PK-2 cells in a six-well tissue-culture plate (Greiner Bio-One), and subsequently maintenance medium containing 1% methylcellulose was added. After 2 days of incubation at 37°C in a humidified atmosphere containing 5% CO_2, the plates were washed in tap water with citric acid; monolayers were then rinsed with tap water and stained with amido-black (0.1% amido-black in 1M acetic acid, 0.09M sodium acetate, and 10% glycerol). Plaques were counted macroscopically. Virus titres expressed as ^{10}log plaque forming units (pfu) per ml and were corrected for the dilution by the medium that was added before storage.

RT-PCR

RNA from the plasma and OPF samples was isolated using the MagNA Pure® system (Roche) and the MagNA Pure® LC kits for total nucleic acid isolation. The isolated nucleic acid was tested in a LightCycler based RT-PCR [9], using primers in the conserved 3D region of the FMDV genome.

Virus Neutralisation Test (VNT)

Neutralising antibody titres (VN-titres) against FMDV O Taiwan in serum samples were measured using the neutralisation assay as described previously [8]. End-point titres were calculated as the reciprocal of the final serum dilution that neutralised 100 $TCID_{50}$ of FMDV in 50% of the wells.

Isotype specific ELISAs

IgM ELISA

ELISA plates (Corning Costar EIA/RIA, 9018) were coated overnight at 37°C with a predetermined dilution in PBS of a monoclonal antibody specific for porcine IgM (MAb 28.4.1) [10]. After washing, we blocked the plates with PBS containing 10%FCS and stored them at -20°C until use. After washing, the coated ELISA plates were incubated for 1h at 37°C with 3-fold serial dilution steps of serum samples, starting with a 1/25 dilution. OPF samples were tested in 2-fold serial dilution steps starting with a 1/2 dilution. After washing, a predetermined dilution of purified FMDV O Taiwan antigen was added and incubated for 1h at 37°C. After washing the plate was incubated with a predetermined dilution of horseradish peroxidase conjugated MAb directed against FMDV type O (MAb

99) [11]. As a final step, the plate was washed and subsequently incubated with a chromogen/substrate solution (0.1 mg/ml tetramethylbenzidin, 0.0006% H_2O_2 (TMB)) for 10 min at room temperature. Colour development was stopped with 0.5M H_2SO_4. The absorbance at 450nm was read with an ELISA reader. Washing was performed with tap water containing 0.05% Tween80. Serum samples, antigen and conjugate were diluted in ELISA buffer (PBS containing 0.5M NaCl, 0.05% Tween 80 and 5% FCS). Antibody titres were expressed as the reciprocal of the sample dilution still giving optical density (OD) values above a predetermined cut-off value. In each test plate a blanco, a negative control sample and a titration of a positive control sample was included. For all ELISAs (except IgA), cut-off values were defined as the mean OD plus 3xSD of pre-serum samples of two previously performed animal experiments (54 pigs).

IgG ELISAs

ELISA plates (Corning Costar EIA/RIA, cat.no. 9018) were coated overnight at 37°C with O Taiwan antigen diluted in 0.5M bicarbonate with pH 9.6. After washing, plates were blocked with PBS containing 10%FCS and stored at -20°C until use. After washing, coated ELISA plates were incubated for 1h at 37°C with 2-fold serial dilution steps of the serum samples starting at a 1/200 dilution. OPF samples were tested in 2-fold serial dilution steps starting at a 1/2 dilution. After washing, a predetermined dilution of horseradish peroxidase conjugated MAb directed against porcine IgG (MAb 23.3.1a) [10] porcine IgG1 (MAb 23.49.1) [10] or porcine IgG2 (MAb 34.1.1a) [10] was added and incubated for 1h at 37°C. Incubation with chromogen/substrate etc. was done as described for the IgM ELISA.

IgA ELISA

The IgA ELISA was performed essentially as described for the IgM ELISA, except that for coating a goat anti-porcine IgA (Serotec, AAI40) polyclonal antiserum was used. Serum samples were tested in a single 1/5 dilution and OPF samples were tested in a single 1/2 dilution. OD values of tested samples were expressed as the percentage of the OD of the positive control sample. Cut-off values were defined as the mean percentage of pre-serum samples of two previously performed animal experiments plus 3xSD.

NSP-ELISA

Antibodies against non-structural proteins (NSP) of FMDV in serum samples were determined using a commercially available ELISA (Ceditest® FMDV-NS) used according to the instructions of the manufacturer.

Statistical Methods

Virus excretion of each individual pig was quantified by calculating the area under the curve (AUC) of the virus isolation data of the OPF samples. For pigs that were euthanized before the end of the experiment, the missing values were replaced by the average value of the remaining pigs of the same (sub)group. Differences in virus excretion between the inoculated groups were analysed using the data of the inoculated pigs only.

Differences in serological responses (VN-titre, IgA, IgM, IgG, IgG1 and IgG2) at day of challenge (7 dpv, 0 dpi) between the three vaccinated groups were also analysed. For this analysis the data of all vaccinated pigs (including vaccine-controls) were used.

Differences in virus excretion, VN-titres, IgA, IgM, IgG, IgG1 and IgG2 responses between the three groups were statistically analysed using the non-parametric Kruskal Wallis test (StatXact®-5) [12]. A non-parametric permutation test (StatXact®-5) was used for pair-wise comparison between groups if the Kruskal-Wallis test gave a significant result.

To estimate the best predictor for (reduction of) virus excretion, stepwise linear regression analysis (S-PLUS® 6.2 for Windows) was used. AUC was entered as response variable whereas VN-titre, IgA, IgM, IgG, IgG1 and IgG2 responses in serum at 0 dpi were evaluated as explanatory variables. In this analysis only the data of the inoculated pigs were included.

Results

Clinical signs

In the non-vaccinated group and the single dose vaccinated group all pigs, both inoculated and contact pigs, showed signs of generalized FMD. In the 4FD vaccinated group, one of the inoculated pigs showed signs of generalized FMD and four inoculated pigs showed FMD lesions only at the inoculated foot. Two of the five contact pigs in this group showed signs of FMD (Table 1).

Virus isolation and RT-PCR

In all inoculated pigs, virus was detected in OPF samples. Virus shedding started from 1-3 dpi. Also all contact pigs in the non-vaccinated and the single dose vaccinated group virus shed virus in the OPF. In the contact pigs of the 4FD vaccinated group in three of the five contact pigs virus was detected in OPF. The RT-PCR results were comparable with the results of virus isolation (Table 1). The combined results of the virus detection in the plasma samples are also shown in Table 1. All samples taken after 14 dpi were negative in by both virus isolation and RT-PCR. The virus excretion of the inoculated pigs differed

Table 1. VI of OPF, RT-PCR, clinical signs, VI/PCR plasma samples, NSP-ELISA and VNT results

	Days post infection											Clinical	VI/PCR	NSP-	rise in
	0	1	2	3	4	5	6	7	8	9	10-14	signs	plasma[e]	ELISA[f]	VN-titre[g]
Non-vaccinated															
Inoculated	-[a]	-	4.1[b]	4.6	3.3	2.7	2.1	-	-	-	-	yes	+	+	+
Inoculated	-	3.0	4.4	3.0	2.8	†[c]						yes	+	-(†)[i]	+
Inoculated	-	3.2	4.5	4.1	†							yes	+	-(†)	+
Inoculated	-	-	2.6	5.1	†							yes	+	-(†)	-(†)
Inoculated	-	-	2.7	4.5	3.5	†						yes	+	-(†)	-(†)
Contact	-	-	3.5	5.2	5.5	3.2	2.4	-	-	-	-	yes	+	+	+
Contact	-	-	4.6	4.7	†							yes	+	-(†)	-(†)
Contact	-	-	4.4	5.7	4.2	1.7	3.0	2.6	†			yes	+	-(†)	+
Contact	-	-	3.0	6.2	3.9	3.4	1.7	-	-	2.5	†	yes	+	+	+
Contact	-	-	3.2	6.3	3.7	2.1	2.8	-	†			yes	+	-(†)	+
Vaccinated -7 dpi															
Inoculated	-	-	1.6	1.8	3.7	4.3	-	-	†			yes	+	+	+
Inoculated	-	-	1.8	-	-	2.5	1.6	n.t.[h]	n.t.	n.t.	n.t.	no (n.t.)	-(n.t.)	+	+
Inoculated	-	-	2.6	2.1	4.5	4.2	2.3	-	-	-	-	yes	+	+	+
Inoculated	-	-	-	2.1	1.8	2.5	-	-	-	-	-	yes	-	+	+
Inoculated	-	-	-	3.3	†							yes	+	-(†)	-(†)
Contact	-	-	-	2.9	2.6	3.0	-	-	†			yes	+	-(†)	+
Contact	-	-	-	2.2	5.3	4.5	1.6	1.5	1.6	-	-	yes	+	+	+
Contact	-	-	-	-	-	-	2.8	-	-	-	-	yes	-	+	+
Contact	-	-	2.3	3.3	4.3	4.0	3.0	2.9	-	-	-	yes	+	+	+
Contact	-	-	2.5	2.6	5.6	†						yes	+	-(†)	-(†)
Vaccinated -7 dpi 4FD															
Inoculated	-	3.7	2.8	-	2.8	2.3	-	-	-	-	-	yes[d]	-	+	+
Inoculated	-	-	-	1.2	2.2	-	-	-	-	-	-	yes[d]	-	+	+
Inoculated	-	-	1.2	2.3	†							yes	+	-(†)	-(†)
Inoculated	-	3.8	-	-	-	-	-	-	-	-	-	yes[d]	-	+	+
Inoculated	-	-	1.8	2.9	-	-	-	-	†			yes[d]	-	-(†)	+
Contact	-	-	-	3.0	3.4	3.3	-	-	-	-	-	yes	+	+	+
Contact	-	-	-	-	-	-	-	-	-	-	-	no	-	-	-
Contact	-	-	-	-	-	-	-	-	-	-	-	no	-	-	-
Contact	-	-	-	-	-	2.4	-	-	2.1	-	-	no	-	-	-
Contact	-	-	-	-	-	1.9	2.3	-	†			yes	-	-(†)	+

[a] log titre VI; - : no virus was detected; [b] in grey RT-PCR positive samples are indicated; [c] euthanasia piglet; [d] only inoculated foot; [e] results of VI and RT-PCR combined; + positive, - negative; [f] results of NSP-ELISA; + positive, - negative; [g] VN-titre at least four times higher than the VN-titre at 0 dpi; [h] not tested; [i] negative test result probably due to early euthanasia of the pig

significantly between the non-vaccinated and the single dose vaccinated group ($p< 0.01$), between the non-vaccinated group and the 4FD vaccinated group ($p<0.01$) and between both vaccinated groups ($p<0.05$). The mean virus excretion in OPF per day for the inoculated and contact pigs of each group is shown in Figure 1.

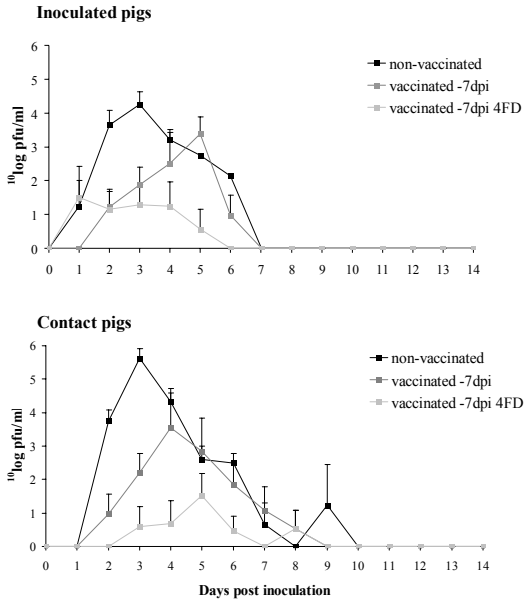

Fig. 1. Virus isolation of OPF: mean virus excretion per day for inoculated and contact pigs of each group. Error bars represent standard error of mean (SEM).

Contact infections

In the non-vaccinated and single dose vaccinated groups, all contact pigs became infected. In the 4FD vaccinated group 3 of the 5 contact pigs became infected of which one was infected subclinically.

The results of the VI of OPF were adequate to determine contact infected pigs since other test results did not detect more or other contact infected pigs.

Virus Neutralisation Test

All vaccinated pigs developed neutralising antibodies against FMDV O Taiwan (Figure 2). Seven days post vaccination (0 dpi) the VN-titres of both vaccinated groups

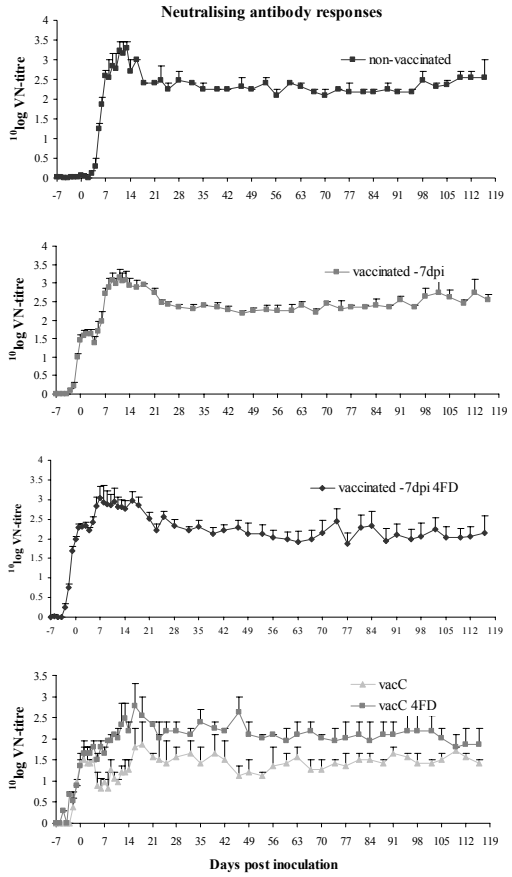

Fig. 2. VN-titres. Error bars represent SEM.

were significantly higher as compared to the non-vaccinated group ($p<0.01$) and also the VN-titres of the pigs of the 4FD vaccinated group were significantly higher as compared to the single dose vaccinated group ($p<0.01$).

