

## Change of Antibody Isotypes in FMD-Vaccinated Sows and Piglets

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### ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals. After the outbreak of serotype O1 FMD virus in Taiwan in 1997, pigs were extensively vaccinated. To investigate the development of antibody isotypes in response to FMD vaccine and the influence of maternally derived antibodies on the production of antibody isotypes in vaccinated piglets, sera were collected over a five month period from twenty vaccinated sows (from two farms) after they were boosted with the FMD vaccine. Sera were also collected from 18 one-month-old piglets from each before and after they were vaccinated. Piglets at farm A were vaccinated when they were three months old; those at farm B were vaccinated at both two and three months. As a control, serum samples were collected from 30 FMD virus-free pigs and 30 FMD vaccinated pigs without booster injection at two additional farms. We analyzed the FMD virus proteins, which contain the 66kD and 13kD antigens recognized by serum antibodies of vaccinated pigs, to find that the IgG1 antibody level increased significantly in vaccinated sows receiving booster injections. The IgG2 antibody was enhanced only in sows at farm A. The IgA levels at both farms were not significantly higher compared to sows without booster injections, but the IgM levels at farm A were constant, which was significantly higher than at farm B or among pigs with no booster. Pigs without booster injections had significantly higher IgG1, IgG2 and IgA levels than those of FMD virus-free pigs, but the IgM antibody levels did not differ. The maternally derived antibodies were IgG1 and IgG2. Serum IgG1 and IgG2 antibodies increased significantly only if piglets were immunized twice. However, the levels of IgM and IgA in piglets did not vary with frequency of vaccination.

**Key words:** antibody, isotype, foot-and mouth disease, vaccination, pig

### Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals, caused by an aphthovirus within the Picornaviridae family (Belsham, 1993). This disease is most commonly spread via aerosols, or contaminated animal products. Although FMD is not a significant zoonosis, it is economically important (Acha and Szyfres, 1987). It spreads rapidly and causes high morbidity, loss in production, and obstacles to the marketing of

livestock and animal products. The FMD virus (FMDV) has a single-stranded positive RNA molecule within an icosahedral capsid. There are seven antigenically distinct FMDV serotypes, identified as types A, O, C, SAT (South African Territories) 1, SAT 2, SAT 3 and Asia 1. Infection with a virus of one serotype does not confer immunity to another. Some FMDV strains show a marked affinity for one animal species. Strains that have caused serious outbreaks in swine have not greatly affected cattle, and other strains isolated from cattle have presented difficulties in

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experimental reproduction of the disease in swine (Acha and Szyfres, 1987).

Animals infected with FMDV develop a state of immunity characterized by high titers of virus serotype-specific neutralizing antibodies, which persist for at least 18 months (Cunliffe, 1964). In contrast to infection, inactivated virus vaccines elicit lower antibody responses for shorter periods (Morgan *et al.*, 1980). Thus, the outbreak of FMD may still occur in vaccinated animals (Gleeson *et al.*, 1995; Woolhouse *et al.*, 1996). Recovery from FMD and protection from reinfection are predominantly associated with the presence of circulating neutralizing antibodies (McCullough *et al.*, 1992). The carrier state, representing a reservoir of potential infection, is characterized by the asymptomatic low-level excretion of FMDV for periods that are species and virus strain-dependent (Gebauer *et al.*, 1988; Wittmann, 1990). As in other viral infections, cell-mediated immunity is believed to be important for the eventual elimination of FMDV infection (Salt, 1993; Sanz-Parra *et al.*, 1999).

In infected cattle, IgA and IgM antibodies are detected before the development of IgG antibody responses. The titers of IgG1 and IgG2 antibodies can be maintained in the sera for longer periods of time. The IgM antibody level is not enhanced in vaccinated cattle (Salt *et al.*, 1996). Although mice are not susceptible to natural infection, they may be experimentally infected. Infected mice show responses dominated by IgG2b, followed by IgG1, IgG2a and IgG3 14 and 60 days post-inoculation (Perez Filgueira *et al.*, 1995).

