Repeated administration of maximum payload emergency vaccines made from inactivated purified antigen concentrates do not induce significant titres of antibodies against non-structural proteins of Foot-and-Mouth disease virus

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Introduction
At the present time, there is not an officially (Office International des Epizooties) prescribed procedure for the routine discrimination of animals which have been infected with foot and mouth disease virus (and have otherwise fully recovered) from those which have received vaccination only. The official serological methods measure only antibodies against the structural proteins of the virus and, under normal circumstances, where there is no supporting clinical picture or other evidence, are unable to differentiate between those antibodies induced by vaccine or previous exposure to live virus. All of this has significant implications for the export of livestock or livestock products from countries which are either free of FMD with vaccination or decide to use vaccine as a control measure for a recent introduction of the disease.

The deficiency with the officially prescribed serological methods led to the examination of other techniques to discriminate vaccinated from infected animals and considerable work has been done over several decades to quantify antibodies against the so-called non-structural (NS) proteins of the virus because these would be particularly prevalent following virus infection. The NS proteins are coded by the FMDV genome and are involved in the replication of the virus within the host cell.

The earliest work concentrated on the VIAA antigen which contains the 3D NS protein (RNA polymerase) of the virus and was for many years incorrectly considered as an indicator of a past virus infection, either as a consequence of field challenge or improperly inactivated vaccine. However, Pinto and Garland (1979) showed that fully inactivated FMDV vaccines induced antibodies against VIAA but their data was to some extent overlooked until relatively recently when studies by a number of groups essentially dismissed VIAA as an exclusive indicator of infection (Bergmann et al, 1993, Mackay, 1998).

Vaccines made from relatively impure but fully inactivated FMDV antigens contain large quantities of some of the NS proteins and, in particular, VIAA (3D) antigens. Indeed, even the purified FMD virus particle contains small amounts of the 3D protein integrated into the viral capsid and absolute purity from this protein is not possible (Newman and Brown, 1997).
Because of the limitations of the assays based on VIAA (3D), more recent studies have focussed on techniques such as immunoblotting or ELISA with other NS proteins, expressed in recombinant systems, and have demonstrated that titres of antibodies against other NS proteins are much more valuable indicators of previous vaccination or infection (Bergmann et al, 1993; Lubroth and Brown, 1995; Mackay et al, 1998).

Thus, research workers and diagnosticians within the public domain are in a substantially stronger position in terms of discriminating between vaccinated and previously infected animals and, indeed, an ELISA based version of the procedure described in this paper and developed by Bergmann and colleagues is now used quite extensively in South America. The success of the public sector in this regard places a responsibility on the commercial FMD vaccine production sector to produce higher purity vaccines which will enable even more the capability of NS protein diagnostic kits to discriminate the infected from the vaccinated animal. In this respect, the focus of Merial has been the development of FMD vaccines made from highly purified antigens such that the induction of antibodies to non-structural proteins is abolished.

One of the most critical scenarios where reliable discrimination would be invaluable would be that of a country, previously free of FMD, and needing to use vaccine to prevent the spread of a new incursion of the disease. Under these circumstances, the unfortunate country would probably use one or two doses of high potency vaccines to limit the spread of the disease from the initial focus(i) and would be subsequently faced with the need to know the clinical status of vaccinated cattle which had apparently resisted infection but could be ‘carrier’ animals. The persistence of FMDV in the upper respiratory tract of otherwise healthy and immune ruminants is a well known phenomenon.

With this scenario in mind, we decided to repeatedly vaccinate cattle with extremely high payloads of purified FMD antigens greatly exceeding those which would be prepared for emergency banks such as the European Union FMD Antigen Bank. While ‘ring’ or zonal vaccination would only be used once or at most twice during an eradication campaign we chose a worst case approach of three rounds of vaccination. Although pigs do not become persistently infected with FMD, we also examined sera from animals given repeated doses of high potency vaccine.

