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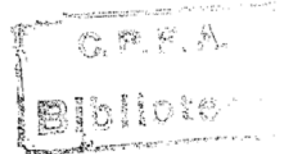
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Bibliote

FOOT-AND-MOUTH DISEASE VIRUS ENZYME-LINKED IMMUNOELECTROTRANSFER BLOT FOR DETECTION OF ANTIBODIES AGAINST REPLICATING VIRUS IN SERA FROM PERSISTENTLY INFECTED ANIMALS

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INTRODUCTION

An enzyme-linked immunoelectrotransfer blot (EITB) assay, was developed at the Pan American Foot-and-Mouth Disease Center, PAHO/WHO, for the detection of antibodies against replicating foot-and-mouth disease virus (FMDV) in sera from cattle with suspected persistent infection. Using only a minute quantity of sera, the test measures the levels of antibodies against some of the proteins produced by the virus when it replicates in the host.

The high specificity and sensitivity of the method, as well as its simple and rapid performance, low cost, easy throughput, which enables the processing of many samples, make it adequate for large-scale epidemiological surveys, as well as for selection of animals during import/export testing.

Since the test discriminates antibodies attributable to infection or vaccination, it allows for:

a) a confirmation of the disease-free status of a region during the last stages of systematic vaccination, contributing effectively to the decision for continuation or suspension of vaccination.

b) a precise evaluation of viral activity in areas where vaccination programs cannot be discontinued due to the risk of foot-and-mouth disease transmission from the neighbouring countries.

A major advantage of the test is that it does not require the handling of infectious FMD virus, making it adequate for its use in disease free regions.

PRINCIPLES OF THE TEST

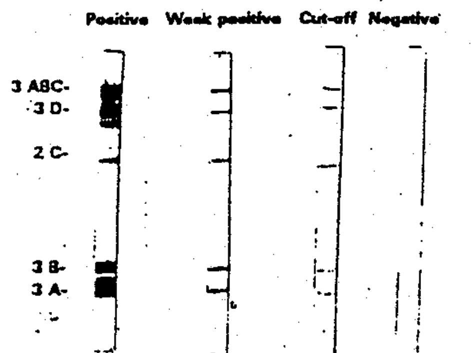
The assay uses a set of purified recombinant DNA-derived nonstructural viral antigens as serologic probes in an EITB method.

A mixture of five FMDV nonstructural proteins, 3A, 3B, 2C, 3D and 3ABC, are resolved on 12.5% polyacrylamide gel electrophoresis, and transblotted to nitrocellulose membrane filters. The presence of antibodies to replicating FMDV in the serum sample will result in the formation of immunocomplexes with the bound antigens, which are subsequently detected by an affinity purified rabbit anti-species IgG conjugated with alkaline phosphatase. Addition of the enzyme substrate produces a dark brown color in proportion to the amount of specific antibody present in the sample. Positive or negative results are determined by visually comparing the intensities of the antigen lines with those of the cut-off control sera.

The assay is used as a single dilution screening test. Alternatively, semi-quantitative information can be obtained through titration of reactive results. By testing serial dilutions of a reactive sample, the dilution required to eliminate reactivity can be determined.

Validity of the individual tests

The identity and location of the antigens after reaction with the control sera are indicated.



For the test system to be valid, the following criteria must be met:

- The positive, weakly positive, as well as the cut-off control strips, must show reaction with all the antigens.

- The negative control strip must not show reactivity.

A set of control sera must be assayed for each nitrocellulose sheet, which defines one transferred gel.

Interpretation

Extensive evaluations have shown that results may be interpreted as follows:

- A sample is negative, if:

  . all lines are below the reactivity of the cut-off control.
  . a maximum of 2 bands are above the reactivity of the cut-off control.

- A sample is positive, if:

  . all five antigens have a reactivity equal or higher than the cut-off control.

- A sample is indeterminate if:

  . the above-mentioned criteria for positive or negative reactivity are not met.

Reference sera

Positive control: The serum stock was derived from a pool of sera from infected animals of proven disease status.

Weakly positive control: The serum stock was derived from a pool of sera from animals which showed the highest EITB antibody levels in regions with very low epidemiological risk (3 years after the last outbreak). In these animals, virus recovery and IDAG determinations were negative.

Cut-off control: The serum stock was derived from a pool of sera with maximal background reactivity observed in animals from FMD free regions, with no vaccination.

Negative control: The serum stock was derived from a pool of sera from animals in FMD free regions, with no vaccination.

All control sera had been aliquoted and stored at -70 °C.