After challenge, a rise in VN-titre was detected in all non-vaccinated, all single dose vaccinated and all 4FD vaccinated inoculated pigs that lived beyond 8 dpi. Of the 4FD vaccinated contact pigs, only the pigs that showed clinical signs of FMD showed a rise in VN-titre after challenge (Table 1). VN-titres of all pigs remained high until the end of the experiment at 116 dpi (Figure 2).

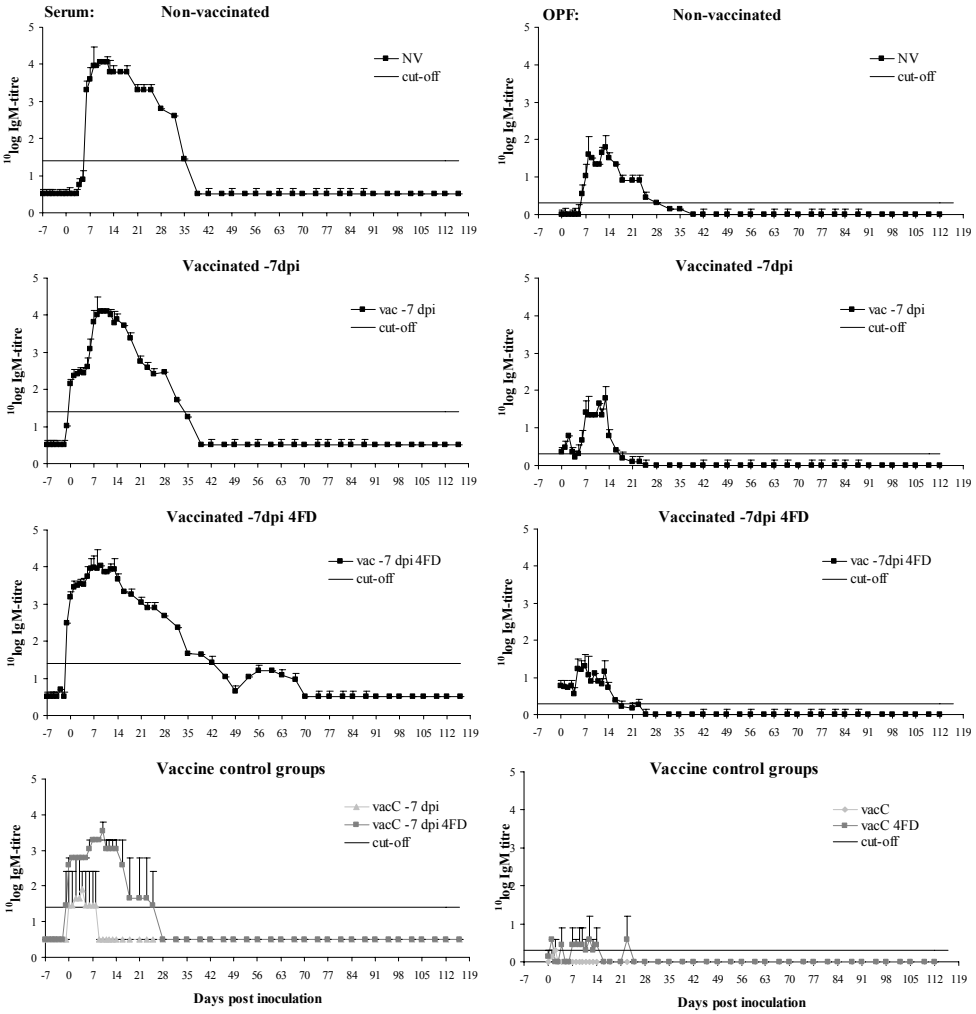

Fig. 3A. IgM responses in serum samples (graphs on the left) and OPF (graphs on the right). Error bars represent SEM.

Isotype specific immune responses

IgM responses

Vaccination resulted in a rapid IgM response. The response in the 4FD vaccinated pigs was higher and lasted longer than the response of the pigs of the single dose vaccinated group.

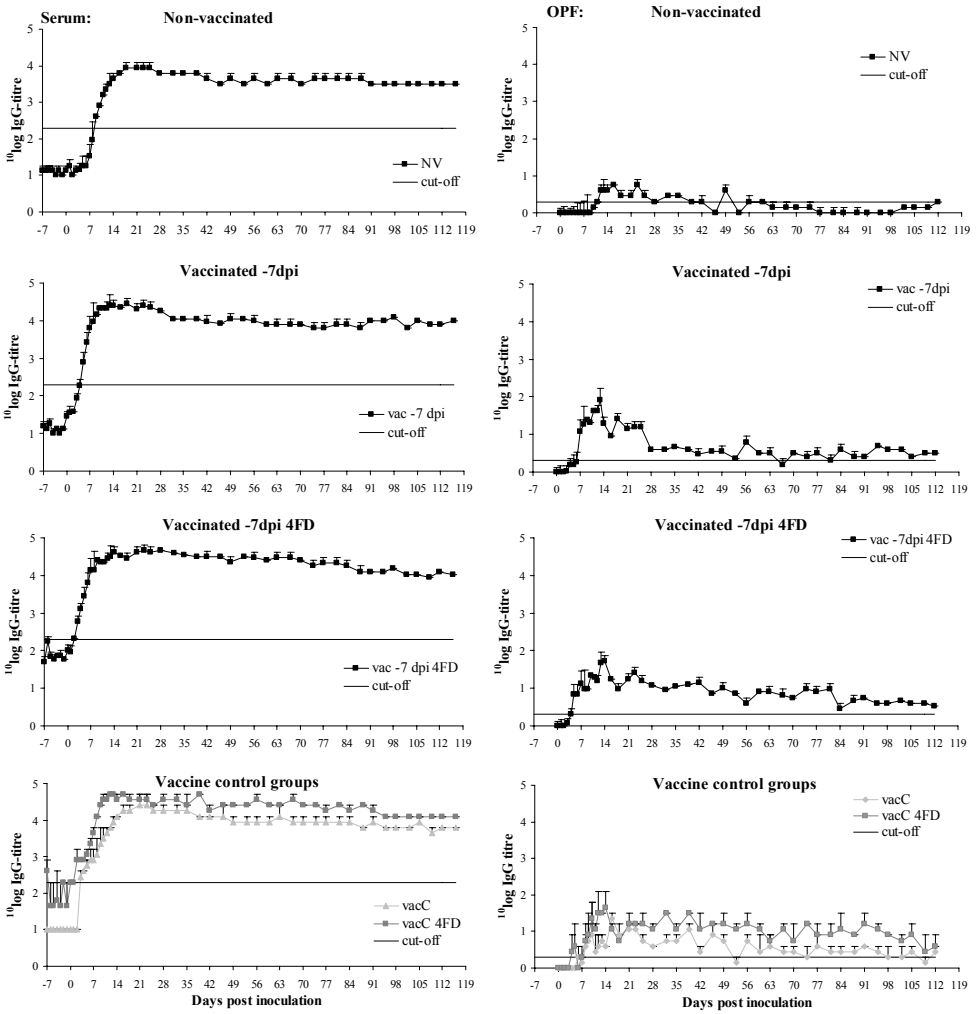

Fig. 3B. IgG responses in serum samples (graphs on the left) and OPF (graphs on the right). Error bars represent SEM.

Seven days post vaccination (0 dpi), significant differences between the non-vaccinated group and both vaccinated groups (serum samples and OPF) and between the two vaccinated groups (serum samples) were detected ($p<0.01$) After inoculation, also a rapid IgM response was seen, detectable from about 7 until 28-35 dpi. The responses of the inoculated pigs were higher than those of the vaccinated pigs. In OPF a similar pattern as in the serum samples was observed (Figure 3A).

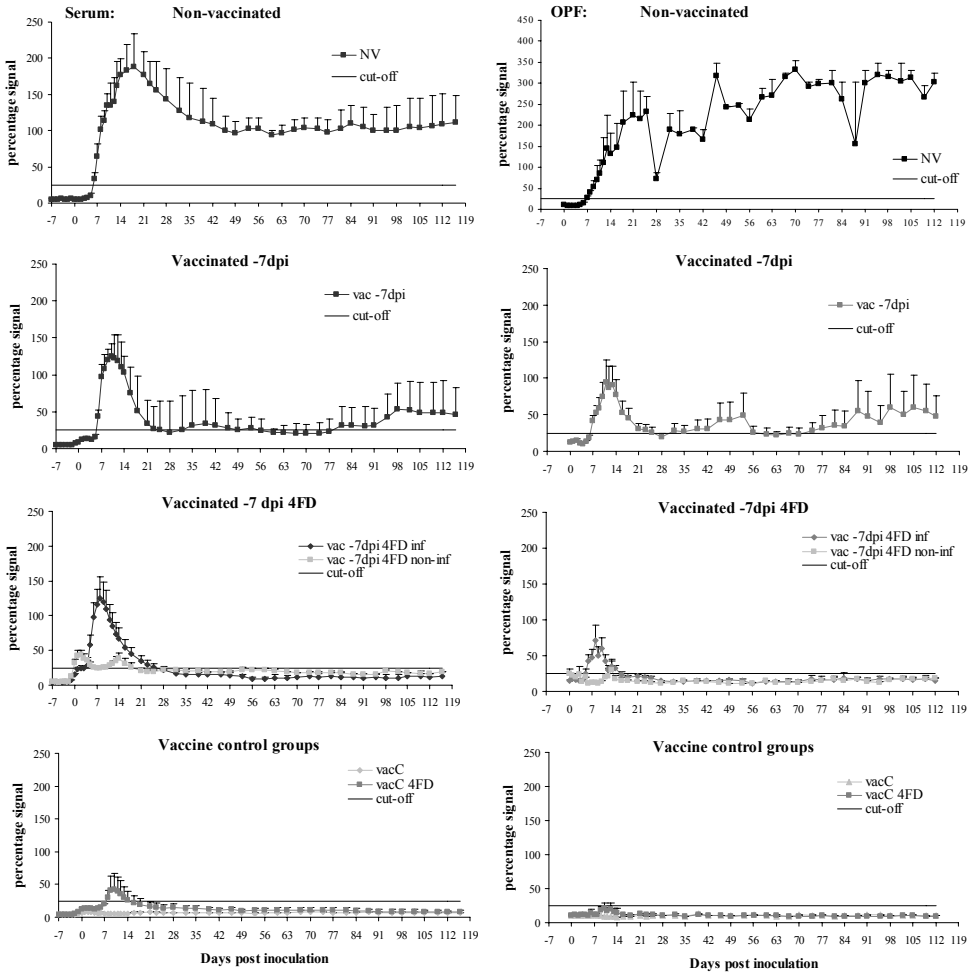

Fig. 3C. IgA responses in serum samples (graphs on the left) and OPF (graphs on the right). Note that the y-axis of the graph of the responses in OPF of the non-vaccinated pigs has a different scale; Error bars represent SEM.

IgG responses

In serum samples, IgG responses rose from 7-10 days post vaccination/inoculation and reached a plateau approximately one week later. Seven days post vaccination (0 dpi) a significant difference was detected between the non-vaccinated group and the 4FD vaccinated group ($p < 0.01$) and between the single dose vaccinated group and the 4FD vaccinated group ($p=0.02$). The IgG responses lasted until the end of the experiment at 116

dpi. In OPF samples a similar pattern was observed, although the responses were lower and in the non-vaccinated challenged pigs very low at the end of the experiment (Figure 3B). IgG1 and IgG2 responses in serum and OPF resembled each other, the only difference being that the IgG2 responses were a little higher than the IgG1 responses. Both IgG1 and IgG2 responses corresponded with the IgG responses (results not shown).

IgA responses

Vaccination resulted in (low) IgA responses, which were highest in the 4FD vaccinated group. At time of challenge (0 dpi), significant differences between the non-vaccinated group and both vaccinated groups and between the two vaccinated groups were detected with regard to IgA response in serum samples ($p<0.01$). In OPF samples, a significant difference was detected between the non-vaccinated group and the 4FD vaccinated group ($p< 0.01$) and between both vaccinated groups ($p=0.02$).

After inoculation, IgA responses rose rapidly in those pigs that became infected but not in the pigs that were protected against challenge. IgA responses of the non-vaccinated and the single dose vaccinated groups were detectable until the end of the experiment and were highest in the non-vaccinated infected pigs. The patterns of the IgA responses in OPF samples were similar as in serum samples but the IgA response in OPF of the non-vaccinated group was very high as compared to the serum response (Figure 3C).

NSP-ELISA

All pigs that became infected, showed clinical signs of FMDV and lived beyond 8 dpi tested positive in the NSP-ELISA. One subclinically infected pig of the 4FD vaccinated group did not respond in the NSP-ELISA. In the vaccinated groups, a tendency towards a lower percentage inhibition and shorter duration of positive test results after infection was observed (Table1, Figure 4).

Correlation of protection

A significant correlation between (reduction of) virus shedding (represented by AUC) and VN-titre at seven days post vaccination (0 dpi) ($p<0.01$) was found. The other explanatory variables (IgA, IgM, IgG, IgG1 and IgG2 responses) did not contribute significantly to the model.

