

Reintroduction of foot-and-mouth disease in Argentina: characterisation of the isolates and development of tools for the control and eradication of the disease[☆]

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Abstract

This paper describes the antigenic and molecular characterisation of foot-and-mouth disease virus (FMDV) strains isolated during the 2000–2002 epidemic in Argentina, and the strategy implemented for disease control. Two different FMDV serotypes, O and A, were involved. Of the various field isolates studied, two distinct O1 lineages (strains Corrientes/00 and Misiones/00) and two serotype A lineages (A/Argentina/00 and A/Argentina/01 prototypes) were identified. The genome sequences of these strains were compared with sequences of previous regional isolates and sequences of vaccine strains. O1 strains were found to be related to regional strains while serotype A strains were found to be more distanced from them. The updating of the antigenic composition of the vaccines used in the emergency was a key issue, since the outbreaks stopped shortly after the implementation of the vaccination programs. The O1 strains quickly disappeared from the field following strict control measures and the use of vaccines containing O1/Campos strain. However, in the case of the A serotype strains, the situation was different, since the use of a vaccine containing strain A24/Cruzeiro yielded acceptable levels of protection only after re-vaccination. Therefore, the new field strains A/Argentina/00 and A/Argentina/01 were incorporated into the vaccine, leading to an effective control of the disease. Viral circulation greatly diminished, as indicated by the significant reduction in the number of outbreaks and in the number of animals with antibodies against non-structural proteins. Satisfactory levels of protective antibodies were subsequently detected in the cattle population (above 75% protection). The absence of outbreaks after January 2002 indicated that the epidemic was controlled.

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1. Introduction

Foot-and-mouth disease (FMD) is a viral vesicular disease of cloven-hoofed mammals that generally causes severe economic losses. Due to its highly contagious nature and economic importance, FMD is included in the list A of the Office International des Epizooties (OIE).

FMD was first recognised in Argentina in 1870. Since then, it has been the most relevant disease affecting animal production. Argentina's total surface area is 2,780,199 km². There are 52.5 million cattle and 13.8 million sheep in the country. The breeding system and the usual movement of cattle within large geographical areas make it difficult to handle FMD outbreaks. Historically, outbreaks occurred twice a year, with peaks in May/June and September/October due to major animal movements and the presence of calves with low antibody levels, owing to recent weaning and the start of vaccination campaigns [1].

The Argentine National FMD Control Programme of 1990–1997 led to the control and eradication of the disease, achieving in 1997 the official OIE status of FMD-free country with vaccination. The success in this case was mainly due to the use of high potency oil-adjuvanted vaccines, large vaccine coverage (>85%) and proactive co-operation between farmers and official institutions. Under these circumstances, the fact that the eradication campaigns were working correctly was indicated by critical epidemiological markers: (a) the progressive decrease in the number of outbreaks, the last of which was detected in April 1994; (b) the evidence provided by serological surveys of both constant diminution of viral circulation and strong and sustained herd immunity. In 1999 vaccination was halted, and in May 2000 the OIE recognised Argentina as a country free of FMD without vaccination.

Regarding vaccine reserves, the Argentine FMD vaccine and antigen bank (AFMDVAB) was created in the year 2000 on a national tender, in order to hold cryopreserved concentrated FMDV antigens and a ready-to-use polyvalent vaccine. Argentine authorities determined that the AFMDVAB should also have the operational capacity to scale up and formulate vaccine from newly produced antigen in case of spread of the disease.

In July 2000, 14 months after vaccination was halted, outbreaks of FMD reappeared. The epidemic lasted 18 months during which more than 2500 outbreaks were reported. The disease mainly affected regions of major livestock concentration. Patagonia remained free of FMD. Infected animals were mostly cattle (96.78%), specially under 2 years of age (80.4%); 2.49% were sheep and 0.65% pigs. Virus serotype O was responsible for only a few outbreaks at the beginning of the epidemic, while serotype A circulated until January 2002. An epidemiological study, conducted in areas with a high density of cattle population shortly before the first outbreak, showed different levels of protection, ranging from adult animals that still possessed high levels of antibodies due to previous systematic vaccination to calves born after

cessation of vaccination that were fully susceptible (B. Robiolo, personal communication).

The initial control measures taken by the National Animal Health Service, SENASA, included stamping out, ring vaccination, restriction of animal movements and several other preventive measures. There were 3563 slaughtered animals (cattle) in the provinces of Formosa, Corrientes and Entre Ríos. Emergency vaccination was initially implemented with available tetravalent vaccines (O1/Campos, A/Arg/79, A/Arg/87, C3/Arg/85) and trivalent vaccines composed of strains O1/Campos, A24/Cruzeiro and C3/Indaial. Since January 2001, the virus strain A/Arg/00 was incorporated into this vaccine replacing the C3/Indaial strain. Twice-yearly systematic vaccination campaigns were started again in April 2001. A tetravalent vaccine was subsequently developed including strains O1/Campos and A24/Cruzeiro and the new strains A/Argentina/00 and A/Argentina/01.

In July 2003 Argentina was recognised by the OIE as free of FMD with vaccination. It is worth mentioning that the status of the South Patagonia Region as free from FMD without vaccination remained unaltered. Recently, an FMD outbreak was reported in non-vaccinated pigs in the Province of Salta, on the border with Bolivia. Special emphasis had been given to border control because of recent FMDV activity in nearby areas of Bolivia and Paraguay. The outbreak was quickly contained. Control measures were, among others, quarantine and animal movement control, stamping out, and vaccination of all susceptible animals in the outbreak area. Intensive FMD surveillance is being conducted in the area.

This paper describes the sequential steps followed to control the disease, including the antigenic and molecular characterisation of the FMD virus isolates, the development of new vaccines, their performance during emergency and systematic vaccination campaigns, and the herd immunity acquired.

2. Materials and methods

2.1. Virus strains

FMDV strains were isolated from field samples by the National Reference Laboratory of SENASA, and were assayed either as epithelial tissue extracts or as first passage in baby hamster kidney (BHK-21) cells (clone 13). FMDV isolates had been characterised by complement fixation, serum neutralisation assays, monoclonal antibodies profile and genome sequencing, as described in the following sections. Strain MC38796 from General Villegas, Province of Buenos Aires, was designated prototype of A/Argentina/00 (A/Arg/00); and strain MC267 from Trenque Lauquen, Province of Buenos Aires, was designated prototype of A/Argentina/01 (A/Arg/01). Representative O1 strains were designated as MC38779 from Mercedes, Province of Corrientes; and as MC38867 from the Province of Misiones, both isolated during the year 2000. FMD vaccine strains O1/Campos/Brazil/58 (O1/Campos), O1/Caseros/Arg/68

(O1/Caseros), A24/Cruzeiro/Brazil/55 (A24/Cruzeiro), A/Argentina/79 (A/Arg/79) and A/Argentina/87 (A/Arg/87), C3/Argentina/85 (C3/Arg/85) and C3/Indaial/Brazil/71 (C3/Indaial) provided by SENASA were also used in this study.

2.2. Serum neutralisation assays

Micro-neutralisation tests were carried out according to the method described in [2], using BHK-21 c13 cell monolayers. Bovine serum from animals vaccinated with oil-adjuvanted vaccines against A24/Cruzeiro, A/Arg/00 and A/Arg/01, containing a total antigenic mass of 10 µg of 146S/dose, was collected 30 days after vaccination. The test was carried out as a two-dimensional neutralisation assay, and antibody titres were calculated as the log₁₀ of the reciprocal antibody dilution required for 50% neutralisation of 100 TCID₅₀ of virus according to [3]. The relationship between strains was determined according to the value of 'r' (r = serum titre against heterologous virus/serum titre against homologous virus) following criteria previously described (from 0.0 to 0.19: strains significantly different; from 0.2 to 0.39: different strains; from 0.4 to 1.0: strains not significantly different) [4].