PERFORMANCE

The standardization of this method, has been achieved during 1990-1992. Its application in the field was evaluated through collaborative studies conducted in Uruguay, Argentina, Paraguay, Venezuela, Colombia and Brazil.

The EITB procedure has been validated by using animals of proven disease status (whether immunized or not), as well as field animals from regions with known epidemiological situation. Results were compared with those obtained with the existing serological method, based on an immunodiffusion in agarose gel test (IDAG), for detection of anti-VIAA antibodies.

Sensitivity

High sensitivity of the method is indicated in **Fig. 1** and **Table 1**.

- Presence of antibodies against nonstructural antigens 3A, 3B, 2C, 3D, and 3ABC in serum samples obtained sequentially from nonvaccinated animals with experimental persistent infection, indicated that the test is suitable for detecting FMDV antibodies persisting at late stages of infection, during which IDAG results were no longer positive, and virus was not recovered from the esophageal-pharyngeal (EP) fluids, or was only occasionally recovered **(Fig. 1)**.

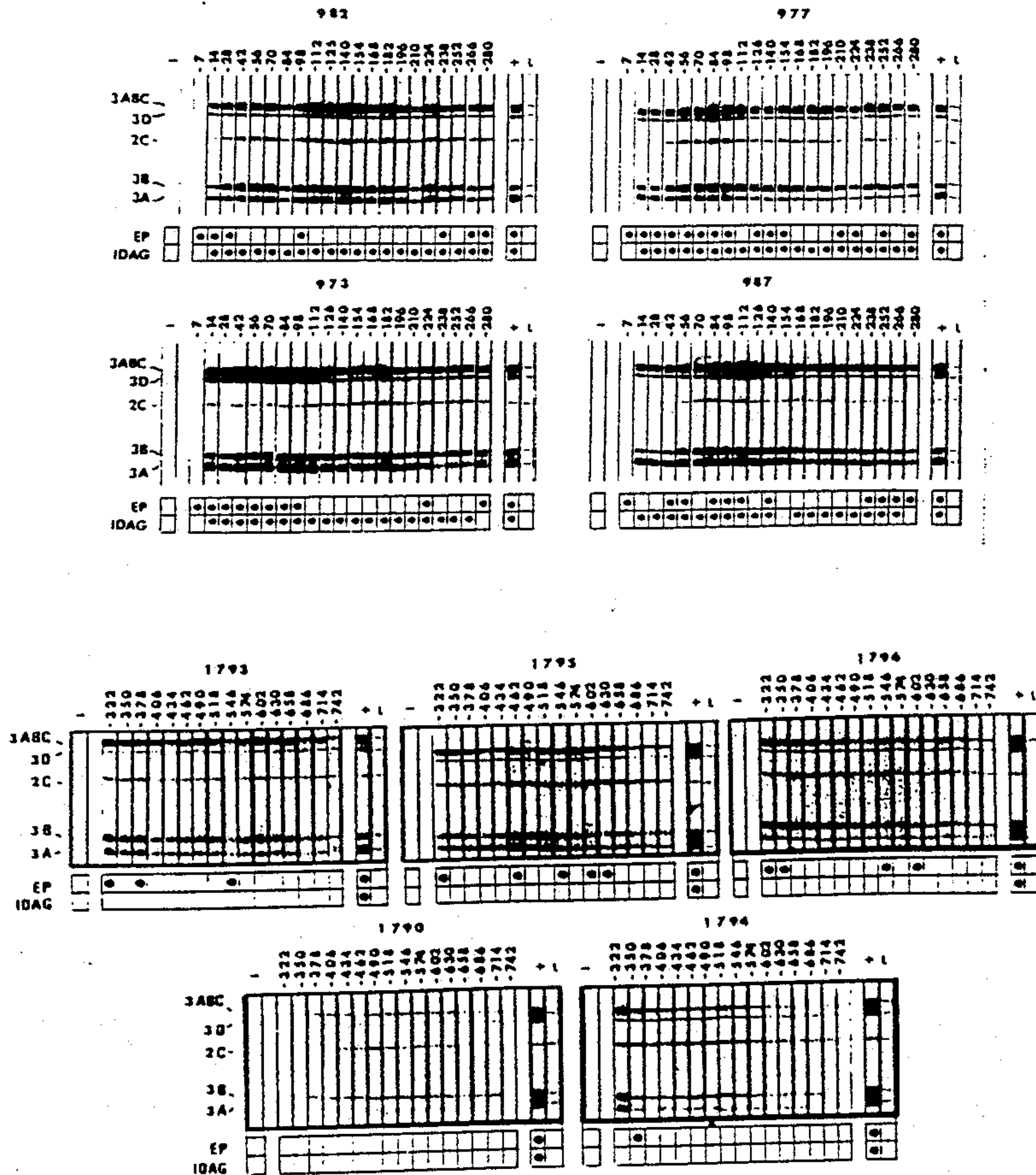


Figure 1-Serum antibody response of infected cattle to recombinant antigens 3A, 3B, 2C, 3D, and 3ABC by EITB. Viral isolation (EP) or positive-VIAA determination (IDAG) is indicated by a dot.
Sera from 4 infected cattle (No. 982, 977, 973 and 987) were tested serially at 14-day intervals between 0 and 280 days after inoculation.
Sera from 5 infected cattle (1793, 1795, 1796, 1790, and 1794) were tested at montly intervals between 322 and 742 days after infection. Control strips included FMDV-negative serum (-); FMDV-positive serum (+), FMDV-cut-off serum (L).
Reference: Bergmann et al, 1993.

- A group of 50 vaccinated cattle, infected under natural conditions, as indicated by recovery of virus from EP fluid, produced the same panel of infectious-specific antibodies as those from the experimental model system. Comparison of the results obtained by EITB and the alternative IDAG test are shown in the following table:

Table 1: EITB and IDAG positive results of vaccinated cattle, infected under natural conditions

FMDV (+) ANIMALS (assessed by virus recovery from EP fluids)	IDAG (+)	EITB (+)
50	28	50

Specificity

- Cattle sera in FMD-free regions such as El Salvador, Panamá, Chile, Patagonia Argentina, and Uruguay were analyzed, and the results are presented in the following table:

Table 2: EITB specificity

FMD-free region	Total Sera	EITB Interpretation			Specificity %
		Positive (%)	Indeter-minate (%)	Negative (%)	
Without vaccination	841	0	0.2	99.8	99,8
With vaccination (animals under 2 years of age)	1459	0	0.4	99,6	99,6
With vaccination (animals over 2 years of age)	509	0	1,2	98,9	98,8

- The response of sera from animals infected with a variety of bovine RNA viruses (bovine diarrhea virus, bluetongue virus, bovine coronavirus, rinderpest virus, vesicular stomatitis virus, bovine ephemeral fever virus) or DNA viruses (bovine papular stomatitis virus, infectious bovine rhinotracheitis virus, bovine herpesvirus type 2, and malignant catarrhal fever virus) indicated lack of cross-reactivity. (Reference: Bergmann et.al, 1993).

Reproducibility

Satisfactory results were obtained when weakly positive samples were assessed for inter- and intra- assay variability.

Discrimination between seropositivity due to infection or vaccination

The assay is capable of discriminating seropositivity due to vaccination or infection.

- Discrimination *is attained even under* conditions of increased immune response, such as occurs with highly concentrated vaccines and/or shorter immunization programs. This observation contrasted with results *obtained* by IDAG/VIAA in which most of *the cattle became* FMDV-*positive between* 15 and *30 days after revaccination*. At these times, several samples showed EITB-reactivity only against polypeptide 3D, major component of the VIAA antigen. **Figure 2** (Reference Bergmann et al, 1993).

