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# Early protection against foot-and-mouth disease virus in cattle using an inactivated vaccine formulated with Montanide ESSAI IMS D 12802 VG PR adjuvant

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SUMMARY

Foot and mouth disease is an acute disease of cattle with a broad distribution around the world. Due to the fast spread of FMDV infections, control measures must be applied immediately after an outbreak, such as the use of vaccines that induce fast protection. Previously, it was shown that mice vaccinated with FMD inactivated virus (iFMDV) formulated with Montanide™ ESSAI IMS D 12802 VG PR adjuvant (802-iFMDV) were protected when they were challenged 4 and 7 days post-vaccination (dpv) with homologous virus.

In this work, we describe the successful use of this formulation in cattle. In addition, adjuvant Montanide™ IMS 1313 VG NPR was also tested. 802-iFMDV vaccine was able to confer 100% protection against viral challenge at 4 and 7 dpv, while eliciting low antibody levels, at 7 dpv. 1313-iFMDV vaccine induced protection in 60% of cattle.

At 4 dpv, 1313-iFMDV vaccinated animals presented increased levels of IFNγ but not of macrophages. At 4 and 7 dpv, macrophages, IFNγ, nasal IgA and IgG1 antibodies against FMDV, and opsonophagocytosis were increased in animals vaccinated with 802-iFMDV indicating that these phenomena could be involved in protection.It is the first time that total protection against FMDV at early stages post-vaccination is reported using a single dose of the formulation iFMDV plus Montanide™ ESSAI D IMS 12802 VG PR adjuvant.



## 1. Introduction

Foot and mouth disease (FMD) is a cloven hoofed disease that occurs in most of the world [1]. The economical losses of infection with foot and mouth disease virus (FMDV) in bovine and swine are due to physical and productive deterioration rather than mortality [2]. Nevertheless, for countries exporting animals and animal products, the most relevant economical impact is connected with restrictions on international trade.

Considerable efforts have been applied to the development of vaccines capable of reducing the time between vaccination and the elicitation of a protective immune response. Until now, vaccines formulated with inactive virus plus adjuvant, have shown to require at least 7 days to produce a complete protective response [3].

Montanide ESSAI IMS are aqueous adjuvants containing liquid particles varying in size between 50 and 500 nm and an immunostimulating compound listed as a GRAS substance (Generally Recognized As Safe). Recently, we reported that inactivated FMDV (iFMDV) plus Montanide ESSAI IMS D 12802 VG PR, rises protection against viral challenge in the murine model [4]. Another aqueous adjuvant, Montanide™ IMS 1313 VG NPR, yielded increased protection against viral challenge when it was incorporated into an experimental FMD vaccine, in the same murine model [5]. These results indicate that adjuvants ESSAI IMS D 12802 VG PR and IMS 1313 VG NPR could be adequate for being tested at early times post-vaccination in cattle.

The present work was designed to assess the efficacy of ESSAI IMS D 12802 VG PR and IMS 1313 VG NPR adjuvants in combination with inactivated antigen (iFMDV) in eliciting protection against

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viral challenge and to study the immune response involved in early protection in cattle. Our data suggest that specific antibodies (Abs) and monocytes could be associated with protection. This is the first time that complete protection is reported at early stages post-vaccination by using inactivated antigen plus Montanide™ ESSAI D IMS 12802 VG PR.

## 2. Materials and methods

### 2.1. Animals

1–2 year-old bovines ($n = 29$) and 14 regular reared pigs (12 kg ± 2 kg) serologically negative for FMDV, were used. Of the total number of pigs, eight were used as donors for the viral challenge and six as positive unvaccinated controls for infection. Experiments with animals were performed according to INTA Ethics Manual "*Guide for the use and care of experimental animals*".

### 2.2. Virus

Binary ethylene-imine (BEI)-inactivated and polyethylene glycol (PEG)-concentrated virus, O1 Campos strain, was used for vaccine formulation and ELISA. For challenge and virus neutralization assays, infective virus from the same serotype (kindly donated by the Argentine National Service of Animal Health, SENASA) was used. All the experiments involving infectious virus were performed in the BSL 3A SENASA facilities and BSL 3A boxes at INTA.