2.3. Monoclonal antibodies profiling

Monoclonal antibodies (MAbs) against reference South American FMDV strains A24/Cruzeiro, A/Arg/79, A/Arg/87, C3/Arg/85, C3/Rezende, C3/Indaial, O1/Caseros and O1/Campos were generated using standard protocols [5]. Each MAb was partially characterised and grouped in panels according to their capacity to neutralise virus infectivity and to bind either intact (146S) or disrupted virions (12S subunits) (Seki et al., manuscript in preparation). The reactivity of each MAb against heterologous reference strain viruses was also tested.

The trapping ELISA test (trELISA) and data processing were carried out as described by Samuel et al. [6]. Briefly, viruses of different origins were trapped by a type-specific rabbit serum and reacted with each MAb and with specific mouse polyclonal serum (PCS). The reactivity was developed by incubation with an anti-mouse serum conjugated with horseradish peroxidase and the substrate/chromophore mixture H₂O₂/ABTS (2,2-azino-bis 3 ethyl-benzothiazoline-6 sulfonic acid diammonium salt, Sigma, USA). In each test were included the reference virus strains from which the MAbs were developed as well as a blank (B) with no virus.

The reactivity of each MAb with a given field virus isolate (X) with respect to the mouse polyclonal serum was calculated by subtraction of the respective blanks (B), using the formula: $R_X = (OD_{MAbX} - OD_{MAbB}) / (OD_{PCSB} - OD_{PCSB})$. Reactivity of the samples, as percentage of the homologous reference strain for each MAb, was calculated using the formula: % React = $R_X / R_{Ref} \times 100$. We adopted the same crite-

ria as Samuel et al. [6] to qualify reactivity differences with respect to the homologous strain. Reaction percentages of 124–76% reflected equal reaction with respect to the homologous reference virus; reactivities of 75–46% and of 45–20% reflected low and high change respectively; and values lower than 20% were considered lack of reactivity.

Sixtythree samples of serotype A and three samples of serotype O1 collected from FMDV outbreaks during 2000 and 2001 in Argentina were tested against a panel of MAbs. The profiling panel was constructed with MAbs raised against different epitopes according to previous characterisation. MAbs generated against O1/Caseros (12H, 3A, 13, 6F, 8G, 2 and 3) and O1/Campos (17 and G8) that did not react to serotype A (A24/Cruzeiro, A/Arg/79, A/Arg/87) or serotype C (C3/Indaial, C3/Arg/85, and C3/Rezende) virus strains were used for characterisation of the O1 field strains. For characterisation of the serotype A virus isolates, a panel of 18 MAbs was used, composed by 4 MAbs derived from A24/Cruzeiro (3C3, 4A2, 2C2 and 3D9), 5 from A/Arg/79 (108, 212, 206, 112 and 7) and 9 from A/Arg/87 (317, 211, 408, 220, 20, 403, 105, 111 and 204) virus strains. These MAbs did not react either to serotype O (O1/Caseros, O1/Campos and O/Taiwan) or serotype C (C3/Indaial, C3/Arg/85 and C3/Rezende). MAbs epitopes have not been mapped yet but their neutralisation capacity is shown in Figs. 1 and 2.

2.4. Nucleotide sequencing

RNA extraction, cDNA synthesis and sequencing of the PCR products was performed as previously described by König et al. [7]. Briefly, RNA was extracted using Trizol[®] (Gibco-BRL) according to the manufacturer's instructions. RT-PCR was performed as described previously [8]. PCR products were purified with the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol. Nucleotide sequences were determined by cycle sequencing using (³⁵S) dATP (New England Biolabs, USA) and the DNA sequencing kits (fmol[®], Promega, USA) following the manufacturer's protocol. Nucleotide sequences of the isolates were determined and primarily aligned with the sequences of the reference strains by using DNAsis[®] for Windows, version 2.5 (Hitachi, 1997) and BIOEDIT[®] version 5.0.9. (Tom Hall, North Carolina State University, 1997–2001). Multiple alignment was performed using CLUSTAL X Multiple Sequence Alignment Programme (version 1.81, 2000 [9]). Parsimony trees were generated with NONA[®], Pablo Goloboff, 1993, version 2.0; and then presented with the WINCLADA[®] programme, Kevin Nixon, 1988–1996, 1997, 0.99 version. 100 random addition sequences (RAS) and tree bisection reconstructions (TBR) were used for tree construction.

2.5. Vaccine formulation

Several experimental and industrial vaccines were used in this study. The experimental vaccines, either monovalent

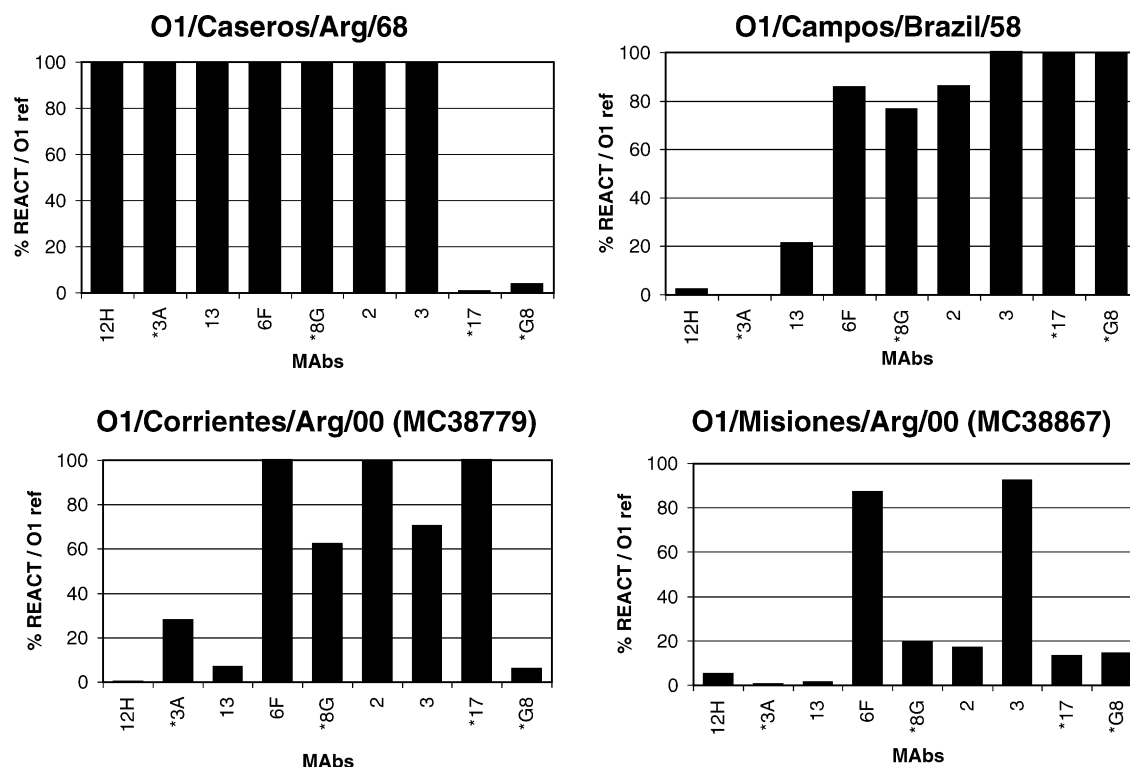


Fig. 1. Reactivity of the new FMDV isolates with a panel of monoclonal antibodies generated to O1 regional reference strains. It shows the different levels of reactivity with a panel of monoclonal antibodies of the O1 strains isolated in Argentina in the year 2000 and with the two O1 vaccine strains used in the region. Reactivity of the isolates is expressed as a percentage of the reactivity of the reference strain against which the antibodies were generated ($\text{OD isolate}/\text{OD Ref} \times 100$). MAbs specific for O1/Caseros strain are 12H, 3A and 13. MAbs specific for O1/Campos strain are 17 and G8. These MAbs do not react with type A (A24/Cruzeiro, A/Arg/79, A/Arg/87), or C (C3/Indaial, C3/Arg/85, and C3/Rezende) viruses. *: Neutralising capacity.