Sera were prepared from the vaccinated cattle at frequent intervals and subjected to analysis of antibodies against the NS proteins using the procedure described by Bergmann et al (1993).

Materials and Methods

**Vaccine and Vaccinations.** Vaccines were prepared from chromatography purified inactivated antigens of 4 strains of FMDV (Asia1 Shamir, A22 Iraq, O Manisa, C Philippines) using aluminium hydroxide and saponin or mineral oil (double oil emulsion, DOE) as adjuvants. Each 2ml cattle dose of vaccine contained 16 µg of each of the 4 strains. Thus, a single dose of vaccine contained 64 µg of purified FMDV antigens. Groups of cattle were vaccinated by the subcutaneous route in the
case of aqueous vaccine or the intramuscular route in the case of oily vaccine with initially either one dose or two doses. All animals were boosted with a single dose at 21 days and 42 days after the initial vaccinations. Serum samples were taken at regular intervals. All of the cattle were shown to be seronegative for FMDV prior to the start of the experiment and had never been vaccinated with FMD vaccine or exposed to FMD virus.

**EITB test.** Essentially, this was as described by Bergmann et al (1993). Briefly, purified recombinant NS proteins, 3A, 3B, 2C, 3D, 3ABC of FMDV were mixed, electrophoretically resolved using 12.5% SDS PAGE and the protein bands electrophoretically transferred to nitrocellulose sheets. Strips of the nitrocellulose sheets were soaked in buffer containing 5% non-fat dry milk to block non-specific adsorption of antibodies and then immersed in 1/200 dilutions of test and control sera, the latter representing positive, weakly positive, cut-off and negative serum samples. After incubation, the strips were washed and bovine or porcine antibodies specifically bound to the recombinant NS proteins detected by alkaline phosphatase-conjugated rabbit anti-bovine or anti-porcine antibodies. After further washing to remove unbound enzyme conjugate, the presence of bovine or porcine antibodies bound to NS proteins was determined by alkaline phosphatase substrate colour development. A serum sample was considered negative if all the NS bands were less than the reactivity of the cut-off control (negative control) or no more than 2 bands were greater than the reactivity of the cut-off control. A serum was considered positive if all 5 NS bands had a reactivity equal or greater than the cut-off control. A sample was deemed indeterminate if the criterion for negativity or positivity was not met.

**Virus Neutralisation Test.** The titres of virus neutralising antibodies in the serum samples were determined in a microneutralisation test in IB-RS2 cells (renal swine). Titres were expressed as the log_{10} of the reciprocal of the serum dilution which neutralised 50% of the infectivity of 100 TCID_{50} of each homologous test virus.

**Results**
The figures (Fig. 1 and 2) show the summarised data from the EITB test analysis of the sera of cattle vaccinated with aluminium hydroxide and saponin adjuvanted vaccine. To facilitate interpretation, we used the following ‘positivity’ scale to allow graphical presentation: Where there was no evidence of antibody against a given NS protein, we scored −1.0; for NS protein bands slightly below or above the cut-off serum controls, we scored +0.1; for NS protein bands significantly above the cut-off serum controls, we scored +1.0. In the figures shown here, a negative serum sample is indicated by ‘neg’ and one serum sample, which was indeterminate, is indicated by ‘ind’.

It can be seen that after 1 administration of a single or double dose of aluminium hydroxide and saponin vaccine there is no evidence of antibody induction against non-structural proteins of the virus and even after the first booster vaccination, only one animal in the double dose group showed evidence of antibody against 3D protein. After the third vaccination of the two groups there was some boosting of the anti 3D response but essentially no reactivity in any of the cattle sera against the other non-structural proteins.
Essentially identical data was observed with the oil adjuvanted vaccines in cattle as shown by Fig. 3 which is the equivalent experiment to that shown in Fig.1. After three doses of vaccine, two animals did show low levels of antibodies against the 3B protein although the overall scoring was still negative.