### 2.3. Vaccines

The adjuvants used in this study were Montanide™ ESSAI IMS D 12802 VG PR (named as 802) and Montanide™ IMS 1313 VG NPR (named as 1313) (Seppic, France). Adjuvants were combined with 20 μg/dose of inactivated antigen (iFMDV), defining three vaccines: 802 plus inactivated antigen (802-iFMDV), 1313 plus inactivated antigen (1313-iFMDV) and inactivated antigen formulated in PBS (iFMDV). Formulations were prepared following Seppic's indications. Briefly adjuvant and virus suspension were mixed 1:1 under moderate agitation during 5 min, using a magnetic stirrer. A commercial vaccine (Bioaftogen®) was provided by Biogénesis Bagó (Argentina) and consisted in a water-in-oil single emulsion, containing 4 FMDV strains (A Arg 2000, A Arg 2001, A24 Cruzeiro and O1 Campos). This vaccine has been approved by SENASA with more than 75% of expected percentage of protection against all vaccine strains (OIE guide [6]).

### 2.4. Immunization

Bovines were vaccinated intramuscularly (i.m.) in the left rear quarter with 2 ml of: 802-iFMDV ($n = 10$), 1313-iFMDV ($n = 10$), iFMDV ($n = 3$) or commercial vaccine ($n = 5$). Five animals in groups 802-iFMDV and 1313-iFMDV were challenged at 4 dpv and the rest at 7 dpv. Animals in group iFMDV and commercial vaccine were challenged at 7 dpv. Control unvaccinated bovine ($n = 1$) and pigs ($n = 6$) were inoculated with phosphate-buffered saline (PBS).

### 2.5. Infections

Pigs ($n = 8$) were used as the source of virus for aerosol challenge of cattle. Pigs were inoculated intradermally into the heel bulb with $10^6$ $TCID_{50}$/ml infective FMDV O1 Campos in 0.5 ml PBS. At 72 h post-inoculation, they displayed clinical signs of FMD and were put in contact with bovines. Vaccinated cattle were divided into the boxes so that there were two infected and two unvaccinated control pigs for every 5–7 vaccinated cattle. Contact was maintained for 4 h and the animals were circulated around the room every 15 min in order to ensure that all animals were exposed to the virus by breathing, contact or both. After challenge, infected donors pigs used to challenge cattle, were euthanized and control unvaccinated pigs were kept separate until the end of the assay. According to OIE guide [6], lesions typical of the disease on tongue and feet were recorded at 7 days post-challenge (dpc). Those bovines which had no vesicles until 11 dpc were considered protected.

### 2.6. Measurement of anti FMDV antibodies

Serum samples were examined for anti-FMDV neutralizing antibodies as described before [30]. Briefly, serial dilutions of inactivated sera were incubated for 1 h at 37 °C with 100 $TCID_{50}$ of infective FMDV. Then virus-serum mixtures were seeded on BHK-1 monolayers. After 40 min at 37 °C, fresh MEM-D/2% fetal calf serum (FCS) was added to the monolayers, which were incubated at 37 °C, under 5% $CO_2$. Cytopathic effects were observed after 48 h.

For total Abs measurement, sandwich ELISAs were performed as described before [4] with minor modifications. These modifications include the use of (HRP)-conjugated anti-bovine Ig (KPL, USA) for total Ig measurement and biotin-conjugated anti-bovine IgA or anti-bovine IgG1 (Caltag, USA) followed by (HRP)-conjugated streptavidin, for IgA and IgG1 measurement in nasal swabs. The cut-off was established as the mean $A_{490}$ of the sera at 0 dpv plus two standard deviations (SD).

### 2.7. RNA extraction and PCR

Viral RNA was extracted using Trizol (Invitrogen) and used as template of a reverse transcription reaction performed with random primers. The resulting cDNA was used to carry out PCR amplification of the FMDV 3D polymerase gene (primers: 1461-GACCCGAAGTTGAGGCTGCC and 1462-GCCGAACTCCGTGCGAAACA) and a host 18S ribosomal RNA as internal control (primers: 18SU-TCAAGAACGAAAGTCGGAGG and 18SD-GGACATCTAAGGGCATCACA). PCR products were separated on a 1.8% agarose gel. Amplicons of 380 pb 480 bp were obtained for 3D viral polymerase and host rRNA, respectively.