In countries where control of FMD relies predominantly on vaccination, young stock ingest specific anti-FMDV antibodies in the colostrum. Although maternally-derived antibodies provide immediate protection against infection, they also interfere with the development of active immunity following vaccination (Sadir *et al.*, 1988). Estimated half-lives of individual maternally-derived antibody isotypes for pigs are 2.1-3.0 days for IgA, 3.6-6.4 days for IgM and 6.6-22 days for IgG (Curtis and Bourne, 1973). It is evident that susceptibility to infection precedes the ability to respond to vaccination in the presence of maternal antibodies (Kitching and Salt, 1995).

After the outbreak of type O1 FMDV in Taiwan in 1997, pigs were extensively vaccinated. The

isotype patterns in the immune response are usually restricted to the immune system by the nature of the antigen presented (Balcovic *et al.*, 1987). Differences in isotype profiles may indicate different conditions of T-cell priming (Perez Filgueira *et al.*, 1995). Therefore, we investigate the development of antibody isotypes (IgM, IgG1, IgG2, IgA) in response to the FMD vaccine and the influence of maternal antibodies on the production of antibody isotypes in vaccinated piglets.

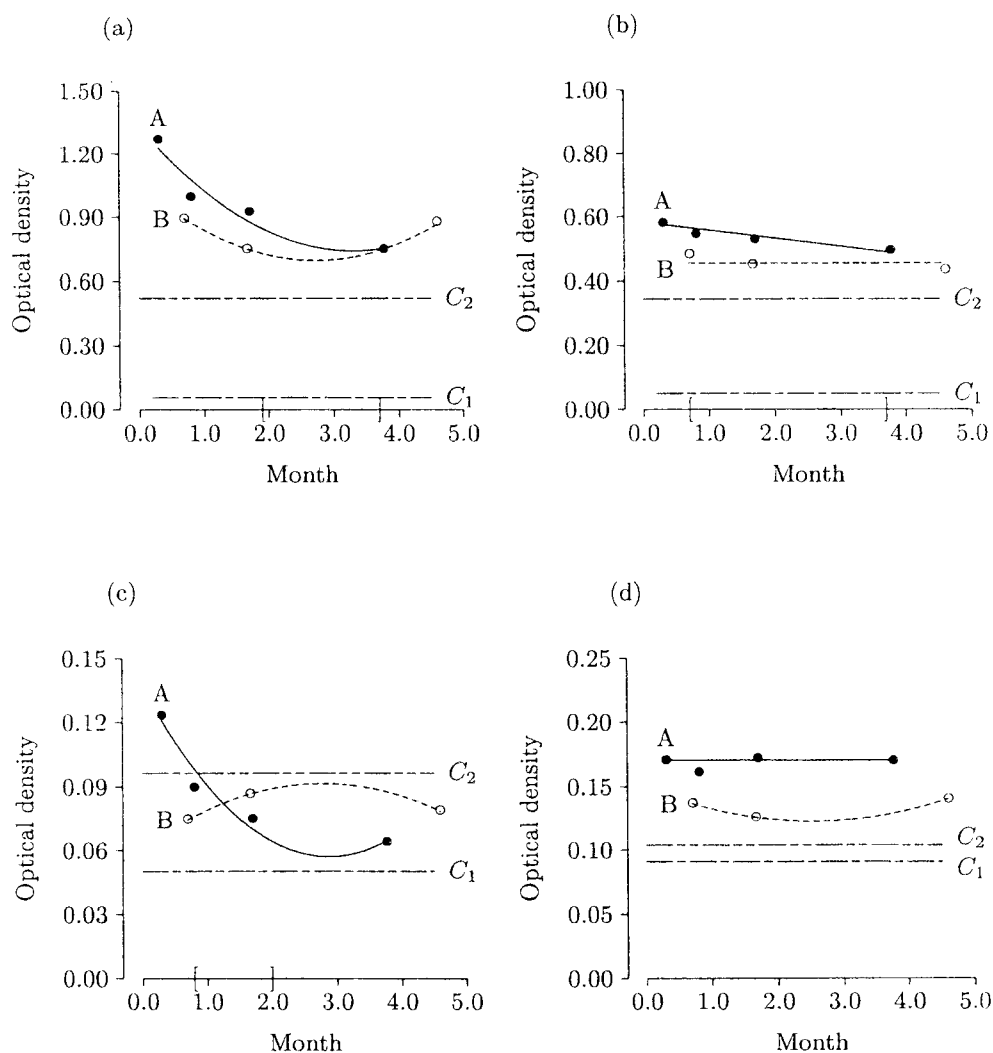