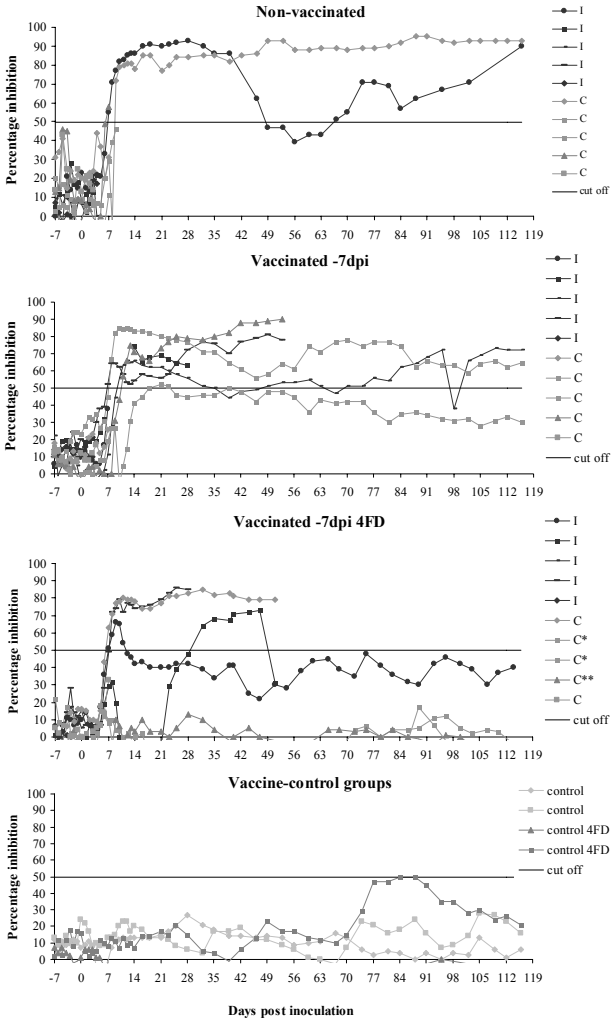

Fig. 4. NSP-responses. I = inoculated pig; C = contact pig; * = not infected pig; **=subclinically infected pig

Discussion

In this study we showed that vaccination of pigs against FMDV with a single dose vaccine one week before challenge significantly reduced virus shedding after challenge, although clinical signs of FMD and virus transmission to contact pigs were not reduced. In contrast, other researchers have reported clinical protection against challenge and reduced virus

transmission in pigs as soon as 4 days post vaccination [5,6]. The difference between their and our studies can probably be attributed to the more severe challenge regime that was used in our experiment. Although after single vaccination clinical signs and number of contact infections were not reduced in our experiment, virus shedding was significantly reduced as compared to the non-vaccinated pigs. Similarly, in cattle [13] and sheep [14] it has been demonstrated that the level of virus replication was reduced in vaccinated as compared to non-vaccinated animals. Vaccination with a 4FD vaccine resulted in better clinical protection of the inoculated pigs and also less contact pigs became infected. Moreover, virus excretion after challenge was reduced even more, not only as compared to the non-vaccinated but also as compared to the single dose vaccinated pigs. The reduction of virus shedding indicates that emergency vaccination of pigs will significantly reduce the amount of virus released into the environment shortly after vaccination and therefore, in a field situation, can contribute to reduction of transmission to other herds.

Vaccination with a 4FD vaccine resulted in significantly higher VN-titres at one week post vaccination as compared to vaccination with a single dose vaccine and moreover, reduction of virus shedding was correlated with induced VN-titres. Similar findings have been reported for other species. Cattle that received a vaccine with 10-fold higher antigen payload developed VN-titres more quickly and significantly higher as compared to cattle that received a single dose vaccine, and fewer animals became persistently infected after challenge [15]. Also for sheep a correlation between the antigen payload of the vaccine, VN-titre after vaccination and inhibition of local virus replication has been demonstrated [16]. Our results show that also for pigs the efficacy of vaccination in an emergency situation shortly after vaccination improves when a higher than standard vaccine dose is used.

We detected isotype specific IgM and IgG responses in serum samples after vaccination of the pigs. The start and duration at which we detected those responses are in correspondence with earlier findings of Cox et al. [17] and Ouldridge et al. [18]. However, inhalation of airborne FMDV, leading to replication in the respiratory tract is considered to be the most common route by which livestock become infected and thus ideally an emergency vaccine should protect the respiratory tract against infection. Therefore, we also examined the effect of vaccination on local (mucosal) immune responses. In our study the isotype specific responses in OPF of the pigs were comparable to those in serum samples. This corresponds with earlier findings of Francis et al., who describe that the neutralising antibody profiles in serum and nasal mucosa samples in pigs were comparable after vaccination and infection, in contrast to results obtained in cattle [19]. Since it is generally considered that good mucosal immunity will contribute to protection against infection with

FMDV, the induction of mucosal immunity by parenteral immunisation needs further research.

We also showed that vaccination against FMDV with a DOE vaccine was able to induce systemic and mucosal IgA responses. To our knowledge, this is the first time that IgA responses after FMDV vaccination are reported. In the 4FD vaccinated group, IgA responses were significantly higher as compared to the single dose vaccinated group. Remarkably, the two pigs that were completely protected against contact challenge were the pigs with highest induced IgA response after vaccination. The induction of IgA responses after vaccination and its possible role in protection against challenge with FMDV merits further investigation and might be used both for vaccine choice and adjuvant research for FMDV vaccines. Whether in our study the improved IgA response after the 4FD vaccination was induced by the higher amount of antigen or the higher dose of adjuvant that the 4FD vaccinated pigs received needs further research.

After challenge, we observed high IgA responses in both serum samples and OPF. In the 4FD vaccinated pigs a clear IgA peak response was seen after infection, but this IgA response decreased and became negative after approximately one month. Surprisingly, in the non-vaccinated and single dose vaccinated pigs after infection the IgA response was prolonged and still present at 4 months after challenge. For cattle it has been documented well that mucosal [20,21,22] and serological [22] IgA responses can be detected after infection with FMDV. However, in cattle, prolonged IgA responses are considered as determinant for carrier animals. It is generally considered that pigs do not become persistently infected with FMDV but our observation might support the finding of Mezencio et al. [23] who demonstrated that in pigs viral RNA could be demonstrated until more than 200 days post infection.

We studied the responses against NSP of FMDV because vaccine-induced immunity might also influence the induction and duration of responses against NSP of FMDV. In our experiment, all except one subclinically infected pig, scored positive in the NSP-ELISA. However, a tendency for a less high (lower percentage inhibition) and shorter duration of responses against NSP could be seen in the vaccinated pigs and was apparent especially in the 4FD vaccinated pigs. This finding corresponds with studies in cattle for which has been demonstrated that high immune cattle can have low responses against NSP of FMDV [24]. This aspect should be taken into consideration when a post-outbreak screening in which NSP-ELISAs are used is undertaken.

In conclusion, we showed that vaccination of pigs could reduce virus excretion significantly and that reduction of virus shedding is correlated with vaccine induced VN-titres. Moreover, IgA response after vaccination plays a possible role in protection against infection. The existence of prolonged IgA responses in pigs after infection needs further

investigation and evoked NSP-responses are influenced by the immune status of pigs at the day of infection.

Acknowledgements

The authors wish to thank the laboratory assistants and staff of the animal isolation unit that participated in the described research for their assistance. This work was supported financially by the European Union (FAIR5-PL97-3665) and the Netherlands Ministry of Agriculture, Nature and Food Quality.

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Chapter 5

Quantification of within- and between-pen transmission of Foot-and-Mouth disease virus in pigs

Phaedra Eblé[a], Aline de Koeijer[b], Annemarie Bouma[c], Arjan Stegeman[c], Aldo Dekker[a]

[a] Central Institute for Animal Disease Control (CIDC-Lelystad), P.O. Box 2004, 8203 AA Lelystad, The Netherlands
[b] Division of Infectious Diseases, Animal Sciences Group Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands
[c] Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, The Netherlands

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Abstract

Quantified transmission parameters of Foot-and-Mouth Disease Virus (FMDV) are needed for epidemic models used for control and surveillance. In this study we quantified the within- and between-pen transmission of Foot-and-Mouth Disease virus (FMDV) in groups of pigs by estimating the daily transmission rate β, i.e. the number of secondary infections caused by one infectious pig during one day, using an SIR (susceptible-infectious-removed) model. Within-pen transmission was studied in four groups of ten pigs in which 5 infected and 5 susceptible pigs had direct contact; between-pen transmission was studied in one group of ten pigs in which 5 infected and 5 susceptible pigs had indirect contact. Daily results of virus isolation of oropharyngeal fluid were used to quantify the transmission rate β, using Generalised Linear Modelling (GLM) and a maximum likelihood method. In addition, we estimated the expected time to infection of the first pig within a pen T_w and in the indirect-contact pen T_b. The between-pen transmission rate β_b was estimated to be 0.59 per day (0.083 – 4.18), which was significantly lower than the within-pen transmission rate β_w of 6.14 (3.75 – 10.06). T_w was 1.6h, and T_b was 16h. Our results show that the transmission rate is influenced by contact structure between pigs.

Introduction

Foot-and-Mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals, and outbreaks in unvaccinated populations can have a devastating socio-economic effect, as was seen during the outbreaks in the UK and The Netherlands in 2001. Foot-and-Mouth disease virus (FMDV) can be spread by various ways, of which direct contact with infected animals is considered the most important, but also transmission of FMD by slurry, transport vehicles and air-borne transmission are described [1]. For control of the disease, it is important that transmission of the virus is quantified. Quantitative information on transmission parameters can be used for the development of surveillance or control programmes, which e.g. can be used to determine the number of expected outbreaks in the high risk period of an epidemic or to evaluate which measures can reduce transmission to such a level that the virus will be eradicated. Mathematical models have for example already been used extensively during the 2001 FMD epidemic in the UK [8, 9, 10].

An often used transmission parameter is the reproduction ratio R, which can be used to predict if an epidemic will spread or fade out [3, 5]. For the transmission of FMDV R has been quantified previously in groups of random-mixing calves [15] and pigs [7]. A limitation of R, however, is that it does not include a time factor, which is important in epidemic modelling when used to analyse the course of the epidemic. Moreover, pigs in

commercial pig herds are housed in pens in compartments. When using estimated within-pen parameters, the rate of transmission within a herd might be overestimated. Quantification of transmission between pens should, therefore, also be determined.

A suitable parameter to use in modelling that does have a time dimension is the transmission rate β, which is defined by the number of secondary infections caused by one infectious individual per unit of time. Although R and β are related, when the estimate of R is infinite, as is often the case in a non-vaccinated population, β cannot be derived from R, and therefore, another method to quantify β is needed. In this study, we quantified the within-pen transmission of FMDV in groups of non-vaccinated pigs and between-pen transmission in a group of pigs in which the infectious and susceptible pigs only had indirect contact. The within-pen transmission rate β_w was quantified using a Generalized Linear Model and the transmission rate between pens β_b was quantified with a maximum likelihood method. We furthermore calculated the expected time to the first transmission in the different groups to visualize the effect of the different transmission rates.

Materials and methods

Animals and experimental design

Conventionally reared, six-week-old pigs were used in the experiments. All experiments were performed with groups of ten pigs, which were housed in animal rooms in the bio-security facilities at CIDC-Lelystad. Following an acclimatisation period of 5 days, at day 0 (0 dpi) five randomly selected pigs were inoculated intradermally in the bulb of the heel of the left hind-foot with 0.1 mL challenge virus. The remaining 5 pigs of each group were contact exposed to the inoculated pigs. At the time of challenge, inoculated and contact pigs were separated and the groups were reunited 24 h later. In the four groups in which the within-pen transmission was observed, the contact pigs were housed in the same pen as the inoculated pigs and thus had direct contact with the inoculated pigs. In the group in which the between-pen transmission was observed, the contact pigs were housed in the same animal room but were separated from the inoculated pigs by a wall of 1.50 m high, so that the only contact of the inoculated and contact pigs was indirect. The airflow in this experiment was directed from contact to inoculated pigs. Before the start of the experiment approval of the Ethics Committee for Animal Experiments of CIDC-Lelystad was obtained. Buprenorfine was administered to reduce pain and pigs that suffered severely were euthanized.

Virus

Challenge virus contained 10^5 $TCID_{50}$/mL FMDV O Taiwan (O TAW 3/97). The used inoculum was a second pig passage of material that was originally derived from the World Reference Laboratory in Pirbright, UK (O Tai 3/97, RS_1 26/3/97).

Sampling procedures

Rectal temperatures and clinical signs (vesicles, lameness) of the pigs were recorded daily. After challenge, oropharyngeal fluid (OPF) was collected daily with cotton mouth-swabs, by placing a forceps with a swab in the buccal cavity for approximately 30 s. In the laboratory, the swabs were incubated for 30 min in 4 mL EMEM containing 5% FBS and 10% antibiotics and then centrifuged, weighed and stored at -70 °C for virus isolation.

Virus isolation

OPF samples were assayed for the presence of virus by plaque titration on monolayers of secondary pig-kidney cells [4]. Ten-fold dilution series (10^0 to 10^{-2}) of the OPF samples (200 µL, tested in duplicate) were allowed to adsorb for 1h on monolayers of secondary pig-kidney cells in a six-well tissue-culture plate (Greiner Bio-One GmbH, Frickenhausen, Germany). After 1 h, maintenance medium containing 1% methylcellulose was added. After 2 days of incubation the plates were washed in tap water with citric acid, monolayers were then rinsed with tap water and stained with amido-black (0.1% amidoblack in 1M acetic acid, 0.09M sodium acetate, and 10% glycerol). All incubations were made at 37 °C in a humidified atmosphere containing 5% CO_2. Plaques were counted macroscopically and virus titres were expressed as ^{10}log plaque forming units (pfu) per mL.