### 2.8. IFNγ measurement

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of blood in a Ficoll-Paque™ plus gradient (GE Healthcare) and incubated in culture medium with or without inactivated FMDV, for 48 h at 37 °C 5% $CO_2$ atmosphere. Culture supernatants were collected and IFNγ was measured using a sandwich ELISA. Briefly, Immulon II plates were coated with mAb anti-IFNγ (kindly donated by Dr. Babiuk) in carbonate–bicarbonate buffer, pH 9.6. Plates were blocked with PBST–0.1% BSA. Dilutions of samples and recombinant IFNγ standard (Serotec, UK) were added. Plates were washed and rabbit polyclonal anti-IFNγ antibodies were added. After incubation, plates were washed and biotin-conjugated antibody anti-rabbit IgG was added. After incubation, alkaline phosphatase-conjugated streptavidine (KPL, USA) was added. Plates were washed, incubated with p-nitrophenyl phosphate as substrate and read at 405 nm. IFNγ concentration was calculated for interpolation of data in the standard curve.

### 2.9. Flow cytometry

Cell suspensions were blocked with normal bovine serum and incubated with the monoclonal antibody CD14 (VMRD) for 15 min at 4 °C. After washing, the secondary PE-conjugated rat antibody against mouse IgG (Jackson) was added and cells were incubated

for 15 min at 4 °C. Cells were washed, fixed with 0.2% paraformaldehyde and analyzed using a FACScan cytometer and CellQuest software (Becton Dickinson). Total numbers of CD14+ cells were calculated multiplying the percentage of positive cells by number of PBMC in each blood sample.

### 2.10. Opsonophagocytosis assay

Inactivated FMD virus was labeled with FITC (Sigma, St. Louis, MO) as described before [7] and opsonophagocytosis of FITC-labeled inactivated virus was analyzed by a previously described technique with minor modifications [7]. Briefly, serum from vaccinated animals was mixed with FITC-labeled inactivated virus. Cells from BoMac cell line were incubated with the opsonized FITC-inactivated virus (moi 10). Extracellular fluorescence was quenched with a 0.2 mg/ml solution of Trypan Blue. Fluorescence was analyzed using a FACScan cytometer and CellQuest software (Becton Dickinson).

BoMac cell line was established from bovine peritoneal macrophages by transformation with SV40 DNA and it was reported that the line retains the morphology of resident peritoneal macrophages and most physiological properties as phagocytic capacity [8].

### 2.11. Statistical analysis

InfoStat program was used. Kruskal–Wallis test and Dunn's multiple comparisons test were performed to assess statistically significant differences.

## 3. Results

### 3.1. Complete protection is achieved at 4 and 7 dpv when inactivated viral antigen is formulated with IMS 802

As shown in Table 1 none of the 802-iFMDV vaccinated bovines challenged at 4 or 7 dpv displayed symptoms of the disease, which determines 100% of protection at 4 and 7 dpv for this group (Fig. 1A).

Percentages of protection in the 1313-iFMDV group were 20% and 60% at 4 and 7 dpv respectively. At 7 dpv, the formulation containing only inactivated antigen (iFMDV) protected only 33% of the animals. At 7 dpv, the commercial vaccine protected 80% of the animals (Table 1 and Fig. 1A). It is worth noting that the unprotected bovine in that group (3511) had FMDV vesicles in one foot, indicating that infection in that animal was milder than those in unvaccinated control group (Table 1).

All non-vaccinated control animals (one bovine and six pigs) presented vesicles typical of FMD in their four feet. Nasal swabs of non-vaccinated control group were titrated in suckling mice. All of them presented titers higher than $10^5$ LD/ml (data not shown) and had positive results by PCR (Fig. 1B).

### 3.2. Vaccines induce low levels of specific antibodies

Bovines vaccinated with inactivated antigen (iFMDV), 802-iFMDV, 1313-iFMDV and the commercial vaccine elicited low levels of serum neutralizing antibodies and total Abs against FMDV (Table 2). Although antibody levels in vaccinated animals seem to be higher, there were no significant differences in the serum neutralizing antibodies nor total Abs elicited in 802-iFMDV, 1313-iFMDV or commercial vaccine groups as compared to the iFMDV group.