It is important to stress that these results are all the more significant when the payload of the vaccine used is considered. Whereas conventional monovalent vaccines made at Pirbright contain between 2 and 10 µg of 146S particles per dose, the quantity depending on the strain, the vaccines used in the present study contained 64 µg of 146S particles per dose and two groups of cattle received twice this concentration at day 0. That is, with the double dose initial group, each animal received the approximate equivalent of 20 monovalent cattle doses at time 0, and 10 monovalent cattle doses at days 21 and 42. Such antigen payloads are considerably greater than would be used even for high potency emergency vaccines and provide a considerable safety margin in the interpretation of the data. Fig. 4 and 5 show the mean virus neutralising antibody titres of the cattle used in the aqueous vaccine experiments (Fig. 1 and 2) and illustrate the very high potency of the vaccines in the current study. Similar data was obtained with the oil adjuvanted vaccines in cattle.

The minimal NS antibody responses of cattle administered high concentrations of purified FMD antigens contrasts with the EITB results seen with non-vaccinated control cattle following live virus challenge. Two of the lanes in Fig. 6 show high levels of antibodies against all of the NS proteins of the virus and correspond to two cattle challenged 8 days previously by intradermolingual injection of A24 Cruzeiro strain of FMD. Six of the lanes immediately above the two control animals correspond to cattle which had been vaccinated twice, each time with approximately 16 µg of A24 viral antigens in aluminium hydroxide/saponin adjuvant, followed by A24 challenge. Eight days after the vaccination, it is clear that the vaccinated animals were completely negative in terms of antibodies against NS proteins. This was supported by the virus neutralising antibody titres which indicated that the immune response to the A24 vaccine was not boosted by the live virus challenge (results not shown).

We have also examined the induction of neutralising antibodies against NS proteins based on more conventional vaccines and vaccination regimens. Sera taken from cattle vaccinated at day 0, day 56 and day 238 with an aqueous vaccine containing 4 µg of 146S particles of each of 4 vaccine strains showed no evidence of antibodies against NS proteins, except for a very weak response to the 3D protein, and were considered to be negative according to the criteria described in this paper (results not shown).

**Conclusions**

The antigenic payloads of the vaccines prepared for the present study greatly exceeded those of vaccines routinely made at Pirbright and were also substantially greater (five to ten fold) than those used for high potency emergency vaccines typical of antigen banks. Furthermore, each vaccine contained 4 strains of FMD virus, all of which would potentially contribute to non-structural antibody responses because of the high level of sequence conservation among the non-structural proteins of the different serotypes (in contrast to the antigenic and sequence diversity among the
structural proteins of the viruses). Clearly, emergency vaccines such as those used recently in South East Asia (South Korea) are exceptionally unlikely to be anything other than monovalent.

Given the repeated use and the very high payloads of antigens within the vaccines, it is noteworthy that the antibody responses to the non-structural proteins of the FMD virus are primarily confined to the 3D (VIAA) protein which, it is widely accepted, is no longer indicative of virus replication without the presence of significant titres against other non-structural proteins of the virus. None of the EITB profiles indicated a positive status for cattle given high potency vaccines in aluminium hydroxide/saponin vaccines (cattle) or oil (DOE) adjuvants. It is concluded that repeated application of high potency, high purity vaccines in an emergency situation would not be expected to induce antibodies against non-structural proteins of the virus other, possibly, than the 3D protein and would not therefore confuse the status of the herd in terms of discrimination between infected and vaccinated animals. This conclusion is further supported by the absence of significant antibody titres against non-structural proteins in serum samples from cattle repeatedly vaccinated with more conventional payloads of 146S particles.

There is also evidence that the use of a high potency monovalent vaccine prevents replication of the virus following challenge and is consistent with a previous report from Doel et al (1994) that high potency vaccines can reduce or even prevent the establishment of the carrier state.

References


OIE Manual FMD Monograph, 2000