Quantification of virus transmission

As the basis of modelling transmission of FMDV, we used a stochastic SIR model. In an SIR model, transmission is described by the change in number of susceptible (*S*), infectious (*I*), removed *(R)* and total number (*N*) of animals. In this model, $dS/dt = -\beta I(t) \cdot S(t)/N(t)$ in which β is the transmission rate parameter. The transmission rate β can be defined as the average number of new infections for a typical infectious animal in a susceptible population per unit of time. In the described model, the probability for a susceptible animal to escape infection during a period Δt is given by $e^{-\beta.I.\Delta t/N}$ and the probability to become infected is therefore $1-e^{-\beta I \cdot \Delta t/N}$. The expected number of new infections (cases, *C*) is then $E(C) = S(1-e^{-\beta I \cdot \Delta t/N})$ [17].

From the experiments, using the data of the virus isolation of OPF samples, for each interval Δt (the interval between two subsequent samplings, i.e. one day) the number of

susceptible pigs at the start of the interval *(S)*, the number of infectious pigs *(I)*, the number of new cases *(C)* and the total number of pigs *N* are known and therefore the transmission rate β can be estimated.

The within-pen transmission parameter β_w was quantified from the above described model, using a Generalized Linear Model (GLM) [14] with a complementary log-log-LINK function and the natural logarithm of ($I \cdot \Delta t/N$) as offset variable. Data of the four within-pen transmission experiments were pooled and daily data of the virus isolation of the OPF samples were analysed using the GLM. Pigs were classified to be infectious from the moment that an OPF samples tested positive by virus isolation. In cases where the first contact animals tested VI-positive simultaneously with the inoculated animals, we used a half day step, assuming that the VI-positive pigs of the inoculated group were already infectious during the second half of the preceding day.

Because the GLM was inappropriate for analysis of the data of the between-pen transmission experiment in which the inoculated-infected (subgroup A) and susceptible pigs (subgroup B) were separated by a barrier, we used a maximum likelihood method to quantify the between-pen transmission. Since the probability for a susceptible animal in the susceptible subgroup (subgroup B) to become infected in time period Δt can be given by

$$p = 1 - e^{-\left(\frac{\beta_w \cdot I_B(t)}{N_B} + \frac{\beta_b \cdot I_A(t)}{N_A + N_B}\right)\Delta t}$$

where $I_x(t)$ gives the number of infectious animals in subgroup x ={A,B}at time t, and $N_x(t)$ gives the number of animals in subgroup x at time t, the log likelihood for β_w and β_b based on the daily data is

$$logL(\beta_w, \beta_b) = -\sum_t C \log\left(1 - e^{-\left(\frac{\beta_b I_A}{N_A + N_B} + \frac{\beta_w I_B}{N_B}\right)}\right) + (S - C)\left(-\frac{\beta_b I_A}{N_A + N_B} - \frac{\beta_w I_B}{N_B}\right) \quad [11]$$

in which the previously quantified value for β_w was used.

Maximizing this function results in a maximum likelihood estimator for β_b. In order to prevent numerical problems, we applied $\beta_b = exp(ln\ \beta_b)$, thus making sure that β_b became strictly positive. The 95% confidence intervals (CI) of the estimated transmission rates β_w and β_b were calculated assuming that asymptotically, the estimators of log β follow a normal distribution, and were obtained through $ln\ \beta_i \pm 1.96\ se(ln\ \beta_i)$. We evaluated the difference between $ln\ \beta_w$ and $ln\ \beta_b$ using a two-sample *t*-statistic.

To illustrate the effect of the differences between the estimated transmission rates, we also calculated the expected time to the first transmission within a pen and between pens in

our experiments with the results of β_w and β_b. Since the rate of transmission is described by $dS/dt = -\beta \cdot S(t) \cdot I(t)/N(t)$, the expected time to the first transmission can be given by $T = 1/(\beta \cdot S_0 \cdot I_0/N_0)$.

Results

Clinical signs

In the experiments studying the within-pen transmission the first day that clinical signs (rectal temperature > 40.5 °C and/or lameness and/or vesicles) were recorded in group 1 in the inoculated pigs was 1-2 dpi and 2-3 dpi in the contact pigs. In group 2, the inoculated pigs started to show clinical signs at 2 dpi and the contact pigs at 2-3 dpi. In group 3, both the inoculated and contact pigs started to show clinical signs at 2-4 dpi, and in group 4 the inoculated pigs started to show clinical signs at 2-3 dpi and the contact pigs at 3 dpi (Tab. 1A).

In the between-pen transmission group, the inoculated pigs started to show clinical signs at 2 dpi, whereas the contact pigs started to show clinical signs at 4-6 dpi (Tab. 1B).

Virus isolation

In the experiments studying the within-pen transmission virus excretion in OPF in groups 1 and 2 of the direct contact groups, in the inoculated pigs started at 1dpi and at 1-2 dpi in the contact pigs. In groups 3 and 4, the inoculated pigs started to excrete virus at 1-2 dpi and the contact pigs at 2 dpi (Tab. 1A).

In the between-pen transmission group, the inoculated pigs started to excrete virus at 1-2 dpi, whereas the contact pigs started to excrete virus at 3-5 dpi (Tab. 1B).

Quantification of virus transmission

The within-pen transmission rate β_w was estimated to be 6.14 (3.75 – 10.06) per day and the between-pen transmission rate β_b was estimated to be 0.59 (0.083 – 4.18), which was significantly smaller than β_w ($p < 0.01$) (Fig. 1).

The expected time to infection of the first pig within a pen T_w was estimated at 1.6h whereas the expected time to infection of the first pig in the between-pen transmission pen T_b was estimated to be 16h.

Table 1. Virus isolation OPF (^{10}log pfu/mL). A. Within-pen transmission groups.

	Days post infection														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group 1															
Inoculated	-[a]	**2.0**[b]	4.0	4.2	-	-	†[c]								
Inoculated	-	2.2	ǂ	4.8	3.3	2.8	†								
Inoculated	-	**3.8**	2.9	3.8	-	†									
Inoculated	-	**3.7**	-	3.6	†										
Inoculated	-	**3.8**	1.8	3.2	-	†									
Contact	-	-	**2.6**	4.7	4.1	2.8	2.0	-	-	-	-	†			
Contact	-	3.3	n.t.	**6.5**	1.5	2.2	-	-	†						
Contact	-	4.3	4.4	**4.0**	3.0	3.0	-	-	-	-	-	†			
Contact	-	2.3	▫	4.1	3.4	3.3	-	-	†						
Contact	-	-	3.2	**4.6**	5.2	3.3	2.6	-	-	-	-	†			
Group 2															
Inoculated	-	3.4	**3.0**	3.4	3.7	3.3	2.6	2.8	2.2	1.8	-	-	-	-	-
Inoculated	-	4.2	**3.4**	3.3	3.0	2.9	2.8	-	3.1	2.0	-	-	-	-	-
Inoculated	-	2.9	**4.8**	3.4	2.9	1.1	3.0	2.3	2.1	2.8	-	-	†		
Inoculated	-	2.9	**4.4**	4.4	†										
Inoculated	-	4.3	**3.5**	5.0	3.6	2.2	2.6	2.9	†						
Contact	-	-	**2.7**	4.1	4.8	3.0	2.4	2.2	1.8	2.1	3.0	-	-	-	-
Contact	-	2.5	**2.1**	4.9	3.2	2.7	-	-	†						
Contact	-	2.9	**3.8**	4.0	3.2	2.0	1.7	-	-	-	-	-	-	-	-
Contact	-	1.8	3.8	**5.1**	3.0	1.8	1.8	1.7	-	-	2.4	-	†		
Contact	-	3.6	4.8	**5.0**	4.1	2.1	1.8	-	†						
Group 3															
Inoculated	-	-	4.3	**5.4**	4.7	†									
Inoculated	-	-	3.2	6.5	**6.6**	†									
Inoculated	-	▫	5.4	3.4	3.4	†									
Inoculated	-	-	**5.6**	4.4	4.4	†									
Inoculated	-	2.5	**5.5**	3.4	2.8	†									
Contact	-	-	3.1	**5.0**	3.5	†									
Contact	-	-	2.7	5.5	**4.6**	4.5	2.8	2.3	1.9	-	-	1.7	-	-	-
Contact	-	-	2.8	**5.3**	4.0	4.3	3.3	1.2	†						
Contact	-	-	2.1	3.9	**5.3**	†									
Contact	-	-	**4.0**	3.3	3.2	†									
Group 4															
Inoculated	-	-	4.1	**4.6**	3.3	2.7	2.1	-	-	-	-	-	-	-	-
Inoculated	-	3.0	**4.4**	3.0	2.8	†									
Inoculated	-	3.2	**4.5**	4.1	†										
Inoculated	-	-	**2.6**	5.1	†										
Inoculated	-	-	**2.7**	4.5	3.5	†									
Contact	-	-	3.5	**5.2**	5.5	3.2	2.4	-	-	-	-	-	-	-	-
Contact	-	-	4.6	**4.7**	†										
Contact	-	-	4.4	**5.7**	4.2	1.7	3.0	2.6	†						
Contact	-	-	3.0	**6.2**	3.9	3.4	1.7	-	-	2.5	†				
Contact	-	-	3.2	**6.3**	3.7	2.1	2.8	-	†						

Table 1. Continued. B. Between-pen transmission group.

	Days post infection														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group 1															
Inoculated	-	-	**3.3**	3.7	-	-	†								
Inoculated	-	-	**3.0**	4.8	1.9	-	-	1.5	2.2	-	-	†			
Inoculated	-	2.3	**+**	5.4	-	-	-	2.5	†						
Inoculated	-	-	**6.3**	3.8	3.5	†									
Inoculated	-	2.5	**4.5**	3.4	†										
Contact	-	-	-	-	-	**5.3**	4.6	3.3	3.0	2.7	-	†			
Contact	-	-	-	3.5	3.2	**6.4**	5.4	3.3	1.9	-	-	†			
Contact	-	-	-	-	2.1	**5.8**	3.4	1.6	2.5	†					
Contact	-	-	-	-	1.5	2.3	**3.3**	3.1	3.0	-	-	†			
Contact	-	-	-	2.0	**3.2**	3.4	2.8	2.8	-	-	-	†			

[a] no virus was isolated; [b] titre expressed as ^{10}log pfu/mL; [c] euthanasia piglet, if not indicated, pigs were euthanized at 14 dpi; **titres in bold** show day of start of clinical signs; + undiluted sample positive (number of plaques too many to count) but insufficient sample left to determine end-point titre

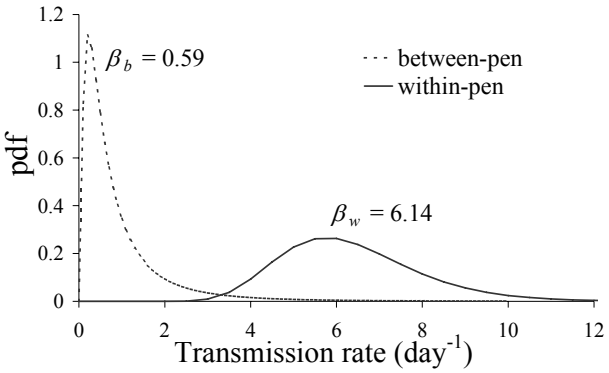

Fig. 1. Probability density functions of the transmission rates β.

Discussion

The purpose of this study was to quantify the within-pen and between-pen transmission rate β of FMDV in groups of non-vaccinated pigs. The within-pen transmission rate β_w was estimated to be 6.14 [3.75-10.06] per day, which was significantly larger than the β_b of 0.59 [0.083-4.18]. These findings indicate that the contact structure affected the transmission of FMDV significantly, and that slower spread between pens should be taken into account

when the course of an FMDV infection in pig herds is analysed or modelled. It probably also means that transmission between different units and, more importantly herds, may develop more slowly than as estimated in within-herd transmission experiments, because the transmission of a pathogen within a herd greatly influences the transmission between herds [16].

Quantified transmission parameters can be of great use during an outbreak of FMDV to estimate the time of first introduction of the virus using the prevalence of FMDV at time of detection, which in turn is important for forward and backward tracing. Moreover, the parameters can help in determining if and when proposed control measures will be sufficient in restricting an epidemic. Although the reduction of transmission caused by limited contact structure by itself might be insufficient to halt an epidemic, the combination with e.g. vaccination might reduce transmission sufficiently [16]. For example, we showed previously [7] that vaccination against FMDV reduced transmission of the virus significantly at 14 dpv, but not at 7 dpv. However, reduction of virus transmission within a herd might be obtained sooner than as estimated in the within-pen transmission experiments since the lower between pen transmission rate indicates that pigs in adjacent pens might have more time to develop a protective immune response.

Although we did find a significant difference in transmission rates between the within-pen and between-pen transmission groups, in all groups ultimately all contact pigs became infected and the transmission of FMDV was reduced but certainly not halted by limited contact structure. Similar results were found in studies with Classical Swine Fever [11, 12, 13]. In contrast to our results, no FMD virus transmission occurred between calves that were housed individually [2]. An explanation for this discrepancy might be that in the calf experiment only one infectious calf was used which had contact with two susceptible calves, whereas in our experiment 5 seeder pigs were used in a group of ten.

The results of the virus isolation of OPF of the individual pigs show, that following experimental infection of pigs, the FMD virus was highly contagious to pigs that were in direct contact, resulting in infection of all the contact pigs within 1-2 days. Infection of the contact pigs in the between-pen transmission group was observed after 3-5 days. In this group, probably one or two of the five contact pigs were infected by indirect transmission and the other pigs were subsequently infected by direct contact. The delay in transmission between pens is not surprisingly, since it is known that pigs are difficult to infect by aerosol [6].