## Materials and Methods

### Swine sera collection

Thirty normal sera (C1) were collected from FMDV-free swine from Pig Research Institute in Miaoli County. Thirty serum samples (C2) were randomly collected from FMD-vaccinated pigs without booster injections at two pig farms in Taipei County. Sera were also collected from 10 FMD-vaccinated sows (10 each from farms A and B in Taichung County) at 10-day to three-month intervals for the five months after being boosted with the vaccine (Figure 1). In addition, sera were collected from 18 one-month piglets each from farms A and B at one to 1.8 month intervals (Figure 2). Piglets at farm A were vaccinated when they were three months old and those at farm B were vaccinated at both two and three months of age. All pigs were vaccinated subcutaneously with the AFTOPOR® FMD vaccine. All sera were collected by the year 2000.

### Characterization of FMDV proteins

The FMDV proteins were characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting assay using a minigel apparatus (Biometra Inc., Tampa, FL). All reagents used were purchased from Bio-Rad Lab (Richmond, CA). The antigen solution was mixed with reducing sample buffer and heated for 4 min at 100°C of which 15µl containing 12.5 µg of proteins or low molecular weight standards were applied to 12% sodium dodecyl sulphate-polyacrylamide gel and electrophoresed at 120 voltages for 90 min. The gel was fixed with 10% trichloroacetic acid + 40% methanol, stained with 0.1% Coomassie blue, and then de-stained with