or polyvalent, were prepared from one or several of the following strains: A24/Cruzeiro, O1/Campos, A/Arg/2000 and A/Arg/2001. In all cases, FMDV strains were propagated in BHK-21 cl 13 suspension cell cultures. Infected tissue culture supernatants were collected, clarified and inactivated twice with binary ethyleneimine (BEI) [10]. Inactivated supernatants were concentrated and partially purified using polyethylene glycol 6000. A commercially available trivalent vaccine (vaccine no. 4, O1/Campos, A24/Cruzeiro and C3/Indaial) was also used.

Vaccines were prepared as water-in-oil emulsions [11]. Briefly, the aqueous phase (40%) was composed of concentrated antigens from the corresponding FMDV vaccine strains, to which 3 mg/dose of saponine was added (Quest International, Ireland). The oil phase was composed of 90% Marcol 52[®] (Exxon, France) and 10% Montanide 888[®] (Sepic, France). The vaccine dose was 2 ml, except for the trivalent vaccine no. 4, for which a 3 ml dose was used. The composition of the different vaccines used in this study is described in Table 1.

Dose/response studies were performed in order to assess the quantitative formulae of the vaccines used for massive vaccination. Decreasing amounts of 146S of the new strains A/Arg/00 (data not shown) and A/Arg/01 strains were also used.

2.6. Challenge studies

One hundred fifty four Hereford breed cattle, aged 18–24 months and free from FMDV antibodies, were used for the trials. They belonged to the FMD-free zone in Argentina, the South Patagonia Region, where vaccination is not practised. After vaccination, animals remained in isolated experimental premises and during the challenge period were kept in controlled pens, under biosecurity conditions in the experimental station of the National Institute of Agricultural Technology (INTA). In vaccination trial no. 1, monovalent vaccines containing an antigenic mass of 10 μg of 146S of the A24/Cruzeiro (vaccine no. 1) or A/Arg/00 (vaccine no. 2) virus strains were used, as well as a polyvalent vaccine (vaccine no. 4, O1/Campos (33 μg)–A24/Cruzeiro (17 μg)–C3 (12.3 μg)). Animals were challenged with the A/Arg/00 FMDV strain.

A general description of trials no. 1–4 is found in Table 1.

Challenge was performed at different times post vaccination or post re-vaccination, as described in Table 1, by inoculation of 10,000 suckling mouse lethal dose 50% ($\text{SMLD}_{50\%}$, trials no. 1–3) or 10,000 bovine infectious dose 50% ($\text{BID}_{50\%}$, trial no. 4) by intradermolingual route. Comparisons between $\text{SMLD}_{50\%}$ and $\text{BID}_{50\%}$ were performed by simultaneous titration of the virus suspensions in suckling mice, BHK-

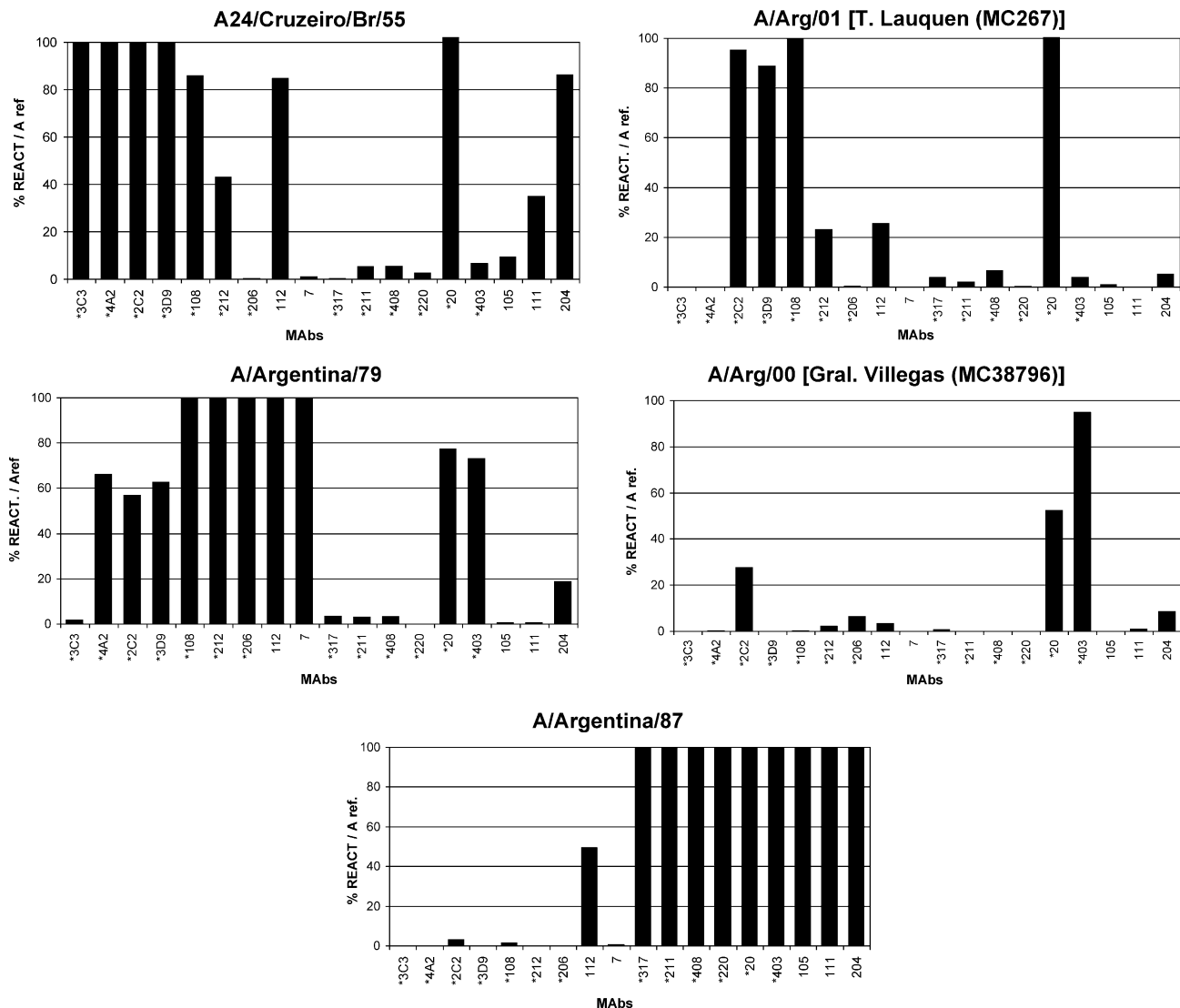


Fig. 2. Reactivity of the new FMDV isolates with a panel of monoclonal antibodies generated to serotype A regional reference strains. It shows the different levels of reactivity with a panel of monoclonal antibodies of the serotype A strains isolated in Argentina in 2000 and 2001 and with three of the serotype A vaccine strains used in the region. Reactivity of the isolates is expressed as a percentage of the reactivity of the reference strain against which the antibodies were generated ($\text{OD isolate}/\text{OD Ref} \times 100$). The MAbs are serotype A virus-specific and do not react with serotype O (O1/Caseros, O1/Campos and O/Taiwan) or serotype C (C3/Indaial, C3/Arg/85, C3/Rezende) viruses. *: Neutralising capacity.