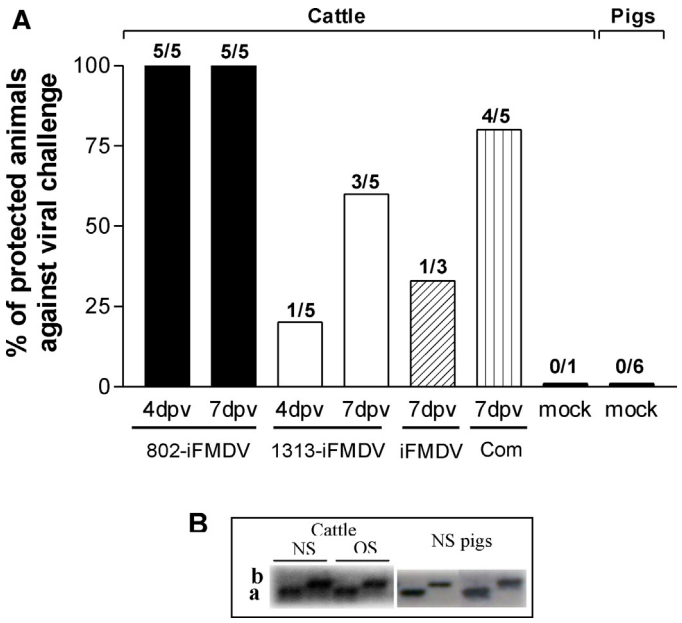


**Fig. 1.** (A) Percentages of protected animals against viral challenge at different times post-vaccination. Vaccines were formulated with 20 μg/dose of inactivated FMDV O1 C. Groups 802-iFMDV, 1313-iFMDV and commercial vaccine (Com) consisted of five vaccinated cattle, group iFMDV consisted of three vaccinated cattle. The mock group corresponds to six pigs and one cattle inoculated with PBS, they were used as positive controls of infection. Numbers of animals protected/challenged are indicated above the bars. Protection was established as total absence of foot or tongue lesions after seven days post-challenge with infective FMDV. (B) Representative data of RT-PCR of nasal (NS) and oral (OS) swabs of control unvaccinated bovine or nasal swabs of control unvaccinated pigs. Primers used amplified a 380 kb FMDV-specific band (a) and a 480 kb internal control (b).

At 7 days post-challenge (dpc) all assayed animals showed an increase in serum neutralizing antibodies and total Abs against the virus (Table 2), suggesting that all animals were properly exposed to the challenge virus.

### 3.3. Vaccines formulated with adjuvant induce FMDV-specific mucosal immunity

Since aerosol transmission is the most common mechanism of spread FMD among susceptible animals and the contact challenge used in the present report includes aerogenous transmission, the induction of mucosal immunity is highly desirable for an effective prevention of infection. We studied the anti-FMDV IgA and IgG1 in nasal swabs taken at 4 dpv from the vaccinated bovines. As shown in Table 3, 50% of animals in the 802-iFMDV group and 33% in 1313-iFMDV group had positive levels of IgA against FMDV in nasal mucosa according to the cut off point. In groups 802-iFMDV and 1313-iFMDV, 75 and 50% of the animals respectively, had positive levels of IgG1 isotype in nasal mucosa. In group inoculated with commercial vaccine IgA was present in 20% of the animals and the IgG1 isotype was detectable in 40%. Animals vaccinated with inactivated antigen alone (iFMDV) did not present IgA or IgG1 against FMDV in nasal mucosa.

### 3.4. 802-iFMDV and 1313-iFMDV increase IFNγ secretion

Since it has been reported that IFNγ efficiently inhibits FMDV replication, we measured this cytokine in the supernatant of PBMC cultures from vaccinated bovines. At 4 dpv groups immunized with 802-iFMDV or 1313-iFMDV reached significantly higher ($p < 0.05$) levels of IFNγ than iFMDV group. At 7 dpv, animals in 802-iFMDV group had significantly higher ($p < 0.05$) secretion of IFNγ