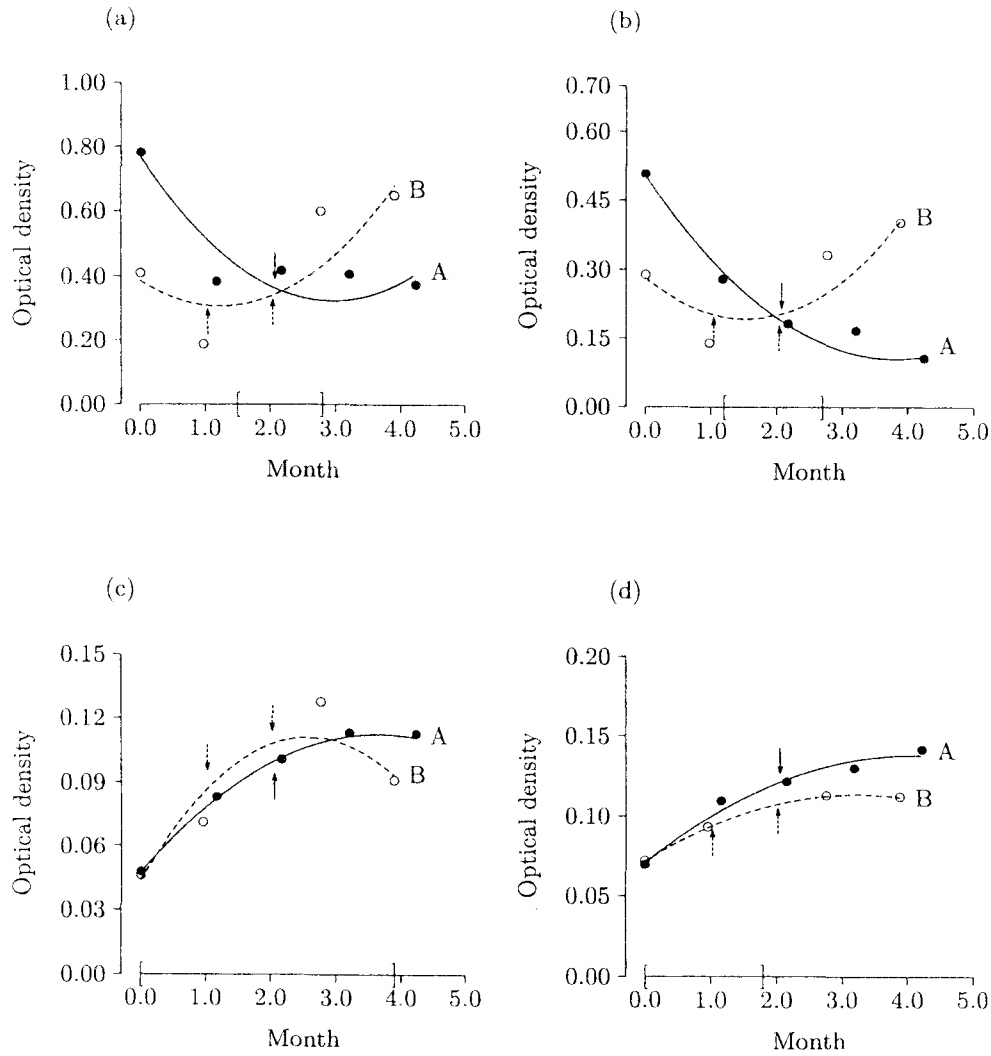


**Figure 1.** Optical density (490nm) response curves for four antibody isotypes for 10 sows at farm A (solid line) and 10 sows at farm B (dashed line) during the five months after receiving booster vaccinations: (a) antibody IgG1, (b) antibody IgG2, (c) antibody IgA, and (d) antibody IgM. Serum samples were collected at intervals of 10 days to three months. Intervals with non-significant response times are indicated by brackets ([ ]). Reference lines are for FMDV-free pigs (C<sub>1</sub>) and pigs without booster vaccination (C<sub>2</sub>). Symbols for farms A (●) and B (○) denote average antibody levels at the time the serum samples were taken.

40% methanol + 10% acetic acid.

For immunoblotting, the sodium dodecyl sulphate gel was transblotted onto a nitrocellulose paper (BA83, Schleicher & Schuell, Germany) using a blotting apparatus (Biometra Inc.). After blocking with 5% skim milk in phosphate-buffered saline overnight at 4°C, nitrocellulose paper was incubated with FMD-vaccinated pig sera diluted to 1:8 in 5% skim milk in phosphate-buffered saline with 0.05% Tween 20 and 1% Triton X-100 (Sigma Chemical Co., St. Louis,

MO) at room temperature for 1 hr. After washing, 50 µl of 1:5,000 goat anti-porcine IgG antibody conjugated with horse radish peroxidase (Serotec Ltd. Oxford, England) in 5% skim milk in phosphate-buffered saline with 0.05% Tween 20 and 1% Triton X-100 were added, followed by incubation at 37°C for 1 hr. The nitrocellulose paper was washed again and added to a substrate containing 4-chloro-1-naphthol (Sigma Chemical Co.) and H<sub>2</sub>O<sub>2</sub>. Positive result was indicated by the appearance of color bands.



**Figure 2.** Optical density (490nm) response curves for four antibody isotypes for 18 piglets at farm A (solid line) and 18 piglets at farm B (dashed line): (a) antibody IgG1, (b) antibody IgG2, (c) antibody IgA, and (d) antibody IgM. Serum samples were collected at the intervals of one to 1.8 months. Piglets at farm A were injected with foot and mouth disease vaccine when they were three months old and those at farm B were vaccinated at both two and three months of age. Intervals with non-significant response times are indicated by brackets ([ ]). Symbols for farms A (●) and B (○) denote average antibody levels at the time the serum samples were taken. Solid arrows ↓↓ and dashed arrow ↓↑ denote the time of piglet vaccinations at farms A and B, respectively.