21 cells and bovine tongue (data not shown). Two unvaccinated cattle were included in each trial as controls. Seven days after challenge, the animals were examined for podal lesions of FMD. Protection against podal generalisation (PPG) was measured. Animals were considered unprotected when typical FMD lesions developed at least in one foot. The unvaccinated control animals showed in all cases podal lesions caused by the disease.

2.7. Detection of antibodies to structural FMDV proteins in cattle sera

Antibody titres against each of the FMD virus strains present in the vaccines were detected by a liquid-phase blocking sandwich ELISA (IpELISA) as described in [12]. Briefly,

sera from vaccinated animals, collected at different times after vaccination, were incubated overnight at 4 °C with a pre-titrated dose of the corresponding virus in a saline buffer liquid phase. The mixtures were then incubated for 1 h at 37 °C on wells coated with specific rabbit polyclonal antibody, where the virus that did not react with the bovine serum in the initial step was trapped. After a washing step, a second incubation was performed with a mixture of monoclonal antibodies specific for each of the viruses being tested. Goat anti-mouse IgG-HRP conjugate was added, and colour development was obtained after addition of the substrate/chromophore mixture ($\text{H}_2\text{O}_2/\text{ABTS}$). This test detects the presence of the virus that did not react with the bovine serum in the initial step and remained trapped in the wells. Antibody titres were expressed as the reciprocal $\log_{10}$ of

Table 1
Protection against challenge with A/Arg/00 and A/Arg/01

Trial number	Vaccine number	Vaccine composition ^a (µg 146S/dose) ^b	Challenge (dpv)		Protection (PPG)%	IpELISA Ab (log ₁₀) ^e ± S.D. ^f		
			A/00	A/01		A24	A/00	A/01
1 ^{g,i}	1	A24 (10 µg)	30	–	30	–	–	–
	2	A/00 (10 µg)	30	–	90	–	–	–
	4	A24 (17 µg)/O1/C(33 µg)/C3 (12,3 µg)	141	–	60	–	–	–
	4 ^d		141	–	100	–	–	–
2 ^{g,i}	1	A24 (10 µg)	–	30	30	2.20 ± 0.42	1.84 ± 0.59	1.80 ± 0.56
	2	A/00 (10 µg)	–	30	0	1.66 ± 0.17	1.66 ± 0.34	1.39 ± 0.35
	3	A/01 (10 µg)	–	30	80	1.96 ± 0.50	2.27 ± 0.49	2.94 ± 0.26
	5	O1/C (15.8 µg)/A24 (6.5 µg)/A/00 (2.7 µg)	–	30	50	2.33 ± 0.59	2.26 ± 0.57	1.99 ± 0.71
3 ^{g,i}	5	O1/C (15.8 µg)/A24 (6.5 µg)/A/00 (2.7 µg)	–	120	60	2.82 ± 0.22	2.52 ± 0.28	2.13 ± 0.21
	5 ^d		–	141	90	2.92 ± 0.31	2.87 ± 0.32	2.35 ± 0.30
4 ^{h,j}	6	O1/C (5 µg)/A24 (6 µg)/A/00 (0.75 µg)/A/01 (6 µg)	–	90	81	–	–	2.56 ± 0.50 ^c
	7	O1/C (8 µg)/A24 (5 µg)/A/00 (3 µg)/A/01(3 µg)	–	90	57	–	–	2.18 ± 0.38 ^c
	8	O1/C (4.4 µg)/A24 (3.2 µg)/A/00 (1.4 µg)/A/01 (1.5 µg)	–	90	31	–	–	1.99 ± 0.30 ^c

dpv: Days post-vaccination. PPG: Protection from Podal Generalisation.

^a FMDV strains: A24 = A24/Cruzeiro; O1/C = O1/Campos; A/00 = A/Arg/00; A/01 = A/Arg/01; C3 = C3 Indaial.

^b 2 ml/dose, except for vaccine no. 4 in which a 3 ml/dose was used. Only one dose per animal. The amount of virus (µg) inoculated per dose for each serotype is shown in parentheses.

^c $P < 0.01$.

^d Re-vaccinated group; second dose was administered at 21 dpv.

^e Detection of antibodies by liquid-phase blocking sandwich ELISA against A24/Cruzeiro, A/Arg/00 and A/Arg/01 in cattle sera collected at day of challenge.

^f Standard deviation.

^g Challenge dose 10,000 SMLD₅₀%.

^h Challenge dose 10,000 BID₅₀%.

ⁱ 10 animals (bovine) per group plus two unvaccinated controls per trial.

^j 16 animals (bovine) per group plus two unvaccinated controls per trial.

Table 2
 Titres of lpELISA, their corresponding expected protection percentage (EPP) values and interpretation of the results

lpELISA titre			EPP ^a	Result
O1/Campos	A24/Cruzeiro	A/Arg/01		
<1.77	<2	<2.1	<75%	Low
1.77–1.91	2–2.1	2.1–2.3	75% to 85%	Medium
>1.91	>2.1	>2.3	>85%	High

^a For each FMDV serotype, EPPs were calculated by comparing lpELISA antibody titres from sera collected at 60 dpv with results from cattle protection in challenge tests, applying the logistic regression fitting.

serum dilutions giving 50% of the absorbance recorded in the control wells (virus without serum). Six control sera of known titres were assayed simultaneously, as internal standards in each ELISA plate.

The expected percentage of protection (EPP) for each FMDV serotype was previously calculated by comparing the levels of lpELISA antibody titres from sera collected at 60 dpv with results from cattle protection in challenge tests, applying the logistic regression fitting (see Table 2). For epidemiological studies, antibodies to structural proteins were also determined by lpELISA and the EPPs were calculated by the same procedure.

2.8. Detection of antibodies against FMDV non-structural proteins (NSP) in cattle sera

Antibodies to NSP were assessed on post-vaccination cattle sera collected at day 0 and at different times post vaccination. Antibodies to 3ABC NSP were determined using the ELISA-3ABC as described by Bergmann et al. [13]. Results were confirmed by EITB (Enzyme-linked immunoelectrotransfer blot assay) following the protocols described in [13]. Ovine and goat sera were tested by agar gel immunodiffusion for detection of the virus infection associated antigen (AGID–VIAA) [14]. Antibodies to NSP were determined using the same methodology for assessment of viral circulation.

2.9. Assessment of herd immunity and viral circulation by sero-epidemiological survey

Since the reintroduction of FMDV in Argentina, serological surveys were performed to evaluate the effectiveness of control measures. The indicators of effectiveness were, among others, herd immunity and virus circulation among susceptible animals.

Herd immunity was evaluated in 2001 by estimating the prevalence of bovines in each of the different levels of expected percentage of protection, according to the values shown in Table 2. A subset of samples negative to NSP were randomly selected for this part of the study in order to exclude animals carrying antibodies induced by infection. Thus only antibodies induced by vaccination were measured.

Surveys for virus circulation were put into effect in 2002 and 2003. Samples were collected between March and May

of each year. The country was divided into three main areas: (a) Central, where 95% of national herd and 28% of national flock are located and more than 99% of FMD outbreaks took place during the last epidemic; (b) Peripheral (West and North-west and North Patagonia Region A), with 4 and 7% of national herd and flock respectively and less than 1% of FMD cases; and (c) North Patagonia B-South Patagonia, with 1 and 65% of national herd and flock respectively and where no FMD outbreaks have been reported. Each area was divided into sub-areas, according to animal density, structure of cattle population, animal movement patterns, and geographic characteristics (León et al., manuscript in preparation). The selection of samples followed a two-stage random process: firstly random selection of farms and secondly random selection of animals. Samples were taken from cattle of two categories defined by age: younger (6 to 12 months old) and older (1 to 2 years old). The amount of samples collected in each survey per species and per area is described in Table 3.