**Table 1**
Outcome of contact challenge at 4 and 7 days post-vaccination.

| Vaccine | dpv[a] | Animal | Foot lesions | Tongue lesions | Protected[e] |
|---|---|---|---|---|---|
| 802-iFMDV | 4 | 3509 | 0[b] | –[c] | Yes |
| | | 3512 | 0 | – | Yes |
| | | 3526 | 0 | – | Yes |
| | | 3530 | 0 | – | Yes |
| | | 3531 | 0 | – | Yes |
| | 7 | 3523 | 0 | – | Yes |
| | | 3524 | 0 | – | Yes |
| | | 3525 | 0 | – | Yes |
| | | 3529 | 0 | – | Yes |
| | | 3537 | 0 | – | Yes |
| 1313-iFMDV | 4 | 431 | 0 | – | Yes |
| | | 3514 | 3 | – | No |
| | | 3516 | 4 | – | No |
| | | 3528 | 4 | – | No |
| | | 3533 | 2 | – | No |
| | 7 | 3501 | 0 | – | Yes |
| | | 3503 | 4 | – | No |
| | | 3507 | 0 | – | Yes |
| | | 3532 | 0 | – | Yes |
| | | 3535 | 3 | – | No |
| Commercial | 7 | 3510 | 0 | – | Yes |
| | | 3511 | 1 | – | No |
| | | 3508 | 0 | – | Yes |
| | | 3513 | 0 | – | Yes |
| | | 3536 | 0 | – | Yes |
| iFMDV | 7 | 420 | 0 | – | Yes |
| | | 3502 | 2 | – | No |
| | | 3515 | 2 | – | No |
| Unvaccinated controls | – | 3522 | 4 | +[d] | No |
| | | Pig 1 | 4 | – | No |
| | | Pig 2 | 4 | – | No |
| | | Pig 3 | 4 | – | No |
| | | Pig 4 | 4 | – | No |
| | | Pig 5 | 4 | – | No |
| | | Pig 6 | 4 | – | No |

[a] Days post-vaccination at which challenge was performed.
[b] Number of feet with FMD lesions.
[c] (–) with no lesions in tongue.
[d] (+) with lesions in tongue.
[e] Animals were considered protected if they had no FMD lesions in feet or tongue.

than those inoculated with the inactivated antigen alone (iFMDV) (Fig. 2A).

### 3.5. Monocytes are increased in group 802-iFMDV

It has been demonstrated that MØs can engulf and destroy FMDV, especially if the virus is opsonized with specific Abs [9–11]. Recently, we have demonstrated that phagocytic monocytes/MØs play a main role in early protection in the murine model [4]. We decided to evaluate this population in the vaccinated cattle.

As shown in Fig. 2B, CD14+ cells, (monocyte/MØs), were significantly increased ($p < 0.05$) in groups inoculated with 802-iFMDV at 4 and 7 dpv, while inoculation with 1313-iFMDV or the commercial vaccine did not rise the total monocytes numbers as compared with inactivated antigen alone (iFMDV) or mock vaccinated animals.

When opsonophagocytosis assays were performed (Fig. 2C), a significant increase in FMDV-FITC uptake was seen in 802-iFMDV group regarding control negative group, although the differences were not significant regarding iFMDV group.

**Table 3**
Percentage of animals with antibodies against FMDV (IgA and IgG1) in nasal swabs at 4 dpv.

| Group | % of animals with anti FMDV-IgA[a] | % of animals with anti FMDV-IgG1 |
|---|---|---|
| iFMDV | 0 | 0 |
| 802-iFMDV | 50 | 75 |
| 1313-iFMDV | 33 | 50 |
| Commercial | 20 | 40 |

[a] According the cut off calculated as the mean OD of swabs taken at 0 dpv + 2SD.

## 4. Discussion

Since the FMD outbreaks that occurred in Europe in 2001, ring vaccination with emergency vaccines around the outbreak zone was incorporated in some countries as a measure to prevent virus spread.

In previous studies, using the murine model, we demonstrated that the use of IMS802 adjuvant yielded protection against viral challenge at 4 and 7 dpv and induced FMDV-specific, but non-neutralizing, antibody titers, while the inoculation of IMS1313 plus inactivated antigen induce a higher protective immune response than the vaccine with inactivated virus alone at 7 dpv [4,5]. Since these findings in the murine model, we decided to evaluate the same formulations in cattle, one of the natural hosts of the virus. Additionally, some of the immune mechanisms involved in such protection were studied.