#### Enzyme-linked immunosorbent assay for antibody isotype detection

For following experiments, reactant liquids were adjusted to room temperature before use and MaxiSorp™ plates (Nunc, Roskilde, Denmark) were placed in a humid chamber for incubation. These procedures were carried out to minimize the 'edge' effect (Oliver *et al.*, 1981). Fifty  $\mu$ l of FMDV antigen, 20  $\mu$ g/ml in 0.1 M bicarbonate buffer pH 9.6, were placed in each well. The plate was then incubated overnight at 4°C and then

washed with phosphate-buffered saline containing 0.05% Tween 20 by enzyme-linked immunosorbent washer (Dynatech Lab., Chantilly, VA). One hundred  $\mu$ l of phosphate-buffered saline containing 0.05% Tween 20 with 3% skim milk were added as a blocking solution. After 1 hr of incubation at 37°C, the plate was washed again. Fifty  $\mu$ l of 1:8 diluted sera in phosphate-buffered saline containing 0.05% Tween 20 with 3% skim milk were added to the wells, followed by incubation at 37°C for 1 hr. After washing, 50  $\mu$ l

**Table 1.** The optical density ( $\hat{y}$ ) response curve of four antibody isotypes found in 10 sows, each, from farm A and farm B in Taiwan at intervals of 10 days to three months ( $t$ ) after being given booster vaccinations for foot and mouth disease. The estimated variance ( $\hat{\sigma}^2$ ) for each observation  $y_{ij}$  and the estimated correlation parameter ( $\hat{\rho}$ ) between two observations of the same subject ( $Corr(y_{i1}, y_{i2}) = \rho^{t_1 - t_2}$ , where  $t_1$  and  $t_2$  are the time points that  $y_{i1}$  and  $y_{i2}$  are observed) are also indicated.

Antibody isotype	Farm	Response curve	$\hat{\sigma}^2$	$\hat{\rho}$
IgG1	A	$\hat{y} = 1.3337 - 0.3616t + 0.0551t^2$	0.0229	0.0247
	B	$\hat{y} = 1.0534 - 0.2643t + 0.0492t^2$	0.0179	0.8104
IgG2	A	$\hat{y} = 0.5834 - 0.0255t$	0.0102	0.7584
	B	$\hat{y} = 0.4555$	0.0410	0.8455
IgA	A	$\hat{y} = 0.1364 - 0.0553t + 0.0096t^2$	0.0009	0.1615
	B	$\hat{y} = 0.0613 - 0.0218t + 0.0039t^2$	0.0009	0.8953
IgM	A	$\hat{y} = 0.1688$	0.0015	0.6530
	B	$\hat{y} = 0.1492 - 0.0208t + 0.0041t^2$	0.0008	0.8181

of 1:500 various mouse monoclonal IgG1 antibodies to porcine IgM, IgA, IgG1, or IgG2 (Serotec Ltd.) were added, followed by incubation at 37°C for 1 hr. After being washed again, 50  $\mu$ l of 1:8,000 affinity purified peroxidase-labeled sheep IgG anti-mouse IgG1 (Serotec Ltd.) were added. The plate was incubated at 37°C for 1 hr, washed, and finally rinsed with distilled water. One hundred  $\mu$ l of the substrate, containing *O*-phenylenediamine (Sigma Chemical Co.) and  $H_2O_2$ , were added and the plate was left in the dark at room temperature for 15 min. The reaction was stopped by 50  $\mu$ l of 1 N HCl. The plate was read at 490 nm by a microplate reader (Dynatech Lab.).