In this study, we quantified the transmission of FMDV within- and between pens among non-vaccinated pigs. The transmission rate that we estimated can be of use in quantitative modelling which is one of the essential tools both for developing strategies in preparation for an outbreak and for predicting and evaluating the effectiveness of control

policies during an outbreak. However, one should keep in mind that many parameters influence transmission, including the strain of the virus, the dose with which the pigs are infected, age, and other factors that will vary under field conditions. This variability must be taken into consideration when mathematical modelling is undertaken or veterinary advice is given for the purpose of practical disease control.

Acknowledgements

This work was supported by the European Union (FAIR5-PL97-3665) and the Ministry of Agriculture, Nature and Food Quality, The Netherlands.

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Chapter 6

Quantification of transmission parameters of FMDV among non-vaccinated and vaccinated pigs

P.L. Eblé[a], A.A. de Koeijer[b], M.C.M. de Jong[b], B. Engel[b], A. Dekker[a]

[a]Central Institute for Animal Disease Control (CIDC-Lelystad), P.O. Box 2004, 8203 AA Lelystad, The Netherlands
[b]Department Quantitative Veterinary Epidemiology, Division of Infectious Diseases, Animal Sciences Group Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands

Preventive Veterinary Medicine (submitted for publication)

Abstract

Scenario studies can be used to determine efficacy of surveillance and control programs for foot-and-mouth disease (FMD). So far, however, limited parameter estimates were available for these studies. The aim of this study was to provide additional estimates of main parameters for the transmission of foot-and-mouth disease virus (FMDV). We used the data of previously performed experiments in non-vaccinated and vaccinated pigs and combined the data of experiments with the same treatment(s). First, we quantified the reproduction ratio R for the various groups using a final size method. Secondly, we used the daily results of virus isolation of oropharyngeal fluid to quantify the transmission rate β, using Generalized Linear Modelling, and the infectious period T, using survival analysis. With the results of β and T estimates for R were made, also for the groups for which a finite estimate could not be obtained using a final size method.

The results of the final size method showed that vaccination with a 4-fold vaccine dose (4FD) at one week before inoculation (–7dpi) reduced R significantly, compared to the non-vaccinated group. The transmission rate β for non-vaccinated pigs was estimated at 6.1 (3.7-10) day^{-1}, β for the –7 dpi vaccinated pigs at 2.0 (1.0-4.0) day^{-1} and β for the –7 dpi 4FD vaccinated pigs at 0.4 (0.1-1.4) day^{-1}. The infectious periods T for the groups were estimated at 6.5 (5.7-7.3), 5.3 (4.7-6.0) and 2.3 (0.9-5.7) days and R at 40 (21-74), 11 (4.9-24) and 1.0 (0.1-7.8) respectively. All estimated parameters, β, T and R, of the vaccinated groups were significantly reduced as compared to the non-vaccinated groups.

We showed that vaccination reduced transmission of FMDV significantly already one week post vaccination. Moreover, we provided estimates of transmission parameters of FMDV which can be used in mathematical models for decision making on control strategies.

Introduction

Since 1991, preventive vaccination against foot-and-mouth disease (FMD) has ended in Europe and thus outbreaks in non-vaccinated populations need to be combated. Outbreaks of FMD in non-vaccinated populations can have huge socio-economic consequences, as was experienced during the 2001 outbreaks in the UK [1] and The Netherlands [2]. For decisions on control of the disease, it is important to have quantitative information on transmission of the virus. As not all situations can be studied experimentally, mathematical models are needed to interpolate and extrapolate to those situations for which decisions need to be made. Mathematical models have for example already been used extensively during the 2001 FMD epidemic in the UK [3] and quantitative modelling has been

recognized as one of the essential tools both for developing strategies in preparation of an outbreak and for predicting and evaluating the effectiveness of control policies during an outbreak [4]. Therefore, quantitative transmission parameters to be used in these models are needed, since these are mostly lacking.

The large number of animals slaughtered during the outbreaks in the UK and The Netherlands in 2001 led to an intense debate about the so-called non-vaccination policy used in the EU. As a consequence, the OIE [5] and the EU [6] adopted new regulations, which make it more favourable to use a vaccinate-to-live policy during an outbreak of FMD. If emergency vaccination will be applied, also quantitative knowledge about the effectiveness of vaccination on virus transmission should be provided. Experimental studies, in which a stochastic susceptible-infectious-removed (SIR) model is used to quantify transmission, have proven to be very suitable for estimating transmission parameters [7,8]. An often used parameter to quantify transmission is the reproduction ratio R, which is the average number of secondary infections per infectious individual during its entire infectious period [9]. As long as $R > 1$ an infection can spread on a large scale (major and minor outbreaks are possible) but as soon as R becomes < 1 the infection will fade out (only minor outbreaks are possible). A limitation of R is that it does not include a time factor, which is needed in epidemic models used to analyse the temporal course of an epidemic. For this purpose the transmission rate parameter β can be used, which is defined by the number of secondary infections caused by one infectious individual per unit of time.

In previous studies [10,11,12,13], we studied transmission of FMDV among non-vaccinated pigs and among groups of pigs to which different vaccination strategies against FMD were applied. We showed that homologous as well as heterologous vaccination protected against challenge at 2 weeks after vaccination. However, in the separate studies we were not able to demonstrate a significant reduction of transmission of FMDV among pigs vaccinated one week (-7 dpi) before challenge, possibly due to the limited power caused by the small experimental groups and the limitations of the final size method that was used to analyze the data. In the present study, we pooled the data of all previously performed experiments and used them to estimate important transmission parameters. First, we used the final size of the experiments to calculate the reproduction ratio R for the non-vaccinated and –7 dpi vaccinated groups and we tested whether transmission differed between the non-vaccinated and the vaccinated groups. Additionally, we used (daily) virus excretion data of all experiments to quantify the transmission rate β for the non-vaccinated and –7 dpi vaccinated pigs after single and 4-fold dose vaccination, using generalized linear modelling. Moreover, we estimated the mean infectious period T for all groups, using survival analysis, and with the results of β and T estimates for R were derived, also for the groups for which no finite estimate could be obtained using the final size method.

Methods

Animals and experimental design

Data were available from ten transmission experiments with groups of ten pigs. Four of the groups were non-vaccinated, two groups were vaccinated at 7 days before inoculation (-7dpi), one group was vaccinated with a 4-fold vaccine dose (4FD) at –7dpi, two groups were vaccinated at –14dpi and one group was vaccinated with an (intra-typic) heterologous vaccine (O Manisa) at –14dpi. Vaccination was done intra-muscularly with a double-oil-in-water emulsion vaccine that contained 3μg of O Taiwan (O Taiwan 3/97) or O Manisa (O Manisa/Turkey/69) 146S antigen per 2 ml dose. In each group, on day 0 (0 dpi), five randomly selected pigs were challenged by intra-dermal inoculation in the bulb of the heel of the left hind-foot with 0.1 ml of challenge virus containing approximately 10^5 $TCID_{50}$/ml FMDV O Taiwan (O Taiwan 3/97). Inoculated and contact pigs of each group were separated before challenge and were reunited again 24h later. From 0-14 days post infection (dpi), oropharyngeal fluid (OPF) samples were collected daily.

More detailed information on the design of the experiments, additional sampling, additional laboratory tests and test results can be found elsewhere [10,11,12,13].

Laboratory tests

OPF samples were assayed for the presence of virus by plaque titration on monolayers of secondary pig-kidney cells [14]. Ten-fold dilution series of the samples (200 μl, tested in duplicate) were allowed to adsorb for 1h on monolayers of secondary porcine kidney cells in a six-well tissue-culture plate (Greiner Bio-One). After 1 hour, maintenance medium containing 1% methylcellulose was added. After 2 days of incubation the plates were washed in tap water with citric acid, monolayers were then rinsed with tap water and stained with amido-black (0.1% amidoblack in 1M acetic acid, 0.09M sodium acetate, and 10% glycerol). Plaques were counted macroscopically. All incubations were made at 37°C in a humidified atmosphere containing 5% CO_2.

Quantification of transmission parameters

Reproduction ratio R (Final Size Method)

In the final size method, the estimation of *R* was based on the final size of the outbreak observed in the experiments. For calculation of *R*, we used a stochastic SIR model [15] in which *R* was calculated using a maximum likelihood estimator [8,16]. Contact pigs were considered infected if virus was isolated in their OPF at one or more days during the experiment.

To determine whether transmission differed significantly between groups, we tested the null hypothesis that there was no difference in transmission between the non-vaccinated and the vaccinated group.

Transmission rate β

The daily data of the virus isolation of OPF samples were used to estimate the parameter for transmission rate, β. Data from experiments with the same treatment(s) were used in a combined analysis and β was estimated using a Generalized Linear Model (GLM) [17]. This GLM was motivated by a stochastic SIR model in which transmission is described by the change in number of susceptible (S), infectious (I), removed *(R)* and total number (N) of animals. In this model, the change of numbers of susceptible and infectious pigs per unit of time is defined as $(S,I) \rightarrow (S\text{-}1, I\text{+}1)$ with rate $\beta \cdot I(t) \cdot S(t)/N(t)$ in which β is the transmission rate parameter. The probability for a susceptible animal to escape infection during a period Δt is given by $e^{-\beta.I.\Delta t/N}$ and the probability to become infected is $1\text{-}e^{-\beta \cdot I \cdot \Delta t/N}$. The expected number of new cases is then $E(C) = S(1\text{-}e^{-\beta \cdot I \cdot \Delta t/N})$ [18].

For each interval Δt (the interval between two subsequent samplings, (i.e. one day) the number of susceptible pigs at the start of the interval *(S)*, the number of infectious pigs *(I)*, the number of new cases *(C)* and the total number of pigs *(N)* was known. Subsequently, the transmission parameter β was quantified using a GLM with a complementary log-log-link function, binomial error distribution (with S the total number of subjects) and log $(I \cdot \Delta t/N)$ as offset variable. All calculations were performed with GenStat Release 4.2.

It was assumed that animals were infectious only at the days that the virus isolation of an OPF sample was positive. If animals that had been positive in the virus isolation assay scored negative again in a later stage, they were no longer considered infectious or susceptible. If a pig was euthanized, the calculation was adjusted by reducing the total group size with one. In cases where the first contact animal became positive in the virus isolation simultaneously with the inoculated animals, we assumed that the pigs in the inoculated group had become infectious half a day earlier. To be consistent, in all cases where the first inoculated animals were positive on the first day, these were assumed to be infectious already half a day earlier.

Infectious period T

The duration of virus shedding of the individual pigs was used to estimate the mean duration of the infectious period T for each group in a survival analysis. The data of the pigs that were euthanized during virus shedding were treated as censored data. To establish the duration of virus shedding of each individual, the same assumptions were used as for

determining the infectious period (see above). The duration of virus shedding of the two pigs in which no virus shedding was observed was set at a value of 0.01 days. The observed periods were modelled with a two-parameter Weibull distribution that fitted the data well. The Weibull parameters were estimated by the maximum likelihood method. The model included provision for right censored observations [19]. From the estimated Weibull parameters, the ML estimators for the mean infectious period T for each treatment group were derived. Standard errors for T and log T were obtained by the Delta method. This is the conventional approach based on large sample asymptotics [20] and Taylor linearization of derived parameters, such as the mean T. All calculations were performed with GenStat Release 4.2.

Reproduction ratio R (with results of β and T)

We calculated an estimate for R from $\log R = \log \beta + \log T$. As an upper bound for the associated standard error, s.e.($\log \beta$) + s.e.($\log T$) was evaluated.

Statistical comparison

Differences between β, T and R for vaccinated and non-vaccinated groups were analysed pair-wise, using the log transformed version ($\log\beta$, $\log T$ and $\log R$) which were assumed to follow a normal distribution.

Results

Reproduction ratio *R* (Final Size Method)

The final sizes of the experiments are shown in Table 1.

Table 1. Results of Final Size Method

Groups	number of exp.	n	S	I	C	FS-Method: *R* (CI)	H_0: R<=1	H_0: R>=1	H_0: $R_{vac}=R_{non\text{-}vac}$
non-vaccinated	4	10	5	5	5	∞ (2.35-∞)	<0.01		
vac -7dpi	2	10	5	5	5	∞ (1.51-∞)	<0.01		
vac -7dpi , 4FD	1	10	5	5	3	1.2 (0.2-5.4)*		0.75	<0.05
vac -14dpi	2	10	5	0^	0	n.a.^	n.a.	n.a.	n.a.
vac -14dpi, heterologous	1	10	5	0^	0	n.a.	n.a.	n.a.	n.a.

***** significantly different ($p<0.01$) as compared to the non-vaccinated group.

^ no virus was detected from inoculated or contact pigs therefore no statistical analysis could be performed.

In the non-vaccinated and –7 dpi vaccinated groups, all contact pigs became infected, and therefore we cannot obtain a finite estimate for the *R* for these groups. However, the lower 95% confidence bound for *R* can be estimated and *R* >= 2.35 for the non-vaccinated and *R* >= 1.51 for the –7dpi vaccinated group. For the –7dpi 4FD vaccinated group, *R* was estimated to be 1.2 (0.2-5.4). When testing H_0: $R_{non\text{-}vac} = R_{vac}$, there was no difference between the –7dpi vaccinated group and the non-vaccinated group, but $p < 0.05$ for the –7dpi 4FD vaccinated group compared to the non-vaccinated group. In the –14dpi vaccinated groups, the inoculated pigs did not become infectious, therefore there were no seeder pigs in these experiments, and consequently the effect of vaccination on virus transmission could not be determined.