Virus circulation was assessed by estimating the prevalence of animals with antibodies against NSPs as described in Section 2.8. Only bovine sera positive for both ELISA 3ABC and EITB were considered a positive result for this purpose.

3. Results

3.1. Antigenic characterisation of FMDV field strains

Antigenic relationships were established by serum neutralisation of sera from animals vaccinated with the A/Arg/00, A/Arg/01 and A24/Cruzeiro FMD virus strains. For the A/Arg/00 virus strain, the *r* values found were 0.21 and 0.24 for the A24/Cruzeiro and A/Arg/01 virus strains, respectively. For the A/Arg/01 virus strain, the *r* values found were 0.30 for both the A24/Cruzeiro and the A/Arg/01 virus strains. For the A24/Cruzeiro virus strain, the *r* values determined were 0.19 and 0.15 for the A/Arg/00 and the A/Arg/01 virus strains, respectively. These results were indicative of low levels of cross-neutralisation between the new serotype A isolates and the serotype A vaccine strain A24/Cruzeiro. Similar results were initially obtained by complement fixation (data not shown). For O1 FMDV strains, which quickly disappeared from the field, the complement fixation analysis showed a close relationship with the vaccine strains O1/Campos and O1/Caseros (data not shown).

Further antigenic characterisation was performed using MAbs developed against unequivocally characterised reference strains, which in the case of the O1 strains were represented by viruses O1/Caseros and O1/Campos. The results in Fig. 1 show the reactivity of the homologous reference and field virus strains with a panel of nine MAbs (see Methods). Reference strain O1/Caseros fully reacted with seven of the MAbs and showed no reactivity with MAbs 17 and G8 which were specific for the O1/Campos virus strain. On the other

Table 3
Monitoring of viral circulation in the field. Sero-epidemiological surveys performed in 2002 and 2003

Area	Bovine Age: 6–12 months		Bovine Age: 1–2 years		Ovine	
	^a Pos/total	(%)	^a Pos/total	(%)	Pos/total	(%)
Survey 2002						
Central	75/11888	0.63	209/9669	2.16	59/91710	0.64
Peripheral	16/7250	0.22	113/7580	1.49	ND	ND
North Patagonia B- South Patagonia	5/3163	0.16	7/2045	0.34	0/17990	0.00
Survey 2003						
Central	^b 23/6173	0.37	46/4117	1.12	ND	ND
Peripheral	^b 10/2573	0.39	15/1718	0.87	ND	ND
North Patagonia B-South Patagonia	ND	ND	ND	ND	NA	

ND: not done. NA: not available at the time of writing this paper.
^a Pos: Positive bovine samples to ELISA 3ABC and EITB [13]. The number of positive animals to FMDV non- structural proteins is shown per area and per species tested.
^b Premises with positive samples were checked as described by the OIE procedures.

hand, reference strain O1/Campos showed full reactivity with six of the MABs and lacked reactivity against MABs 12 H, 3A and 13, which were specific for O1/Caseros virus strain. The two field isolates O1/Corrientes-Arg/00 (MC38779) and O1/Misiones-Arg/00 (MC38667) showed two clearly different reactivity profiles when reacted with the serotype O MABs panel, indicating that although the viruses belong to serotype O, they had undergone important genetic and antigenic variations upon circulation in the field (see below). Moreover, the

circulation of these two isolates was transient as judging by their rapid disappearance from the field. No virus isolates of this serotype were detected later on.
The reactivity of serotype A field virus isolates with a panel of 19 different MABs raised against virus reference strains A24/Cruzeiro, A/Arg/79 and A/Arg/87 is shown in Fig. 2. With this panel we were able to clearly differentiate each reference strain, as well as the two representative field virus isolates, A/Arg/00 and A/Arg/01.

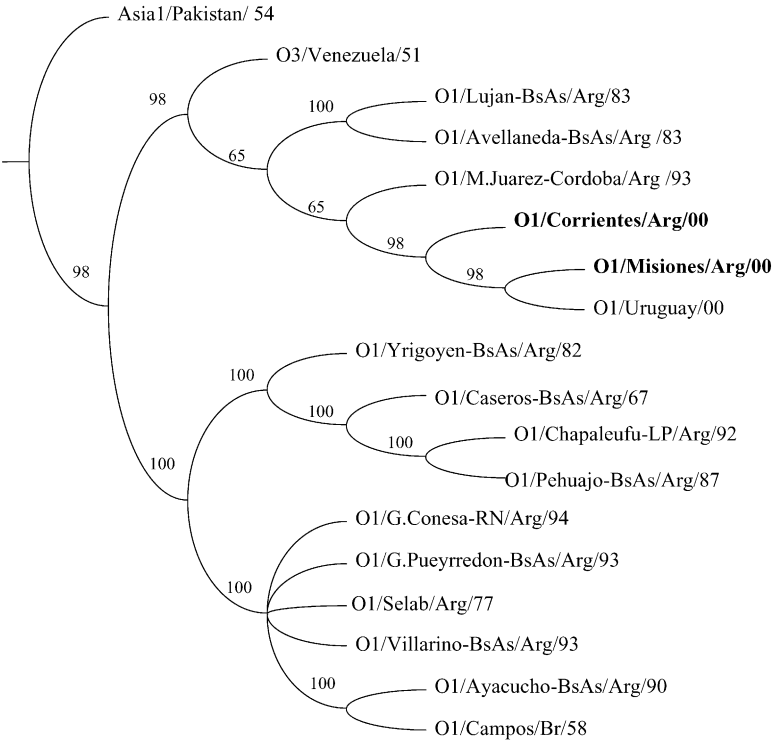


Fig. 3. Cladogram constructed using the UPGMA algorithm, showing the genetic relationships between Argentine and South American FMDV serotype O strains, based on the complete VP1 encoding sequence. Viruses analysed in this study are in bold. Numbers indicate the relative frequency of each node. Pakistan 54 virus strain (serotype Asia 1) was used as a tree root. Genebank accession numbers or sequence source from top to bottom are AJ251478, AJ004645, AJ308706, König et al. (unpublished) [17]; N. Knowles (unpublished); Z21862, U82271, AJ308708, König et al. (unpublished); AJ292206, AJ292209, AJ308705, AJ292205, AJ308696 and AJ320488.

While A/Arg/00 and the type O virus strains had a restricted circulation in the field, the A/Arg/01 strain showed massive circulation and a surprisingly high virulence.

3.2. Sequence and genetic relationship

Two different lineages of O1 viruses are shown in the cladograms of Fig. 3; their ancestor is probably not far away in time. From six virus isolates from year 2000 sequenced so far, five are grouped in one branch (including O1/Corrientes-Arg/00 (MC38779)) and one is set apart (O1/Misiones-Arg/00 (MC38867)).

The cladograms in Fig. 4 show that type A viruses isolated during the 2000 and 2001 outbreaks could be grouped into one of two different branches of the phylogenetic tree. From all the isolates sequenced so far, only two representative isolates were selected for inclusion in this cladogram because of their divergence: a virus strain isolated in Trenque Lauquen (MC267) and another virus isolated in General Villegas (MC38796), both in the Province of Buenos Aires. All other virus isolates had subsequently been classified into ei-

ther the A/Arg/00 FMDV group or the A/Arg/01 FMDV group. All the viruses isolated in 2001, one from the year 2000 and the only virus strain isolated in 2002 belonged to the latter group. On the other hand, it was not possible to find a close relationship between the viruses belonging to both groups and the ones circulating during the previous years in Argentina or the reference serotype A virus strains. However, a close genetic relationship between the A/Arg/01 virus strain and the viruses isolated during the same year in Uruguay and Brazil is apparent.