### Statistical analysis

Since three or more serum samples were taken from each pig at different times during the five months investigation period, the data are not independent but represent repeated measures. Therefore, correlation between consecutive measurements should be considered. Statistical analysis was done using a repeated measures analysis of variance (SAS Institute Inc., 1999). The response of each antibody isotype was modeled by a second order polynomial:  $y_{ij} = \beta_0 + \beta_1 t_{ij} + \beta_2 t_{ij}^2 + \varepsilon_{ij}$  where  $y_{ij}$  is the observed optical density of  $i$ th pig at  $j$ th time point,

$t_{ij}$  is the time point (month) and  $\varepsilon_{ij}$  is the random error term associated with the  $j$ th serum sample of the  $i$ th pig. If a higher order term was not significant then a model of lower order term was adopted. For example if the coefficient of quadratic term  $\beta_2$  did not differ significantly from zero then a linear model ( $y_{ij} = \beta_0 + \beta_1 t_{ij} + \varepsilon_{ij}$ ) was adopted. We assumed that the random error  $\varepsilon_{ij}$  was normally distributed with mean at zero, variance ( $\sigma^2$ ) and correlation ( $Corr(y_{ij}, y_{ij'}) = \rho^{t_{ij} - t_{ij'}}$ ).

### Results

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of FMDV proteins revealed two significant bands (66kD and 13kD) recognized by the serum antibodies of vaccinated pigs. The response curves of IgG1, IgG2, IgA, and IgM antibodies of sows at farms A and B are shown in Figure 1 and Table 1. The IgG1 antibody increased dramatically in sows receiving booster injections compared to those without booster injections (Table 2). The IgG2 antibody was significantly enhanced only in sows at farm A. Except at farm A, nine days after booster vaccinations, the levels of IgA were not significantly higher for sows receiving booster

**Table 2.** Comparisons of the optical density response curves for four antibody isotypes for foot and mouth disease in Taiwan for four different adult pig groups (sows): farm A, farm B, foot and mouth virus-free (C<sub>1</sub>) and non-booster injected (C<sub>2</sub>). Serum samples were collected at the intervals of 10 days to three months. Significant differences are indicated for the entire five-month test period (—), and non-significant differences by the time interval ( $[x_1, x_2]$ ) and for the entire period (ns). For piglets, comparisons are made only between farms A and B. Serum samples were collected at the intervals of one to 1.8 months. Piglets at farm A were injected with foot and mouth disease vaccine when they were three months old and those at farm B were vaccinated at both two and three months of age.

Age Group	Comparison	Antibody isotype			
		IgG1	IgG2	IgA	IgM
Sows	A vs. B	[1.9, 3.7]	[0.7, 3.7]	[0.8, 2.0]	—
	A vs. C <sub>1</sub>	—	—	[2.0, 4.6]	—
	A vs. C <sub>2</sub>	—	—	[0.3, 1.9]	—
	B vs. C <sub>1</sub>	—	—	—	—
	B vs. C <sub>2</sub>	—	[0.7, 3.7]	[0.7, 4.6]	[1.8, 3.4]
	C <sub>1</sub> vs. C <sub>2</sub>	—	—	—	ns
Piglet	A vs. B	[1.5, 2.8]	[1.2, 2.7]	[0.0, 3.9]	[0.0, 1.8]

vaccinations. Interestingly, the IgM antibody in sows at farm A was maintained at a constant level throughout the study period, a level significantly higher than for sows at farm B or among sows without booster injections (Figure 1). The sows without booster vaccinations had IgG1, IgG2 and IgA antibody levels significantly higher than those of FMDV-free pigs, but the IgM antibody, did not vary between these two groups (Tables 2 and 3).

In piglets, the antibody response curves are shown in Figure 2 and Table 4. The major maternal antibodies were IgG1 and IgG2. Piglets at farm A had higher maternal IgG1 and IgG2 antibody levels than those at farm B. Serum IgG1 and IgG2 antibodies were significantly increased only if piglets (farm B) were immunized twice. The levels of IgM and IgA did not vary between piglets vaccinated once or twice.