Table 2. Cumulative daily number of pigs found positive in the virus isolation assay of OPF samples

			Days post inoculation										# VI
Groups	Exp.	I/C	0	1	2	3	4	5	6	7	8	9-14	pos
Non-vaccinated	1	I	0[1]	5	5	[2]							5
		C	0	3	5								5
	2	I	0	5	5								5
		C	0	4	5								5
	3	I	0	1	5								5
		C	0	0	5								5
	4	I	0	2	5								5
		C	0	0	5								5
Vac -7dpi	1	I	0	4	5								5
		C	0	2	5								5
	2	I	0	0	3	$5^{\#}$	$4^{*\#}$	4	$4^{\#\#}$	[2]			5
		C	0	0	2	4	4	3^{*}	$4^{\#}$				5
Vac -7dpi,4FD	1	I	0	2	$4^{\#}$	$5^{\#\#}$	$4^{*\#\#}$	$4^{\#\#\#}$	$4^{\#\#\#\#}$	$4^{\#\#\#\#}$	$3^{*\#\#\#}$	$3^{\#\#\#}$	[3] 5
		C	0	0	0	1	1	3	$3^{\#\#}$	$3^{\#\#\#}$	$2^{*\#}$	$2^{\#\#}$	3
Vac –14dpi	1	I	0	0	0	0	0	0	0	0	0	0	0
		C	0	0	0	0	0	0	0	0	0	0	0
	2	I	0	0	0	0	0	0	0	0	0	0	0
		C	0	0	0	0	0	0	0	0	0	0	0
Vac –14dpi, heterologous	1	I	0	0	0	0	0	0	0	0	0	0	0
		C	0	0	0	0	0	0	0	0	0	0	0

[1] cumulative number of pigs found positive in the virus isolation assay; [2] end of input GLM (all contact pigs infected); [3] end of input GLM (all infectious pigs stopped shedding virus); [*] euthanasia pig, therefore number of pigs decreased by 1; [#] earlier infectious pig scores negative in VI, therefore number of infectious pigs as input in GLM minus 1

Transmission rate β

In the four non-vaccinated groups, all inoculated and contact pigs started to shed virus at 1-2 dpi. In the –7dpi vaccinated groups, the inoculated pigs started to shed virus from 1-3 dpi and the contact pigs from 1-6 dpi. In the group that was vaccinated at –7dpi with a 4-fold vaccine dose, the inoculated pigs started to shed virus at 1-3 dpi. Only 3 of the 5 contact pigs shed virus and started shedding virus at 3-5 dpi. At 9 dpi, all pigs had stopped shedding virus. In the –14dpi vaccinated groups, both homologous and heterologous vaccinated, no pigs were determined that shed virus (Table 2).

In the non-vaccinated pigs, β was estimated to be 6.1 (3.7-10.1) day $^{-1}$ in the –7 dpi vaccinated pigs β was estimated to be 2.0 (1.0-4.0) day $^{-1}$ and in the –7 dpi 4FD vaccinated pigs, β was estimated to be 0.4 (0.1-1.4) day $^{-1}$ (Table 4).

Infectious period *T*

The duration of the virus shedding of all individual pigs of all performed experiment are shown in Table 3. The mean length of the infectious period *T* was estimated to be 6.5 (5.7-7.3) day in the non-vaccinated pigs, 5.3 (4.7-6.0) day in the –7dpi vaccinated pigs and 2.3 (0.9-5.7) day in the –7dpi 4FD vaccinated pigs (Table 4).

Table 3. Duration of virus shedding of individual pigs (days).

		Inoculated					Contacts				
Groups	Exp.	I	I	I	I	I	C	C	C	C	C
non-vaccinated	1	3*	5†*	3*	2†*	3*	5	4	5	4	5
	2	9*	8*	9*	3†*	7†*	9	5	6	8	6
	3	3†	3†	3†	3†	4†*	3†	8	6†	3†	3†
	4	5	4†*	3†*	2†	3†	5	2†	6†	6†	5
vac –7dpi	1	6†	3†*	6*	5*	6*	6	5	7	6	6
	2	4*	3†*	5*	3	1†	3	6	1	6	3†
vac –7dpi, 4FD	1	4*	2	2†	1*	2	3	0^	0^	2	2

† euthanized during virus shedding, therefore censored in analysis; * half day extra in analysis; ^ as input in analysis 0.01 was used

Reproduction ratio *R* (with results of β and *T*)

Using the estimates for β and *T*, we estimated *R* for the non-vaccinated pigs at 40 (21-74), *R* for the –7dpi vaccinated pigs at 11 (4.9-24) and *R* for the –7dpi 4FD vaccinated pigs at 1.0 (0.1-7.8) (Table 4).

Statistical comparison

For as well the single as the 4FD –7 dpi vaccinated groups β, *T* and *R* in the vaccinated groups were significantly reduced as compared to the non-vaccinated group (Table 4).

Table 4. Results of transmission rate β, infectious period T and reproduction ratio R

Groups	number of exp.	β (CI)	T (CI)	R (CI)
non-vaccinated	4	6.1 (3.7-10.1)	6.5 (5.7-7.3)	40 (21-74)
vac -7dpi	2	2.0 (1.0-4.0)*	5.3 (4.7-6.0)*	11 (4.9-24)*
vac -7dpi , 4FD	1	0.4 (0.1-1.4)*	2.3 (0.9-5.7)*	1.0 (0.1-7.8)*
vac -14dpi	2	n.a.^	n.a.	n.a.
vac -14dpi, heterologous	1	n.a.	n.a.	n.a.

^ no virus was detected from inoculated or contact pigs therefore no statistical analysis was performed; * significantly different ($p<0.01$) as compared to the non-vaccinated group

Discussion

The aim of this study was to quantify reduction of transmission by vaccination and to provide transmission parameters of FMDV for groups of non-vaccinated and vaccinated pigs. Previously, we showed that both homologous and heterologous vaccination at –14 dpi completely protected against challenge with FMDV [10,12], although in these studies it was not evaluated whether in this time-span also protection against direct contact challenge with non-vaccinated pigs would be obtained, but it was assumed that this situation would not occur during an epidemic because of the obligatory stand-still.

If vaccination is used as intervention tool during an epidemic, the interval between vaccination and infection might be shorter than 2 weeks. Therefore, we also performed transmission experiments with a vaccination-challenge interval of one week. In these experiments, the contact pigs were not (all) protected yet [10,11]. Beside knowledge on how soon after vaccination animals are fully protected, for decision making regarding control of the disease, it is also important that transmission in non-vaccinated and vaccinated pigs is quantified.

Using a final size method we showed that in pigs vaccinated at one week before challenge with a 4-fold vaccine dose transmission was significantly reduced as compared non-vaccinated pigs, although R was not significantly < 1. For pigs vaccinated one week before challenge with a single vaccine dose and in non-vaccinated pigs, a finite estimate of R could not be obtained using this method. In another study, we observed that virus shedding of the –7 dpi vaccinated pigs was significantly reduced as compared to the non-vaccinated pigs [11] and since the reproduction ratio R incorporates both infectiousness and susceptibility and virus excretion can be seen as a measure of infectiousness, differences in R between the groups could be expected. However, the final size method is not a suitable method to detect these kinds of differences.

For modelling purposes it is useful that other transmission parameters than R are quantified. In some models estimates for R will suffice, but for models that specifically

incorporate time the transmission rate β is a more suitable parameter. Therefore, we analysed the data again, using more information on the infectious process as compared to the final size method, and we estimated the daily transmission rate β, the infectious period T and subsequently R for the various groups. We found that all the estimates (β, T and R) of the –7 dpi vaccinated pigs were significantly reduced as compared to the non-vaccinated pigs. For the group vaccinated at -7dpi with a 4-fold vaccine dose R was estimated to be 1.0, thus major outbreaks could still occur. However, we probably overestimate the within-herd transmission by using the within-pen transmission estimates. Between-pen transmission is reduced as compared to within-pen transmission [13] and within-herd transmission is probably reduced even more. Moreover, even if the within-herd reproduction ratio is slightly above 1, if taken together with other implemented measures during an outbreak (stand-still, hygienic measures) this will probably be enough to reduce transmission to other herds, as for example has been evaluated for Aujeszky's disease [21].

Transmission parameters as quantified in this paper are essential in studies that assess the effect of vaccination programs in controlling epidemics. The 2001 UK FMD outbreak for example has been analysed by various groups using available field data in which they assessed the effect of the applied control measures, as is summarized by Kao [3]. Moreover, some groups have extended these models by evaluating the possible effect of vaccination in controlling this epidemic. Lacking other quantitative data on the effect of vaccination on transmission, Keeling [22] and Kao [23] both assumed that the transmission is 0 at four days post vaccination but that no reduction in transmission will be found until then. This assumption is based on results from experiments in which the vaccinated animals were infected by short during exposure (hour(s)) to virus excreted by donor-pigs [24]. Although suitable to simulate airborne spread from an infected holding, the severity of the challenge used in these studies is relatively low. Data such as summarized in this paper are a valuable addition to the transmission data available until now for the models, since they quantitatively show the effect over time of vaccination in groups of vaccinated animals. Preferably, our quantitative data should be checked or extended with field data on farm to farm transmission. Moreover, for a further combat of the disease the data should be extended with data for other strains of FMD and data provided for other species.

Conclusion

Vaccination is an effective tool to reduce the transmission of FMDV.
Vaccination reduces the transmission rate β, the duration of virus shedding and consequently the reproduction ratio *R*, of FMDV in pigs as soon as one week post vaccination.
Experimental data from transmission studies can be used to provide parameter estimates which can be of importance in models for decision making on control strategies.

Acknowledgements

We thank Dr. J.A. Stegeman and Dr. A. Bouma for their advice and helpful comments on the manuscript. This work was supported financially by the Netherlands Ministry of Agriculture, Nature and Food Quality.

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Chapter 7

Summarising Discussion

Summarising Discussion

Since the foot-and-mouth epidemics in Europe in 2001 the use of emergency vaccination, if an outbreak occurs, has become more prominent in EU legislation. In this thesis, the efficacy of emergency vaccination against FMD in pigs was studied experimentally. The aim was to establish if and how soon (herd-) immunity is induced by vaccination and, furthermore, to study the correlation between immune response after vaccination and protection against challenge.

Efficacy of vaccination

Conventional vaccines against FMD are based on chemically inactivated virus, and are being used at large scale as prophylactic control measure against FMD. Also, they have been used extensively to eradicate FMDV in several areas of the world where FMD used to be endemic. Many FMD-free countries now stopped vaccination but might use vaccination as intervention tool in the event of an epidemic, as was for example done in The Netherlands in 2001 [1]. If vaccination is used as intervention tool during an epidemic, the induction of rapid protective immunity against the circulating strain is of paramount importance. Development of immunity after vaccination takes time and factors that might influence the rate at which sufficient immunity is induced are various, amongst others the homology between field- and vaccine-virus and the dose of vaccine applied. Previously performed studies showed that high-potency emergency vaccines can protect cattle [2], sheep [3] and pigs [4] against clinical disease within 4 days of immunisation.

Vaccines, however, should not only induce sufficient clinical protection, but most of all a level of herd-immunity to such an extent that transmission of the virus within the population in the vaccinated area is stopped. The experimental design that was used in the studies described in this thesis is suitable to quantify virus transmission and reduction of transmission caused by vaccination within a pen, and was used previously to study the effect of vaccination on transmission of other viruses [5,6]. The results reported in this thesis indicate that in a field situation, homologous as well as intra-typic heterologous vaccination will be effective in controlling an outbreak of FMD in a pig population within two weeks after vaccination (Chapter 2, 3). A shorter vaccination-challenge interval of one week led to significant reduction of transmission of the virus as compared to the non-vaccinated pigs, but the reproduction ratio R for the vaccinated group was estimated to be 11 and thus (considerably) above one, therefore the effect of vaccination one week before challenge will not be sufficient to stop transmission of the virus within a pen. Vaccination did lead to reduced virus excretion of the vaccinated-infected pigs, thereby reducing the amount of virus released into the environment which will help in reducing the transmission

of the virus to other herds (Chapter 4, 6). Application of a four-fold vaccine dose improved the efficacy of vaccination at one week after vaccination, since it reduced transmission and virus excretion more than vaccination with a single vaccine dose. However, even in the four-fold dose vaccinated pigs, after one week post vaccination, the within-pen transmission ratio was not significantly < 1, thus within pens, large outbreaks could still be expected. However, with an estimated reproduction ratio of 1, there is a reasonable chance that between-herd transmission will be reduced sufficiently (Chapter 4, 6). The positive effect of increased vaccine dose was in accordance with results of studies in other species in which was shown that by using emergency vaccines with a higher antigen-payload, the induction of specific antibodies was faster and higher antibody titres were obtained [7,8]. In order to increase the amount of antigen administered to the pigs, in the study described in this thesis the application of an increased vaccine volume was used instead of the use of a vaccine batch with a higher antigen-payload. This was done to avoid differences in immune responses between the standard and high dose vaccinated groups due to differences in vaccine composition. However, the application of a four-fold vaccine volume is not practical in the field, and it needs to be evaluated whether increase of antigen-payload in the vaccine alone would have the same effect and, if so, what antigen-payload would be optimal to be used in emergency vaccines for pigs. Also it should be evaluated whether the use of other adjuvant and/or the use of another application method might lead to improved immune responses after vaccination.

In transmission experiments, the infection chain has to be started artificially. The infection in the described transmission studies was induced by intra-dermal injection of virus into the bulb of the heel of the foot. The use of a needle to challenge the pigs is an artificial method to infect pigs, but simulates infection through damaged skin. Moreover, this inoculation route is known as a reliable and sensitive method for the establishment of an infection, usually with a very short incubation period [9]. Using this inoculation method resulted in infection of all inoculated and subsequently all contact-exposed in the non-vaccinated pigs. Moreover, no differences in clinical signs and virus excretion between inoculated and contact-infected pigs were observed. Therefore, the used method was considered as a reliable method to induce an infection chain and to study vaccine efficacy.