Air/contact transmission is the most common infection route for FMDV. For this reason contact challenge was the infection strategy in this study. Since it has been reported that infected pigs excrete 1000–3000 fold more aerosol virus than cattle [12] they were used as donors of challenge virus. After challenge, control unvaccinated animals presented the typical disease lesions and were positive to viral detection by PCR and virus isolation. In addition, the antibody titers were increased after viral challenge. These data strongly indicates that the circulation of virus was sufficient to infect cattle.

From the early 1990s, a large amount of research efforts were devoted to find an emergency vaccine capable of efficiently protects animals from the disease in a short time. Doel and collaborators [12] achieved 100% protection in cattle by indirect contact challenge, at 4 and 8 dpv, using adjuvants Montanide™ ISA206, Montanide™ ISA25 and aluminum hydroxide in combination with inactivated O1 Lausanne FMDV. On the other hand, Golde and collaborators [3] achieved 100% protection of cattle at 7 dpv, using a commercial monovalent vaccine and an intradermal lingual viral challenge

**Table 2**
Serum FMDV neutralizing antibodies and total antibody titers against FMDV elicited in cattle pre and post-challenge. The seroneutralizing titer was calculated using the fixed virus–variable serum method. Total antibodies were measured by ELISA. Each number represents the mean titer ± SD.

| Vaccine | Neutralizing antibodies | | | Total specific antibodies | | |
|---|---|---|---|---|---|---|
| | 4 dpv | 7 dpv | 7 dpc | 4 dpv | 7 dpv | 7 dpc |
| 802-iFMDV | 1.33 ± 0.63[a] | 1.34 ± 0.53 | 2.92 ± 0.53 | 1.36 ± 0.68 | 1.55 ± 0.38 | 3.96 ± 0.04 |
| 1313-iFMDV | 1.02 ± 0.57 | NT[b] | 2.88 ± 0.52 | 1.58 ± 0.19 | NT | 2.88 ± 0.52 |
| Commercial | 1.06 ± 0.59 | NT | 3.01 ± 0.46 | 1.46 ± 0.42 | NT | 3.01 ± 0.46 |
| iFMDV | 0.91 ± 0.35 | 0.85 ± 0.27 | 2.61 ± 0.94 | 0.9 ± 0.05 | 1.04 ± 0.21 | 2.61 ± 0.94 |

[a] Mean ± standard deviation.
[b] Sample not taken.