## Discussion

Foot-and-Mouth Disease is an extremely contagious disease, with a respiratory infection of <10 units initiating the disease (Acha and Szyfres, 1987). In Taiwan, infected pigs were slaughtered and those showing no signs of FMDV infection were vaccinated. Although vaccination can offer animals some degree of protection against FMDV challenge (Salt *et al.*, 1998; Sanz-Parra *et al.*, 1999; Mayr *et al.*, 2001), there are two disadvantages associated with vaccination (Doel *et al.*, 1994). Firstly, all vaccinated animals will be seropositive for FMD and cannot be readily distinguished from animals that have recovered from the infection. Secondly, the FMDV is able to persist in animals (the carrier state) regardless of whether or not they were vaccinated prior to

**Table 3.** Mean and standard error of optical density response curves for four antibody isotypes for foot and mouth disease in Taiwan for foot and mouth disease virus-free (C<sub>1</sub>) and non-booster injected (C<sub>2</sub>) pigs. Each entry is computed from a sample of 30 observations.

Group	Statistic	Antibody isotype			
		IgG1	IgG2	IgA	IgM
C <sub>1</sub>	Mean	0.0581	0.0506	0.0501	0.0909
	SE	0.0016	0.0010	0.0011	0.0050
C <sub>2</sub>	Mean	0.5210	0.3436	0.0965	0.1041
	SE	0.0288	0.0282	0.0129	0.0063

**Table 4.** The optical density ( $\hat{y}$ ) response curve of four antibody isotypes found in 18 piglets each, from farm A and farm B in Taiwan. Serum samples were collected at the intervals of one to 1.8 months ( $t$ ). Piglets at farm A were injected with foot and mouth disease vaccine when they were three months old and those at farm B were vaccinated at both two and three months of age. The estimated of variance ( $\hat{\sigma}^2$ ) of each observation  $y_{ij}$  and the estimated correlation parameter ( $\hat{\rho}$ ) between two observations of the same subject ( $Corr(y_{i_1}, y_{i_2}) = \rho^{h_1 - h_2}$ , where  $t_1$  and  $t_2$  are the time points that  $y_{i_1}$  and  $y_{i_2}$  are observed) are also indicated

Antibody isotype	Farm	Response curve	$\hat{\sigma}^2$	$\hat{\rho}$
IgG1	A	$\hat{y} = 0.7701 - 0.3027t + 0.0510t^2$	0.0241	0.4660
	B	$\hat{y} = 0.3804 - 0.1217t + 0.0515t^2$	0.0449	0.3001
IgG2	A	$\hat{y} = 0.5030 - 0.2109t + 0.0278t^2$	0.0302	0.6138
	B	$\hat{y} = 0.2808 - 0.1152t + 0.0380t^2$	0.0185	0.5098
IgA	A	$\hat{y} = 0.0476 + 0.0355t - 0.0049t^2$	0.0023	0.6105
	B	$\hat{y} = 0.0413 + 0.0537t - 0.0101t^2$	0.0009	0.0531
IgM	A	$\hat{y} = 0.0709 + 0.0330t - 0.0040t^2$	0.0003	-0.0032
	B	$\hat{y} = 0.0720 + 0.0258t - 0.0040t^2$	0.0004	-0.0040

infection. Different antibody isotypes are produced in response to vaccination; and maternally-derived antibodies can interfere antibody production in the piglets (Francis and Black, 1986). In infected and vaccinated cattle (Mulcahy *et al.*, 1990; Salt *et al.*, 1996) and models using mice (Perez Filgueira *et al.*, 1995), the IgG class dominated the antibody response. This was also the case for all sows receiving booster vaccinations. In addition, the IgG1 titers tended to dominate IgG2. The IgG1 and IgG2 antibodies are the most common antibody isotypes produced when porcine lymphocytes are stimulated in vitro with recombinant FMDV polypeptides (Rodriguez *et al.*, 1995). Furthermore, animal studies have indicated that both FMDV-specific IgG1 and IgG2 antibodies are enhanced in FMD-vaccinated swine (Mayr *et al.*, 2001) and cattle (Perez Filgueira *et al.*, 1999; Sadir *et al.*, 1999). An independent study suggested that the specific serum IgG1, but not IgG2, isotype response of vaccinated animals correlates almost linearly with their capacity to pass the challenge of FMDV (Capozzo *et al.*, 1997).