A more natural way to start the infection chain would have been the use of an aerosol challenge, but in pilot experiments it was observed that even application of high doses of an individual aerosol challenge (spray) failed to induce infection in all inoculated pigs (unpublished results, Eblé). Also the use of intranasal instillation was not successful (unpublished results, Orsel). Therefore, these methods were not applied in the experiments described in this thesis. In previous studies, intra-dermally infected donor pigs have been used as seeder pigs through indirect-contact [4,10]. Seeders pigs (housed in cages) and

contact pigs were held in the same room during 1-4 hours. Although this method is appropriate to mimic air-borne spread the variable exposure times applied by the authors indicates that this method is hard to standardise, and since pigs are relatively resistant to aerosol challenge [11,12], the challenge method might be not severe enough to simulate the effect of virus introduced via another route. The continuous exposure to non-vaccinated infected seeder pigs could have been another possible challenge method. However, long exposure to infected non-vaccinated pigs is not in accordance to what might happen during an epidemic. When emergency vaccination is applied, all susceptible animals will be vaccinated and because of the obligatory stand-still, the introduction of non-vaccinated infected pigs will probably not occur.

Whether or not pigs will become infected after exposure with FMDV will depend on route of infection, infection dose and duration of exposure. The intradermal inoculation of the bulb of the heel that was used mimicked the introduction of virus, in a sufficient dose to establish infection in non-vaccinated pigs, through short during contact, which was assumed to be a realistic approximation of the introduction that might occur during an epidemic. Moreover, the constant dose used throughout the experiments allowed for comparison between experiments.

Correlation between immune responses and protection

Before (new) vaccines will be accepted for use in the field, the vaccine(candidate)s need to be selected and they need to be evaluated for their efficacy. Efficacy of vaccination is usually established by carrying out challenge-experiments, which are harmful for the animals involved, time-consuming and expensive. It would be convenient if correlation(s) between induced immune responses and protection against challenge were established because these could be used to assess the efficacy of vaccines without challenge experiments, during research for improved vaccines, and for choice-making between vaccines. During an epidemic, vaccine choice is complicated because of the antigenic variability of the virus. To enable the quick formulation of vaccine in case of an epidemic, vaccine manufacturers usually have an antigen-bank in which several selected antigens are stored that give (cross-) protection to most circulating viruses. Because the number of antigens stored is limited, the vaccine used during an emergency is usually (intra-typic) heterologous. The usual way to select an appropriate vaccine candidate is by carrying out serological tests in which the field-isolate and a panel of sera against the candidate vaccine strains are used to determine the serological relationship between the field isolate and the vaccine strain. This serological relationship can be quantified by the ratio ‘r1’ [13], which reflects whether or not a (reasonable) relationship between the vaccine strains and the field strain can be expected.

Although for cattle a correlation between level of antibodies and protection against challenge at 21 days post vaccination has been established [14,15], it is still largely unknown if and how the immunoglobulin isotypes, mucosal immunity and cell-mediated immunity induced by vaccination contribute to protection shortly after vaccination. In this thesis the humoral and cellular immune responses after homologous and (intra-typic) heterologous vaccination were compared, and the serological and mucosal antibody responses after vaccination and infection were studied.

Humoral immune responses

Due to the large number of cattle challenge tests, there has been overwhelming evidence of correlation between the level of FMD specific antibodies and protection against FMD. However, no correlation between antibody-titres and protection has been proven for short vaccination-challenge intervals and in pig challenge studies. Concerning the pig challenge studies, this was probably due to the paucity of data but also to the difficulty in carrying out meaningful challenge experiments in pigs, because a factor that will influence the outcome of a pig challenge experiment is whether and when clinically infected test animals will be removed from the remaining, clinically healthy group in order to prevent a secondary challenge [16,17]. In this thesis it was shown that also for pigs, when challenged one week post vaccination, reduction of virus excretion after challenge of the individual pigs correlates with neutralising antibody titres at the day of challenge (Chapter 4).

In order to study (protective) immune responses in more detail, the induction of isotype specific immune responses (IgG, IgG1, IgG2, IgM, IgA) by vaccination was evaluated. Vaccination resulted in IgM and IgG responses and these were consistent with the responses described in previous studies [18,19], but for the first time also IgA responses were demonstrated after vaccination. The induced IgA responses were highest in the group that was vaccinated with the highest vaccine-dose and the pigs with the highest IgA responses post vaccination were protected against contact-challenge. Therefore, the IgA response post vaccination might be indicative of protection against challenge (Chapter 4). The prolonged IgA response after challenge that was detected in non-protected pigs also merits further investigation. Prolonged IgA responses are well known in carrier cattle but are hardly detected in non-carriers [20]. However, it is generally considered that pigs do not become carriers of FMDV. Although immune responses differ between different species, one can speculate that the IgA response that was detected is indeed induced by persistence of FMDV or viral RNA in pigs as was observed by Mezencio et al. [21].

As inhalation of airborne FMDV, leading to replication in the respiratory tract is considered an important route by which livestock become infected, ideally, a vaccine should also induce mucosal immune responses. In this thesis it was demonstrated that in

pigs the isotype specific antibody responses (IgM, IgG and IgA) in OPF samples, appeared at approximately the same time and had a similar duration as in serum, both after vaccination and infection. This corresponds with earlier findings that the neutralising antibody profiles in serum and nasal mucosa samples in pigs were comparable after vaccination and infection, in contrast to results obtained in cattle [22]. Therefore, in contrast to cattle, vaccination in pigs does not only lead to a systemic antibody response but also to antibodies present locally on mucosa, probably enhancing protection against infection.

Cellular responses

The aim of the study in Chapter 3 was to evaluate the cell-mediated immune responses that contributed to (cross-) protection. Heterologous vaccination with O Manisa vaccine resulted in cross-reactive levels of antibodies against FMDV O Taiwan and with the sera obtained in the study, 'r_1' was calculated to be 0.4. Despite this relatively low 'r_1'-value, all pigs were protected against challenge, which might be explained by the high level of antibodies that was induced, since high potency vaccines might provide sufficient protection against a heterologous challenge [23]. After both homologous as heterologous vaccination, lymphocyte proliferation was demonstrated after re-stimulation of cultured PBMC with FMDV O Taiwan. Cross-reactive proliferation after FMD vaccination has been described before in cattle [24] and pigs [25] and is probably caused by recognition of conserved epitopes within or even between serotypes. It was also demonstrated that single vaccination, homologous as well as heterologous, induced a comparable systemic Th1 (IFN-γ) and Th2 (Il-10) response. Unfortunately, because all vaccinated pigs in the study were protected against challenge, a possible correlation between protection and the induced responses could not be determined. Interestingly, Parida et al. [26] recently reported that vaccination of cattle resulted in a detectable IFN-γ response, and showed that the quantity of the post-vaccination produced IFN-γ correlated with the ability to control pharyngeal virus replication. They suggest that a combination of VN-titre and IFN-γ production might be used in vaccination studies to provide a better correlation with the ability to control virus replication. In their study the induction of IFN-γ was serotype specific and they suggest that it might be relevant to test the ability of future vaccines to elicit a more broadly cross-protective cell mediated immune response. In this respect, the fact that intra-typic heterologous vaccination with O Manisa (which is a known immunodominant strain) resulted in a comparable IFN-γ response as homologous O Taiwan vaccination is very interesting, and further studies are needed to evaluate whether the IFN-γ response might be suitable as indicator of immunodominance.

Note: Immunodominance or broad cross-reactivity can be described as a serological relationship between pairs of FMDV strains where the relationship is not always symmetrical and one strain can be dominant over another [27] and is a highly sought after characteristic during the selection of vaccine strains.

Implications

In the EU, a non-prophylactic vaccination policy is applied. Therefore, the livestock population is highly susceptible for an infection with FMDV, and outbreaks can be expected in the future. Should an outbreak of FMD occur, control measures will be implemented immediately to prevent further spread of the infection. The obligatory control programme include the establishment of an immediate stand-still, zoo-sanitary measures, culling of infected herds and forward and backward tracing of dangerous contact herds [28]. In the recent past, also (large scale) pre-emptive culling of contiguous herds was implemented.

When an outbreak is detected, it takes time to implement all control measures and in the meantime the virus may continue to spread. Especially in densely populated livestock areas the situation might soon run out of control and, moreover, limitations in manpower, destruction capacity etc. might be envisaged. Then, other intervention tools, such as vaccination, may be needed, as was for example implemented during the 2001 epidemic of FMD in The Netherlands. However, in 2001 in The Netherlands a vaccination-to-cull policy was applied, meaning that all vaccinated animals were killed and the carcasses disposed of in a rendering plant. Now that the regulations of the EU [28] and the OIE [29] have changed, the use of a vaccinate-to-live policy has (economically) become more favourable, reacting to the fact that public opinion had turned against the destruction of huge numbers of healthy animals. A vaccinate-to-live policy in which vaccinated animals can finish their normal production life, should reduce destruction of huge numbers of healthy animals, and supports the political view of the Dutch Minister of Agriculture who stated after the outbreak in 2001 in The Netherlands, that it was his intention to control a future epidemic of FMD with minimal culling and the possibility to use emergency vaccination-to-live has been implemented now in the contingency plan of The Netherlands.

The implementation of an emergency vaccination campaign will take time but, if organised well, can probably be carried out faster than (large-scale) pre-emptive culling. If vaccination is going to be used as intervention tool during an epidemic, the induction of rapid (herd) immunity is very important. The results in this thesis showed that vaccination is effective from two weeks onwards within groups of pigs. It should be kept in mind that it was not evaluated whether in this time-span also protection against direct contact challenge

with non-vaccinated pigs would be obtained, but it was assumed that this situation would not occur during an epidemic because of the obligatory stand-still.

In the vaccination-challenge studies described in this thesis, the within-pen transmission was estimated. In addition to the vaccination trials, also the within- and between-pen virus transmission in groups of non-vaccinated pigs was compared. It was shown that between-pen transmission is slower than within-pen transmission, as was also shown for Classical Swine Fever [30], and between-herd transmission will probably be reduced even more [31]. Therefore, by using the estimates of the within-pen transmission, the efficacy of the vaccine to reduce between-herd transmission is probably underestimated. As between herd-transmission is the most important aspect during an epidemic, transmission of FMDV will probably be sufficiently reduced in a shorter notice than two weeks. This can be illustrated by data from field outbreaks in pigs in The Netherlands in 1966-67 in which vaccination was used as intervention tool and where on vaccinated farms outbreaks were only detected within 10 days after vaccination [32]. However, one should keep in mind that the pig industry and the vaccines used have changed in the meantime.

Beside the aimed induction of herd-immunity and reduced culling of healthy pigs on contiguous herds, another advantage of vaccination is that only one visit of a vaccination team is needed per herd, which is desirable because strict hygienic measures are essential during an outbreak. Therefore, in case of an epidemic, quick implementation of vaccination at appropriate spatial scale should be considered and the preparation and the availability of the necessary sources to do so should be arranged beforehand.

For decision making regarding control of the disease, it is also important that transmission in non-vaccinated and vaccinated pigs is quantified, which can be used in quantitative modelling which is recognized as one of the essential tools both for developing strategies in preparation of an outbreak and for predicting and evaluating the effectiveness of control policies during an outbreak [33]. Mathematical models have for example already been used extensively during the 2001 FMD epidemic in the UK [34]. However, quantitative transmission parameters that can be used in these models were mostly lacking. In this thesis, estimates for the transmission rate, the infectious period and R for both non-vaccinated and vaccinated pigs are provided (Chapter 5,6). Data such as summarized in this thesis are a valuable addition to the data used until now in the models, since they quantitatively show the effect over time of vaccination in groups of vaccinated animals. Preferably, the data should be checked or extended with field data on farm to farm transmission. Moreover, for a further combat of the disease the data should be extended with data for other strains of FMDV and data provided for other species.

In this thesis, it was shown that the currently used vaccines in pigs worked very well in inducing protective immunity within groups of pigs from two weeks after vaccination and

emergency vaccination might reduce between-herd transmission even earlier. Beside research for alternatives of the currently used vaccines, new research should also focus on how to improve current vaccines. These vaccines should induce (even) higher levels of protection, achieved more quickly and should block transmission from vaccinated animals. Results of this thesis suggest that the correlation between protection and administered vaccine dose, post-vaccination IFN-γ response and post-vaccination IgA response might be worthwhile to investigate further.

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Samenvatting

Mond-en-klauwzeer (MKZ) is een zeer besmettelijke virusziekte die voornamelijk voorkomt bij evenhoevigen. De belangrijkste gedomesticeerde dieren die besmet kunnen raken zijn runderen, varkens, schapen en geiten. Runderen en schapen kunnen al met een lage dosis virus via de ademhalingswegen worden besmet. Varkens zijn ongevoeliger voor infectie via deze route, maar als ze eenmaal besmet zijn spreiden ze grote hoeveelheden virus en ze worden dan ook beschouwd als vermeerderaars van het virus in een populatie. Het MKZ-virus (MKZV) kan zich snel verspreiden door een dierpopulatie, m.n. door directe diercontacten, maar ook door indirecte contacten via vervoermiddelen, mensen, dierlijke producten of via de lucht.

MKZ komt nog steeds voor in grote delen van de wereld, met name in ontwikkelingslanden. Binnen de EU is MKZ door grootschalige vaccinatiecampagnes uitgeroeid, en sinds 1992 wordt er een zogenaamd non-profylactisch vaccinatiebeleid gevoerd. Dat wil zeggen dat er niet meer routinematig gevaccineerd wordt, waardoor de hele dierpopulatie erg gevoelig is geworden voor MKZ. Vanwege economische en veterinair technische redenen, werden na 1992 uitbraken binnen de EU voornamelijk bestreden met behulp van ‘stamping-out’, waarbij alle dieren van besmette bedrijven en contactbedrijven werden gedood en dit werd eventueel nog aangevuld door ruimen van dieren op buurtbedrijven.