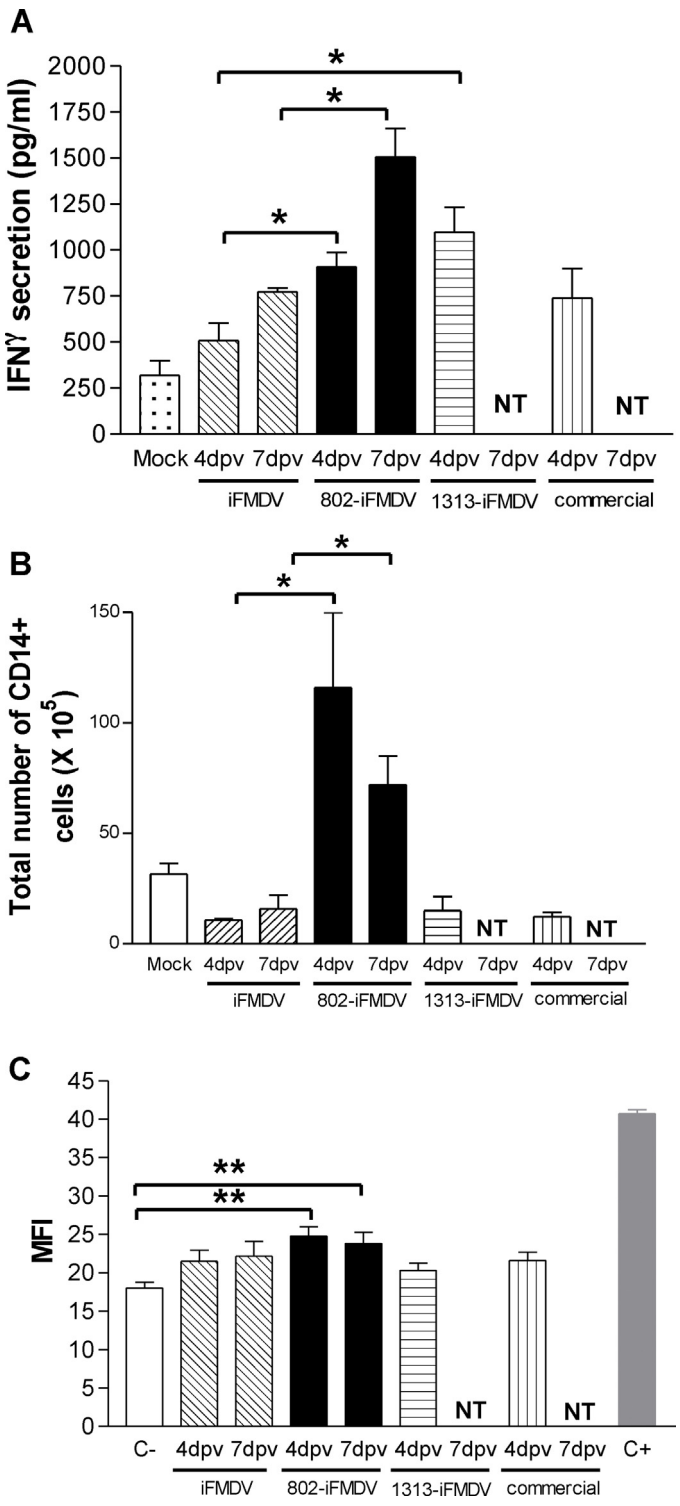


**Fig. 2.** (A) Secretion of IFNγ (pg/ml) in PBMC isolated from vaccinated cattle. Cells were incubated for 48 h in 96 wells plates, supernatants were collected and IFNγ was measured using a sandwich ELISA. (B) Flow-cytometry analysis of CD14+ cells (phagocytic monocytes) isolated from peripheral blood of vaccinated animals. Mean numbers ± SD from mock vaccinated or animals vaccinated with iFMDV, 802-iFMDV, 1313-iFMDV or Commercial vaccine are shown. Numbers are expressed as cells × $10^5$ and were calculated using the percentage of positive cells and then referring to the total number of PBMC isolated in each blood sample. (C) Opsonophagocytosis assay. FITC-FMDV was incubated with pre-immune sera (C−), sera from multi-vaccinated bovines (C+), or sera from FMDVi, 802-iFMDV, 1313-iFMDV or commercial vaccine vaccinated bovines. Then the mixtures were put in contact with BoMac cells. The mean numbers + SD of mean fluorescence intensity (MFI) from three assays are shown. *Significant differences ($p < 0.05$); **significant differences ($p < 0.001$). NT: sample not taken.

of $10^4$ $TCID_{50}$/dose, although protection at 4 dpv was only partial (25%). We demonstrate that 802-iFMDV containing a 20 μg/dose of inactivated O1 C virus, was capable of protecting 100% of the vaccinated cattle at 4 and 7 dpv. To our knowledge, this is the first time that total protection is achieved in cattle using inactivated antigen plus IMS12802PR adjuvant.

In the present study the commercial polyvalent vaccine currently used in vaccination programs in Argentina, was evaluated at 7 dpv and it protected 80% of vaccinated cattle. Although protection levels at earlier times were not investigated, the high level of protection obtained at 7 dpv indicates that the commercial vaccine could be useful as an emergency vaccine in case of a FMDV outbreak.