3.3. Challenge studies and animal immune response after vaccination

The aim of the trials was to assess the degree of protection induced by the available vaccines against the field viruses isolated in Argentina in 2000 and 2001. In addition, vaccines containing the two serotype A FMDV strains, A/Arg/00 and A/Arg/01, were developed. It took three to four months to perform cross-neutralisation assays and cross-protection tests, while, simultaneously, the new A Arg strains

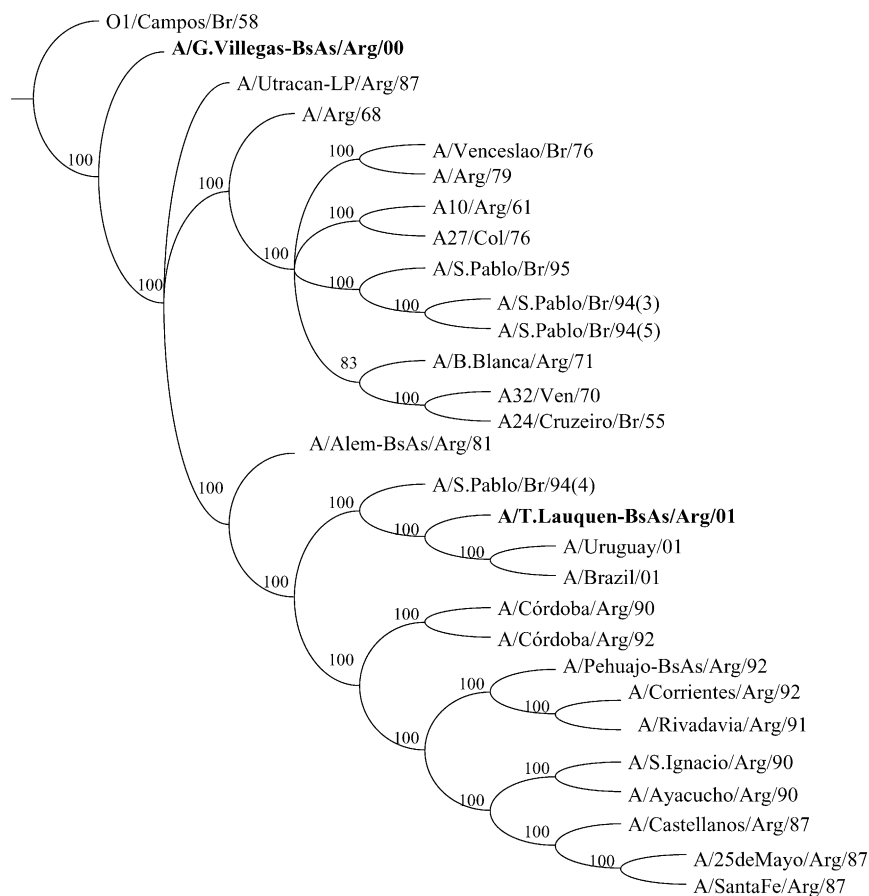


Fig. 4. Cladogram constructed using the UPGMA algorithm showing the genetic relationships among Argentine and South American FMDV serotype A strains, based on the complete VP1 encoding sequence. Representatives of the A/Arg/00 and A/Arg/01 virus strains are in bold. Numbers indicate the relative frequency of each node. The O1/Campos virus strain was used as a tree root. Genbank accession numbers or sequence source from top to bottom are AJ320488, König et al. (unpublished), AJ306221, AJ308694, M12905, K03345, X00429, König et al. (unpublished), [15], [15], [15], AJ308695 [16], K03340, AJ306219, [15], König et al. (unpublished), Knowles (unpublished), Knowles (unpublished), AJ308697, AJ308700, AJ308702, AJ306701, AJ308699, AJ308696, AJ308698, U62255, AJ306220 and AJ306222.

were being adapted for large-scale production and vaccine use.

Table 1 shows the composition of several vaccines used in these studies. It is worth mentioning that protection studies were performed with available experimental and industrial vaccines, and monovalent vaccines were prepared at laboratory-scale [11].

The Animal Health Service in Argentina evaluates potency and immunity duration of FMD oil vaccines based on a challenge test at 90 days post vaccination (dpv). However, in several trials reported in this study, early challenge at either 21 or 30 dpv was performed due to urgency reasons. Thereby, it is possible that vaccines challenged at 30 dpv were tried in more strict conditions than others challenged at longer times.

3.3.1. Cross-protection study for the A/Arg/00 virus strain—vaccination trial no. 1

As shown in Table 1, protection data from trial no. 1 showed that cattle vaccinated with one dose of monovalent A/Arg/00 vaccine (vaccine no. 2) induced a higher percentage of protection against challenge with the homologous strain (90% protection) than that provided by vaccines prepared from heterologous strains, including a monovalent A24/Cruzeiro vaccine (30% protection) and a polyvalent vaccine (60% protection, vaccine no. 4). Two doses of vaccine including only heterologous strains (O1/Campos, A24/Cruzeiro, C3) were necessary to induce protection levels similar to that achieved by one dose of homologous monovalent vaccine when challenged with A/Arg/00 virus strain (100% protection, obtained later with two doses of vaccine no. 4).

3.3.2. Cross-protection study for the A/Arg/01 virus strain—vaccination trials nos. 2 and 3

Protection data from trials no. 2 and 3 are also shown in Table 1. Cattle vaccinated with a monovalent A/Arg/01 vaccine (vaccine no. 3) induced a higher percentage of protection against homologous virus challenge than cattle given one

dose of vaccines made with heterologous A24/Cruzeiro or A/Arg/00 virus strains. However, as in trial no. 1, two doses of polyvalent vaccines made from heterologous FMDV strains induced strong immunity and protection (see data from trial no. 3). The lowest antibody titres were detected in cattle vaccinated with monovalent A/Arg/00 vaccine (vaccine no. 2). These animals were not protected against A/Arg/01 virus challenge.

3.3.3. Dose-response study for A/Arg/01—vaccination trial No. 4

Since A/Arg/01 was the main circulating virus strain since March 2001, a trial was conducted to determine the minimum amount of this strain to be included in polyvalent formulations in order to induce protection. Under the experimental conditions of the trial, a minimum of 6 µg of 146S of A/Arg/01 was found necessary to induce levels above 80% protection against homologous challenge after a single dose. The results showed that IpELISA antibody titres to A/Arg/01 increased significantly ($P < 0.01$) with 146S concentration (Table 1). The contribution of other serotype A strains present in polyvalent vaccines, which might have played a role in cross-protection, was not assessed.

3.4. Assessment of herd immunity by IpELISA using sera from cattle under the FMD vaccination programme

Results from herd immunity studies performed in 2001 are shown in Fig. 5. Cattle 1–2 years of age evidenced high immunity levels against all FMDV strains included in the vaccine, revealing a satisfactory vaccine coverage in the age category with the highest risk of disease. The EPP values were calculated as described in the Section 2, using the titre ranges shown in Table 2.

3.5. Monitoring of viral circulation in the field

The results are presented in Table 3.