MKZ is een aangifteplichtige dierziekte en uitbraken kunnen enorme economische maar ook grote sociale gevolgen hebben, zoals gedurende recente uitbraken in Taiwan (1997), Nederland (2001) en Groot-Brittannië (2001) duidelijk werd. Binnen de EU leidde de epidemieën van 2001 en de massale doding van dieren die ermee gepaard ging tot discussies over hoe te handelen bij uitbraken in de toekomst. De maatschappelijke wens was vooral om het gebruik van vaccinatie (voor het leven) te gebruiken als bestrijdingsmaatregel, mede omdat er tegenwoordig testen zijn ontwikkeld die gevaccineerde van geïnfecteerde dieren kunnen onderscheiden. Dit heeft geleid tot vernieuwde regelgeving die het gebruik van vaccinatie als interventiemaatregel tijdens een uitbraak (economisch) aantrekkelijker heeft gemaakt.

Als vaccinatie gebruikt zal gaan worden als interventiemaatregel tijdens een uitbraak, is het van groot belang dat er inzicht is in hoe effectief (nood-)vaccinatie is tijdens een uitbraak. Het belangrijkste aspect is dan dat vaccinatie transmissie van het virus in de populatie voldoende zal reduceren en het liefst kort na vaccinatie.

In het onderzoek dat in dit proefschrift beschreven wordt, werd de werkzaamheid van noodvaccinatie tegen mond-en-klauwzeer bij varkens experimenteel onderzocht. De transmissie van MKZV en de invloed van vaccinatie daarop, werd bepaald in

dierexperimenten, waarvan de uitkomsten met behulp van een wiskundig model werden gekwantificeerd.

Allereerst werd onderzocht of vaccinatie transmissie van MKZV kon reduceren en hoe snel na vaccinatie dit het geval was. Uit het onderzoek dat beschreven staat in hoofdstuk 2 bleek dat twee weken na vaccinatie met een homoloog vaccin (vaccin- en challengevirus identiek) de varkens beschermd waren tegen infectie (challenge) met MKZV. Eén week na vaccinatie werd echter nog wel transmissie gezien naar alle contactdieren en er kon met de gebruikte mathematische methode (zgn. final-size methode) geen verschil worden gevonden tussen de niet gevaccineerde groep en de groep die was gevaccineerd één week voor challenge.

Omdat er maar een beperkt aantal MKZV stammen ter beschikking staan als vaccinstam, zal er tijdens een uitbraak veelal gevaccineerd gaan worden met een (binnen het serotype) heterologe vaccinstam (vaccin- en veldvirus niet identiek, maar wel binnen hetzelfde serotype MKZV). Daarom werd er in hoofdstuk 3 gekeken naar het verschil tussen homologe en heterologe vaccinatie. Beide groepen werden gevaccineerd twee weken voor challenge. Er werd geen verschil gevonden tussen beide groepen, twee weken na vaccinatie waren zowel de homoloog als de heteroloog gevaccineerde varkens beschermd tegen challenge.

Omdat tijdens een uitbraak introductie van virus binnen een gevaccineerde groep dieren wellicht sneller plaats zal vinden dan na twee weken, werd verder onderzocht in hoofdstuk 4 of bescherming sneller kon worden geïnduceerd door de toegediende vaccindosis te verhogen. Hiertoe werden een groep varkens die een enkelvoudige dosis vaccin en een groep varkens die een 4-voudige dosis vaccin hadden gekregen vergeleken en gechallenged op één week na vaccinatie. Verhoging van de vaccindosis leidde tot vermindering van het aantal contactinfecties. Verder werd ook aangetoond dat vaccinatie leidde tot een significante afname van de virus excretie van de gevaccineerd-geinfecteerde dieren, wat zal leiden tot reductie van MKZV in de omgeving. Na vaccinatie met een 4-voudige vaccindosis werd de virusexcretie van de gevaccineerd-geinfecteerde dieren nog minder, hetgeen aansluit bij resultaten die eerder werden gevonden bij runderen en schapen.

Voor goede besluitvorming omtrent controlemaatregels bij introductie van MKZV, is het belangrijk om de transmissie van MKZV te kwantificeren, in zowel niet gevaccineerde als gevaccineerde dieren. Deze parameters kunnen worden gebruikt in mathematische modellen, die op hun beurt weer worden gebruikt om het verloop van een uitbraak te voorspellen of om controlemaatregels op effectiviteit te toetsen. In 2001 zijn in Groot-Brittannië dit soort modellen veelvuldig gebruikt.

In het onderzoek dat beschreven staat in hoofdstuk 5, werd de transmissie-rate β (aantal dieren dat door één infectieus dier per tijdseenheid worden besmet) in niet-gevaccineerde

varkens bepaald. Daarbij werd de transmissie-rate binnen en tussen hokken vergeleken. Hierbij werd aangetoond dat de tussen-hok transmissie-rate significant kleiner was dan de transmissie-rate binnen een hok.

Met de resultaten van de eerder uitgevoerde proeven werden vervolgens in hoofdstuk 6 drie belangrijke transmissie parameters geschat. De transmissie rate β, de infectieuse periode T (duur van virus uitscheiding) en met behulp van deze twee parameters werd de zogenaamde reproductie ratio R geschat voor zowel de niet gevaccineerde als de gevaccineerde varkens. R is gedefinieerd als het aantal secundaire infecties dat wordt veroorzaakt door één infectieus individu in een gevoelige populatie. Wanneer R kleiner is dan 1, zal een infectieus dier gemiddeld minder dan één ander dier infecteren en zal de infectie uitsterven in een populatie. Wanneer R groter is dan 1, kan een dier gemiddeld meer dan één ander dier infecteren en kan de infectie spreiden in een populatie.

Bij de dieren die één week voor challenge gevaccineerd waren, zowel die met enkel- als die met 4-voudige dosis, waren alle drie de parameters significant kleiner dan bij de niet gevaccineerde dieren. Voor de groep die gevaccineerd was met een 4-voudige vaccindosis werd R geschat op 1. Bij een dergelijke waarde van R, kan aangenomen worden dat dan de transmissie tussen bedrijven, hetgeen het meest belangrijk is tijdens een uitbraak, voldoende zal worden gereduceerd ($R < 1$).

Naast kennis over effectiviteit van de huidige vaccins, is ook meer kennis nodig over de immuunresponsen die worden geïnduceerd door vaccinatie. Correlatie tussen de geïnduceerde immuunresponsen en protectie zou kunnen worden gebruikt om vaccins te testen op hun werkzaamheid zonder dierproeven, of kunnen worden gebruikt bij de ontwikkeling van een nieuw type MKZ-vaccin.

In het onderzoek dat beschreven staat in hoofdstuk 4 werd aangetoond dat bij individuele varkens, de reductie van virus excretie was gecorreleerd met de neutraliserende antilichaamtiter op het moment van infectie. Ook werd aangetoond dat er een IgA respons werd geïnduceerd na vaccinatie, en werden er aanwijzingen gevonden dat de hoogte van de IgA respons mogelijk gecorreleerd is met protectie. Daarnaast bleek dat bij de duur van de IgA respons na infectie langdurig aanwezig kon blijven (>4 maanden na infectie), hetgeen tot u toe alleen bekend was bij zgn. carrier runderen die lange tijd virus bij zich kunnen dragen.

Verder werd gevonden dat voor wat betreft de isotype specifieke antilichaam responsen (IgG, IgM en IgA), de mucosale immuunrespons na vaccinatie en infectie op ongeveer hetzelfde moment opkwamen en gedurende dezelfde tijd aanwezig bleven als de systemische responsen, in tegenstelling tot hetgeen voor runderen beschreven is.

Er werden geen verschillen gevonden in immuunresponsen na homologe en heterologe vaccinatie (hoofdstuk 3). Beiden induceerden een humorale en vergelijkbare cellulaire

(lymphoproliferatie, Th1 (IFN-g), Th2 (IL-10)) immuunrespons. Hierbij kon geen correlatie met protectie worden aangetoond omdat al de gevaccineerde dieren beschermd waren tegen challenge.

Concluderend kan worden gesteld dat vaccinatie bij varkens een belangrijke tool kan zijn om transmissie van MKZV te reduceren. De resultaten die in dit proefschrift beschreven staan, duiden er op dat in een veld situatie, zowel homologe (vaccin- en veldvirus identiek) als heterologe vaccinatie (vaccin- en veldvirus niet identiek, maar wel binnen hetzelfde serotype MKZV) binnen twee weken na vaccinatie een uitbraak van MKZ in een varkenspopulatie onder controle zou kunnen krijgen. Verhoging van vaccindosis leidt tot snellere bescherming en er zou nader onderzoek moeten plaatsvinden naar de optimale vaccindosis of verhouding antigeen/adjuvant in een noodvaccin. Verder zijn nu transmissieparameters geschat die voorheen ontbraken. De conclusies in dit proefschrift zijn gebaseerd op binnenhoktransmissie experimenten en zouden moeten worden uitgebreid met gegevens over tussenbedrijfstransmissie. De correlatie van VN-titers en mogelijk IgA respons na vaccinatie met protectie zou kunnen worden gebruikt voor het inschatten van vaccin effectiviteit zonder challenge experimenten en in toekomstig vaccinonderzoek.

Dankwoord

Poeh hé. Het is nu ècht helemaal af. Vanaf nu zijn er geen ‘verboden’ vragen meer (‘is je artikel al klaar’, ‘hoe staat het met de statistiek’ en ‘heb je al een datum’). Dat hier nu uiteindelijk een voltooid proefschrift ligt is, ondanks het feit dat alleen mijn naam op de voorkant van dit proefschrift staat, te danken aan de inzet, hulp en medewerking van heel veel verschillende mensen. Een aantal van hen wil ik graag bij name noemen.

Allereerst mijn promotoren, beste Arjan en Mart, dank jullie wel dat jullie mij hebben willen begeleiden gedurende het promotietraject. Ondanks jullie beider zeer druk bezette agenda’s werd er altijd ruimte gevonden voor overleg over het onderzoek, en het onderzoek dat er al lag is zeker completer geworden met jullie begeleiding. Daarnaast heb ik veel geleerd van onze discussies. Ook wil ik graag Jan bedanken, die mij heeft begeleid tijdens de eerste fase van het onderzoek.

Dan mijn co-promotoren. Aldo, jouw enthousiasme, hulp bij van alles en nog wat (vrouwen en computers/statistiek/techniek…) en onverminderd positieve stimulans waren werkelijk onmisbaar. Ik hoop dat we in de toekomst samen nog een heel aantal onderzoeksideeën uit zullen mogen werken! Annemarie, jij was onvervangbaar bij het corrigeren en becommentariëren van de teksten maar ook onze mail- en telefoongesprekken over de ‘verkens’ en allerhande andere zaken werden zeer gewaardeerd.

Daarna alle medewerkers en oud-medewerkers van het mond-en-klauwzeer lab. Froukje, Klaas, Elma, Karin, Peter, Heleen, Edwin, Hans, Kor, Gilles, Yolanda, Tiny, Christine, Annemarie, Sylvia, Reina, Annelies en Brenda dank jullie wel voor al jullie werk, hand-en-spandiensten, ‘tips-van-de-dag’ (het valt niet mee, een veterinair op het lab opvoeden..) en vooral voor de gezelligheid op het lab!

Aline, dank je wel voor al je uitleg, hulp en geduld bij het modellenwerk, Elly dank voor je hulp bij de final size berekeningen en Joop, Bas en Willem bedankt voor de ondersteuning bij de statistiek.

Ook ben ik grote dank verschuldigd aan de dierverzorgers waarvan vooral Arie, Simon, Meindert, Roelof, Roel en Nico veel in ‘mijn’ proeven hebben gelopen, maar ook alle andere DBers bedankt voor al het werk in de stallen en de koffie ‘achter’.

Norbert, Ralph, Ad en Ad dank voor de hulp bij de secties.

Fred, Herma en Diny, bedankt voor de hulp bij de lay-out en het drukklaar maken van het manuscript.

Verder alle andere niet bij name genoemde collega’s van de HRW ‘binnen’ en ‘buiten’, collega’s van de EHW, familie, vrienden en kennissen dank voor jullie steun en belangstelling.

Dan het thuisfront. Reinder, jou kan ik niet genoeg bedanken voor je vertrouwen, inzet, hulp en aanmoedigingen. Lieve Sybren en Tjalle, dank jullie wel dat jullie nooit mopperden als mama weer eens ‘aan werk’ moest. Nu komt er weer meer tijd voor met z’n vieren!

En voor iedereen die op één of andere wijze een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift nogmaals: heel erg bedankt! Ik ben blij met het resultaat en ik hoop jullie ook!

Curriculum Vitae

Phaedra Lydia Eblé werd op 20 februari 1967 geboren te ’s-Gravenhage. In 1985 haalde zij het VWO-diploma en na een jaar studie Gezondheidswetenschappen aan de Rijksuniversiteit Leiden, begon zij in 1986 met de studie Diergeneeskunde aan de Rijksuniversiteit Utrecht. In oktober 1994 behaalde zij het dierenartsexamen. Na bijna twee jaar gewerkt te hebben als praktiserend dierenarts in verschillende gezelschapshuisdierenpraktijken, trad zij in september 1996 in dienst bij het DLO-instituut voor Dierhouderij en Diergezondheid (ID-DLO), het latere ID-Lelystad. De divisie Wettelijke Onderzoekstaken van ID-Lelystad verzelfstandigde in het Centraal Instituut voor DierziekteControle (CIDC-Lelystad), waar zij nu werkzaam is als wetenschappelijk medewerker bij de divisie Virologie. Binnen het instituut heeft zij gewerkt op achtereenvolgens het laboratorium voor klassieke varkenspest, bij het project kleine exoten en bij het project vesiculaire ziekten, waar het onderzoek beschreven in dit proefschrift werd uitgevoerd.

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