Since FMDV enters the animal through mucosal surfaces, the induction of mucosal immunity is highly desirable for an effective prevention of infection. Cubillos and collaborators [13] have reported a positive correlation in pigs, between protection and IgA induction conferred by a peptide vaccine. Interestingly, we detected FMDV-specific IgA in nasal swabs as soon as 4 days after the immunization with 802-iFMDV and 1313-iFMDV vaccines. In other species different from cattle, it has been demonstrated that the interaction of dendritic cells, follicular dendritic cells and stromal cells with B cells are involved in the production of T cell independent Abs and class switching to IgG and IgA. These interactions involve IgA inducing factors as BAFF, APRIL and TGF-β which secretion can be stimulated by TLR activation [14–19]. Since the adjuvants used in the present work include immunostimulating complexes, different TLRs could be activated. These phenomena would explain why only the groups 802-iFMDV, 1313-iFMDV and commercial vaccine have IgA and IgG in nasal swabs as early as 4 dpv. In contrast to other vaccines, 802-iFMDV and 1313-iFMDV induced specific mucosal immune responses by parenteral administration. Several mechanisms have been proposed to explain the production of secretory antibodies after parenteral administration of the antigen, including direct diffusion of soluble or phagocytosed antigens to mucosa-associated lymphoid tissue or activation of antigen-presenting cells at draining lymph nodes, which then migrate to mucosa-associated lymphoid tissue [13,20–23]. However, regulation of IgA production in cattle is not completely understood and further studies should be aimed at establishing the association between these immunoglobulins and protection.

It is widely accepted that an effective immune response against FMDV requires a specific and neutralizing humoral response [24]. We observed that serum neutralizing antibodies and total Abs titers were low but detectable in all groups but not significantly different than those induced by the inactivated antigen alone.

It is well known that some animals with serum neutralizing antibodies titers below the levels that are considered as protective, are in fact protected against viral challenge [10,25]. This phenomenon has been previously observed using emergency vaccines in natural hosts [3,12,26,27] and in the murine model [4]. For these reasons it has been proposed that other mechanisms might provide immunity against the disease [28].

Hence, we studied the role of cellular components of the immune response in early protection. It has been reported that monocytes/MØs can engulf and destroy FMDV, especially if the virus is opsonized by specific antibodies [9–11]. Recently, we have demonstrated, using the murine model, that depletion of MØs in animals vaccinated with 802-iFMDV, hampers protection against FMDV showing the main role of this population in early protection [4]. In the present report, we demonstrate in cattle that monocytes/MØs were increased by vaccination with 802-iFMDV, when compared to iFMDV, in peripheral blood mononuclear cells. Changes in blood leukocyte populations can indicate acute systemic inflammatory reactions [29]. On the other hand, it is expected that vaccination will induce a local inflammatory response and a

consequent increase in chemotactic activity, thus mobilizing phagocytic cells from the bone marrow [30]. Aucouturier and collaborators [31] reported that IMS802 adjuvant induces local inflammation recruiting antigen-presenting cells as MØs and dendritic cells. It has been proposed that through migratory activity, phagocytic function would be enhanced, both in terms of the individual cell recruited and due to the increased number of phagocytes [32]. Our results are consistent with those reported by Rigden and collaborators [11] who in spite of not being able to prove the increase in the number of monocytes after inoculation of pigs with an emergency vaccine against FMDV, were able to demonstrate an increased chemotactic activity in phagocytes. They concluded that the increased chemotaxis of phagocytes is a key factor in developing early protection.

The levels of secreted IFNγ were increased in PBMC from bovines vaccinated with 802-iFMDV and 1313-iFMDV with respect to those from the group vaccinated with inactivated antigen (iFMDV). 1313-iFMDV group had lower levels of protection at 4 dpv despite the fact that its IFNγ levels were higher; this led us to think that the role of this cytokine would be more related with macrophages activation than with an antiviral activity itself.

When opsonophagocytosis was assayed, 802-iFMDV group had a significantly higher virus uptake than negative control group (pre-immune sera) demonstrating that antibodies elicited by vaccine 802-iFMDV have opsonizing activity.

We think that the increase in the activated monocytes numbers and the presence of antibodies with opsonizing activity in these animals, could be enough to achieve protection at early times post-vaccination, as we have demonstrated before in the murine model [4].

In conclusion, IMS802 plus inactivated antigen is able to protect cattle against challenge with FMDV at early times post-vaccination. At present, the mechanism for such protection remains unclear, although Abs, IgA and IgG1 isotypes in nasal mucosa, an increase in monocytes levels and an increase in opsonizing capacity could be involved.

The high levels of protection achieved for 802-iFMDV vaccine at 4 and 7 dpv, indicate that this vaccine could be useful as an emergency vaccine.

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