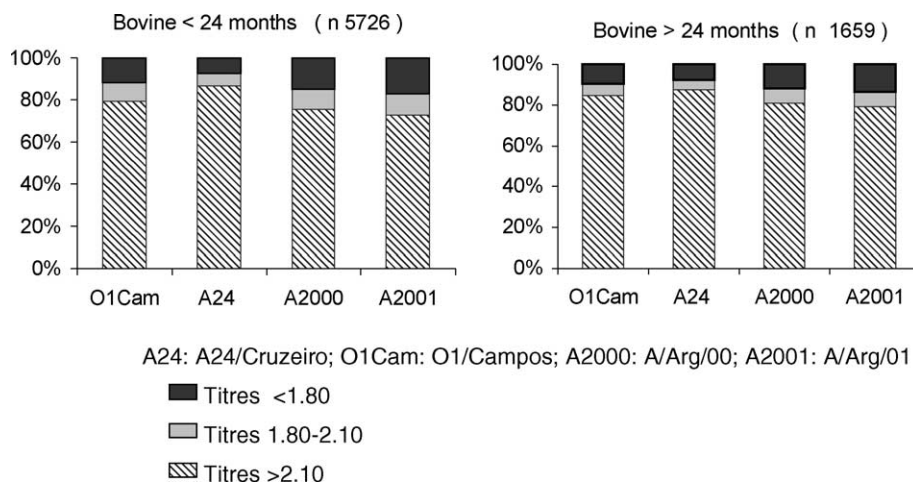


Fig. 5. Herd immunity assessed in 2001 in different age animal categories. The 7385 animals were bled and the serum analysed for structural proteins by IpELISA. Results are shown in the categories of animals younger or older than 24 months.

Table 4
Number of FMDV outbreaks, virus strains, and vaccine strains used in Argentina between 2000 and 2002

Step	Year	Virus strain	No. of outbreaks	Vaccine strains ^a
Emergency step 1 (July–December)	2000	O1	9	O1/Cas-A/79-A/87-C/85
	2000	A/Arg/00	115	O1/C-A24-C3 Indaial ^b
Emergency step 2 (December–March)	2000/2001	A/Arg/00	269	O1/C-A24-A/Arg/00
National campaign 1 (March–July)	2001	A/Arg/01	2075	O1/C-A24-A/Arg/00
National campaign 2 (August–December)	2001	A/Arg/01	93	O1/C-A24-A/Arg/00/A/Arg/01
National campaign 3 (February–June)	2002	A/Arg/01	1	O1/C-A24-A/Arg/00-A/Arg/01
National campaign 4 (September–December)	2002	–	0	O1/C-A24-A/Arg/00-A/Arg/01

Emergency Step: Only acute measures were taken in order to control the outbreaks. National Campaign: Official massive vaccination campaigns and systematic vaccination, and epidemiological measures.

^a O1/Cas: O1/Caseros; A/79: A/Arg/79; A87: A/Arg/87; C/85: C3/Arg/85; O1/C: O1/Campos; A24: A24/Cruzeiro.

^b For details of vaccine composition see text.

Survey 2002: In younger cattle as well as in sheep the level of prevalence of individuals with NSP was in general very low. In older cattle prevalence was significantly higher than in younger cattle and sheep ($P < 0.01$). The frequency of positive reactions in sheep was similar to that observed in younger cattle. The highest prevalence was detected in the Central Area while the lowest was observed in the North Patagonia B and South Patagonia regions, where no outbreaks were reported.

The twelve cattle samples that tested positive for NSP antibodies in North Patagonia B/South Patagonia (Table 3) were obtained from eight farms. Clinical signs of the disease were not observed either in the sampled farms or in the region. Therefore, the eight farms were re-checked and a total of 703 serum samples were collected including the 12 animals mentioned. All of them tested negative for NSP antibodies. Considering that the time span between the two sampling procedures was short, it is not probable that the antibody

response had waned to undetectable levels when re-sampled. We concluded that the discrepancy was due to false positive results from the test. No sheep from Patagonia tested positive.

The lower prevalence in younger cattle as compared to older cattle indicates a reduction of the incidence of new infections during this year.

Survey 2003: Values of prevalence were consistently low and no major differences between areas were observed. As in the previous survey, the prevalence in younger cattle was significantly lower than in older animals, indicating that the incidence of new infection was decreasing. Furthermore, according to the OIE procedures, 60 bovines were individually identified in each positive premise and serum samples were tested for NSP antibodies as previously described. At the time of writing this paper, all samples taken from the premises where positive results were detected, tested negative to NSP antibodies in the re-checking assay.

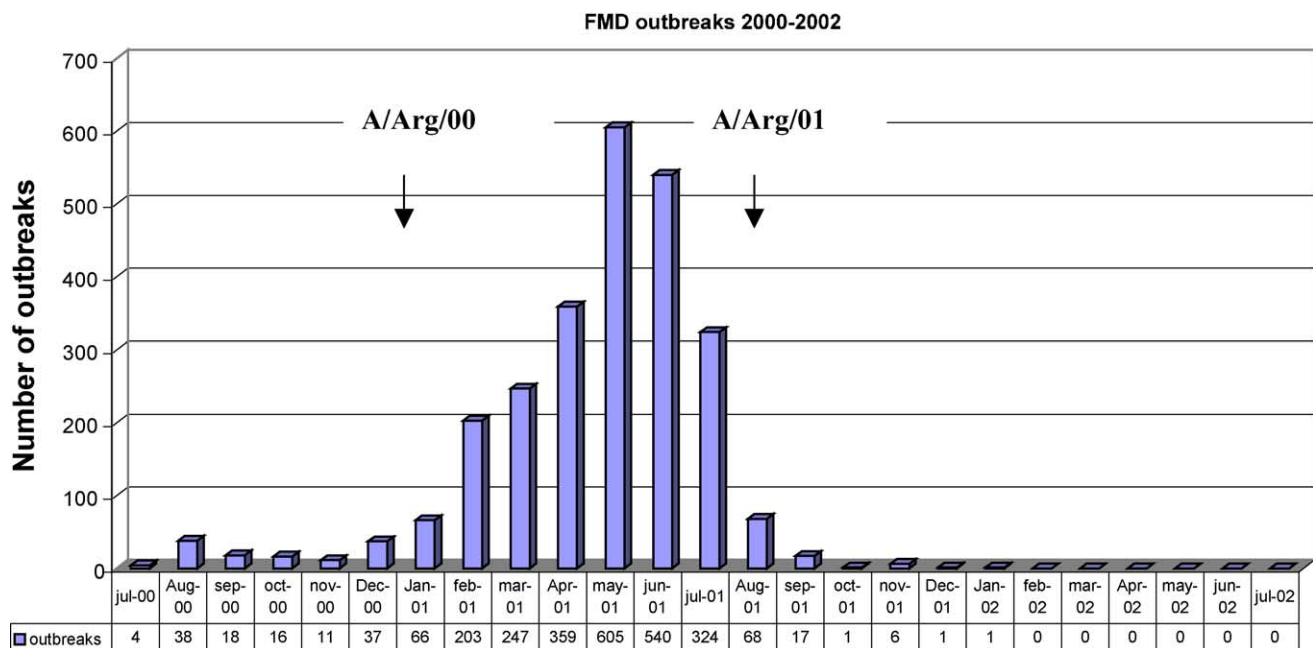


Fig. 6. Number of outbreaks notified in Argentina in the 2000–2002 period. The total number of outbreaks is shown (all serotypes included). The arrows mark the time when the field virus strains A/Arg/00 and A/Arg/01 were incorporated into the vaccines.

3.6. FMDV outbreaks and vaccination procedures

Table 4 shows the number and serotype of FMD virus outbreaks per year and the strains present in the vaccine used during the FMD epidemics. The nine outbreaks of FMD due to O1 strains occurred during the year 2000, whereas the 115 outbreaks of A/Arg/00 virus strain took place during 2000 and the beginning of 2001. The A/Arg/01 virus strain circulated from October 2000 to January 2002.