Salt *et al.* (1996) reported that the IgM antibody was not detected in vaccinated cattle. We found that pigs without booster vaccinations had the same IgM levels as FMDV-free pigs and farm B pigs with booster vaccinations. However, IgM

antibody levels of sows at farm A were higher than for pigs at farm B and pigs without booster vaccinations. The reason for this difference is not known. Booster vaccination did not significantly influence IgA antibody production.

Factors such as age and maternal antibodies have been associated with diminished response to FMD vaccination in newborn animals (Sadir *et al.*, 1988). Interestingly, calves 20 days old or older with high maternal antibody titers actually respond well to vaccination (Spath *et al.*, 1995). In piglets born to FMD vaccinated sows, maternally derived antibodies had a suppressive effect on vaccination response. This suppression, which was correlated with the titer of maternally derived antibodies present in the piglets at the time of vaccination, was complete in animals less than one-month old and partial in two-month-old piglets (Francis and Black, 1986). In one-month-old piglets, the major maternal antibodies were IgG1 and IgG2. The IgG1 and IgG2 antibody responses significantly increased only if piglets were vaccinated twice: at the ages of two and three months. Apparently, the time and frequency of vaccination greatly influences the antibody response in piglets. However, the levels of IgM and IgA antibodies in piglets were not increased by multiple vaccinations.

In conclusion, serum antibody isotypes vary among sows with or without booster vaccination

and in vaccinated piglets. Study of the antibody isotypes developed in response to infection or immunization is important when considering the mechanisms of *in vivo* protection. Further studies are required to clarify the relationship between isotypes of induced antibodies and protection against FMD.

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## 抗體 isotypes 在口蹄疫疫苗注射母豬及仔豬的變化

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### 摘 要

口蹄疫為偶蹄類之一高度傳染病。臺灣於 1997 年爆發 O1 型口蹄疫病毒之後，所有豬隻皆注射口蹄疫疫苗。本研究乃要探討抗體 isotypes 在口蹄疫疫苗注射豬隻的變化以及母體移行抗體對疫苗注射仔豬之抗體 isotypes 產生的影響。為了探討抗體變化情形，在五個月期間於不同時間點採集二十頭疫苗補強注射過豬隻 (A 及 B 場各十頭) 血清。同時自 A 及 B 場各十八頭一月齡仔豬在疫苗注射之前與之後收集血清。A 場仔豬在三月齡時接受疫苗注射，而 B 場仔豬共接受兩次疫苗注射分別於二及三月齡時。對照組血清樣品乃採自三十頭 specific pathogen-free 豬隻，以及自兩場三十頭注射過口蹄疫疫苗但尚未補強豬隻。使用口蹄疫病毒蛋白質 (含有可被疫苗注射過豬隻血清抗體認識的 13 kD 及 66 kD 抗原) 於本試驗。結果發現，接受疫苗注射且有補強豬隻，其 IgG1 抗體量比沒補強注射者顯著增加。IgG2 抗體只在 A 場豬隻有上升情形。和沒補強注射豬隻比較，IgA 量在 A、B 兩場並沒顯著增加。A 場豬隻 IgM 抗體維持一常值，且顯著比 B 場和沒補強注射豬隻高。沒補強注射豬隻比 specific pathogen-free 豬隻有較高的 IgG1, IgG2 及 IgA 量；而 IgM 抗體在此兩組並沒顯著差異。主要的母體移行抗體為 IgG1 和 IgG2。血清 IgG1 及 IgG2 抗體只在接受兩次疫苗注射之仔豬顯著增加。然而，IgM 和 IgA 量在接受一次或兩次疫苗注射之仔豬並沒顯著不同。

**關鍵詞：**抗體，Isotype，口蹄疫，疫苗注射，豬