The monthly epidemic evolution of outbreaks for all strains is shown in Fig. 6. Arrows indicate the dates when the new serotype A strains were introduced into the vaccine formulation.

4. Discussion

Argentina was recognised by the OIE as a country free of foot-and-mouth disease without vaccination in April 2000. Fourteen months after vaccination was halted (May 1999), several outbreaks of the disease were reported in cattle in the North-east region. Due primarily to cattle movements, the disease spread rapidly to several provinces, (all outside the Patagonia region), affecting the main cattle production area.

Four different lineages of FMDV circulated during the 2000–2002 epidemics. Two O1 FMDV strains circulated during 2000. Serotype A strain A/Arg/00 circulated in 2000 and part of 2001 and strain A/Arg/01 from October 2000 to January 2002. Primary diagnosis and preliminary serotype identification was performed by ELISA and complement fixation tests and through serial passages in BHK-21 cell cultures or suckling mice. Further characterisation was performed by nucleotide sequencing and monoclonal antibodies profiling.

Monoclonal antibodies profiling indicated that field strains of serotypes O and A were clearly different from the reference and vaccine strains used in Argentina.

Sequencing data indicated that both O1 FMDV strains were related to previous South American isolates, and belonged to two different lineages (O1/Misiones-Arg/00 and O1/Corrientes-Arg/00). In average, a 6% difference in the nucleotide sequences of VP1 was found between both lineages. O1/Misiones-Arg/00 isolated in October 2000 was closely related to an O1 virus strain from a previous outbreak in Rio Grande do Sul, Brazil [18] and a simultaneous outbreak in Artigas, Uruguay [19]. The results suggest that both represent different independent FMDV introductions in Argentina, since it is not probable that two contemporary viruses could have evolved so much in the short time they were present in the field [20].

Outbreaks caused by O1 strains quickly disappeared without further dissemination, due to the immediate application of efficient control measures—mainly stamping out and ring vaccination—strict control of animal movements, and the use of vaccines from the Argentine Vaccine Bank containing an O1 virus strain of recognised broad protection capacity. Despite the different reactivity with monoclonal antibodies

shown in Fig. 1, previous results of cross-protection and virus-neutralisation among different O1 strains showed that highly potent type O1 vaccines are likely to protect against other O1 virus strain variants [21,22].

As shown in Fig. 4, the two serotype A virus strains that circulated during 2000 and 2001, A/Arg/00 and A/Arg/01, also belonged to two different lineages. For serotype A viruses, antigenic studies indicated low levels of molecular and antigenic relatedness between field strains and previous vaccine strains used in Argentina and in neighbouring countries, showing 'r' values lower than 0.4. Further studies are ongoing to establish the statistical significance of the neutralising antibodies data presented here, and to investigate relationships of these strains with other vaccine strains.

Outbreaks caused by the A/Arg/00 virus strain were not reported after the first months of 2001. Thereafter, all field isolates belonged to the A/Arg/01 virus strain lineage. The two serotype A strains had different clinical behaviour. A/Arg/00 strain showed low virulence. Field veterinarians reported that the clinical signs caused by this strain in the field and in the challenge trials were mild, and only few and small lesions were to be seen in the infected animals. This strain had low dissemination and was rapidly controlled.

On the other hand, the A/Arg/01 FMDV strain was highly virulent and caused severe lesions in the affected animals in the field and in the challenge trials. Moreover, in two farms calf deaths due to viral infection were reported.

The origin of the A/Arg/00 strain could not be related to any of the other virus strains previously circulating in the region (Fig. 4), at least considering the sequence information available from the genebank. In this regard, we support the considerations made in the report of the recent session of the research group of the European Commission's Standing Technical Committee for the control of foot-and-mouth disease (Gerzensee, Berne, Switzerland, 16–19 September, 2003) regarding the relative lack of genotype information of virus strains from many regions.

In fact, the A/Arg/01 strain was found to be closely related to strains that caused outbreaks in Uruguay and Brazil in the same year, and probably have a common origin with the viruses isolated in 1994 in San Pablo, Brazil. Therefore, there is evidence that at least two virus strains (A/Arg/01 and O1/Misiones-Arg/00) also circulated in different countries of South America (see Figs. 3 and 4).

The results obtained in cross-challenge studies indicated that protection against the A/Arg/00 and A/Arg/01 virus strains after primary vaccination was induced by vaccines containing homologous strains. vaccines containing heterologous serotype A strains achieved satisfactory levels of protection only after re-vaccination (trials no. 1 and 3). Therefore, in order to induce a high level of protection in animals vaccinated with only one dose, the inclusion of the new strains in the vaccines was mandatory. This was achieved for the A/Arg/00 strain in December 2000 and for the A/Arg/01 strain in August 2001, from the second vaccination campaign to the present day (Fig. 6 and Table 4).

The importance of having actualised strains in the vaccines is a key issue for countries like Argentina, where the production system and the large land areas involved make it impossible to have animals vaccinated twice within a short period of time. In these countries there is intense movement of animals from breeding to fattening areas, which in most cases are separated by great distances. The protection of young calves, the most susceptible animals (approximately 20% of the bovine population), has to be achieved either with two doses of vaccine or with very specific vaccines capable of inducing high protection after one dose. It is important to point out that this animal category was totally negative at the time when the outbreaks began. This was due to the halting of vaccination in May 1999. In this regard, we strongly state that the inclusion of the new strains in the vaccines was one of the most important factors which allowed for the rapid control of the disease in Argentina.

Moreover, the appearance of new variants resistant to neutralisation due to selective pressure under sub-neutralising conditions has been previously reported [23]. In this regard, if the strains in the vaccines had not been actualised, it is possible that the use of the vaccine strain A24/Cruzeiro, with only partial protection and partial virus neutralising capacity, may have generated conditions for the selection of new variants resistant to neutralisation.

Assessment of herd immunity to FMD and detection of viral activity have been shown to be reliable indirect indicators of the progress of the FMD control campaigns and useful in the detection of at-risk areas [24,25].

Herd immunity was found to be above 75% in the sampled animals, assuring satisfactory protection levels (Fig. 5). It should be noted that in this case only antibodies due to vaccination had been considered, as positive reactors to NSP were not included. Good vaccine coverage was demonstrated by the dramatic decrease in the number of outbreaks since September 2001, after the second round of vaccination of the whole cattle population (Fig. 6). The vaccines used in Argentina during the epidemics of 2000–2001 did not induce antibodies against non-structural proteins, as assayed by different available methods (results not shown), which is highly desirable in order to assess viral activity in the vaccinated cattle population.

Results from Survey 2001 (not shown) indicated that FMDV circulation outside the affected premises and its contiguous ones was very low, suggesting that the control measures implemented to avoid the spread of the virus were effective. Results from surveys 2002 and 2003 showed that virus circulation declined consecutively year after year. Moreover, the relationship between prevalence in younger and older cattle suggested that incidence of new infections was decreasing. Preliminary data of Survey 2003 confirms this finding.

The prevalence observed in the different areas is directly related to the frequency of outbreaks during the 2000–2002 epidemics, being greater in the Central Area and lower in Peripheral Area.

The decline of virus circulation was caused by several factors, of which the most significant was probably the systematic and massive vaccination campaigns.

Finally, we would like to remark a major point gleaned from this particular experience: Given the complexity of the breeding system in Argentina, if the initial contingency measures fail, a quick answer with a massive immunisation programme is critical in a situation where outbreaks involve a large number of animals distributed in an extensive geographical area.

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