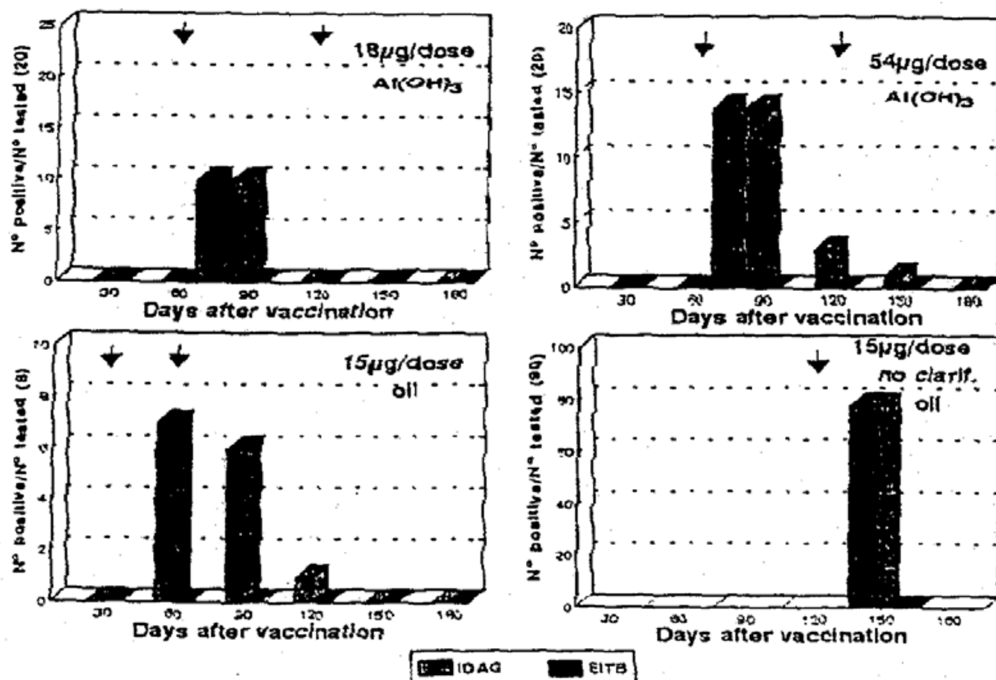


Figure 2- *Sera from cattle vaccinated and revaccinated at the times* indicated by the arrows were analyzed by IDAG and EITB; results of the EITB assay were negative in all cases, so only positive results are shown for the IDAG. The IDAG test using the *conventional VIAA, was carried out as described* (Alonso et. al, 1988).

No clarif.: no clarification.

- Analysis of sera from animals immunized with several vaccine preparations obtained from different laboratories in Argentina, Brasil, Paraguay, and Uruguay indicated the induction of antibodies agains 3D and/or 2C, in some of the sera taken at 30 days after immunization.

FIELD STUDIES

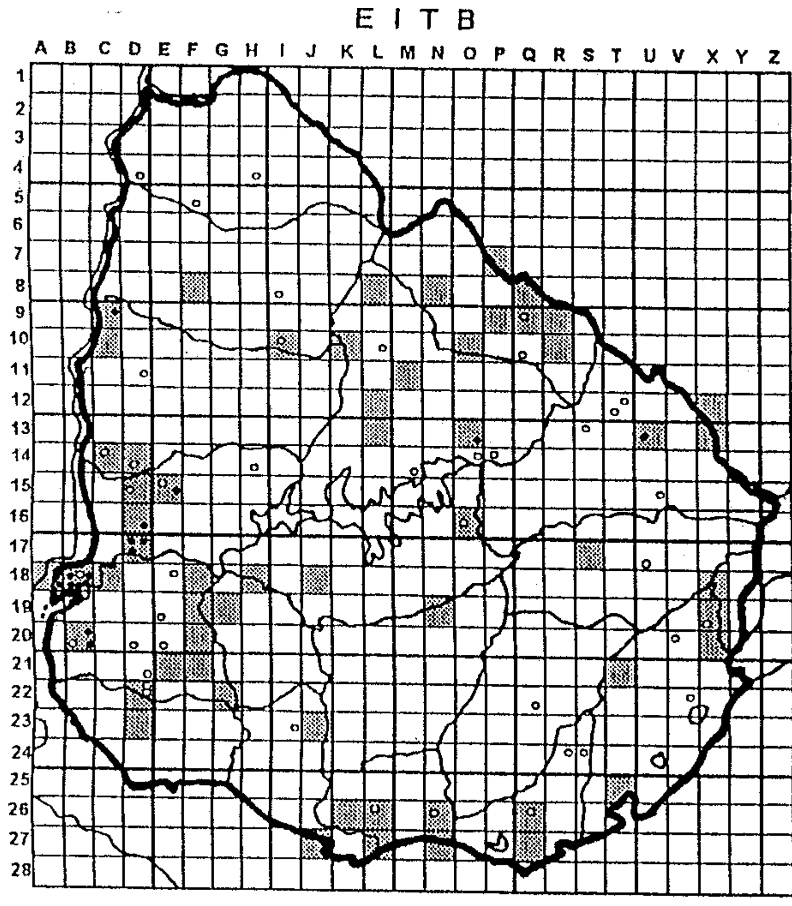
Identification of FMD-free areas

A sampling of 2.194 cattle sera was collected in Uruguay, in 1992, two years after the last outbreaks of FMD. EITB-positive reaction was restricted to sera from cattle in squares with the last outbreaks of FMD during 1989-90, and to animals over 2 years of age. In contrast, the IDAG test yielded a rather homogeneous distribution of positive results when squares without FMD during the last four years preceeding the sampling were compared with those affected during 1989-1990. (Table 3, Fig.3).

Table 3: Distribution of EITB and IDAG reactivity in free and affected squares

SQUARE STATUS	NUMBER OF SQUARES				
	EITB +	EITB -	IDAG +	IDAG -	TOTAL
FREE	0	28	9	19	28
AFFECTED IN 1989-1990	9	11	12	8	20
TOTAL	9	39	21	27	48

(Reference: Bergmann et al, 1995)



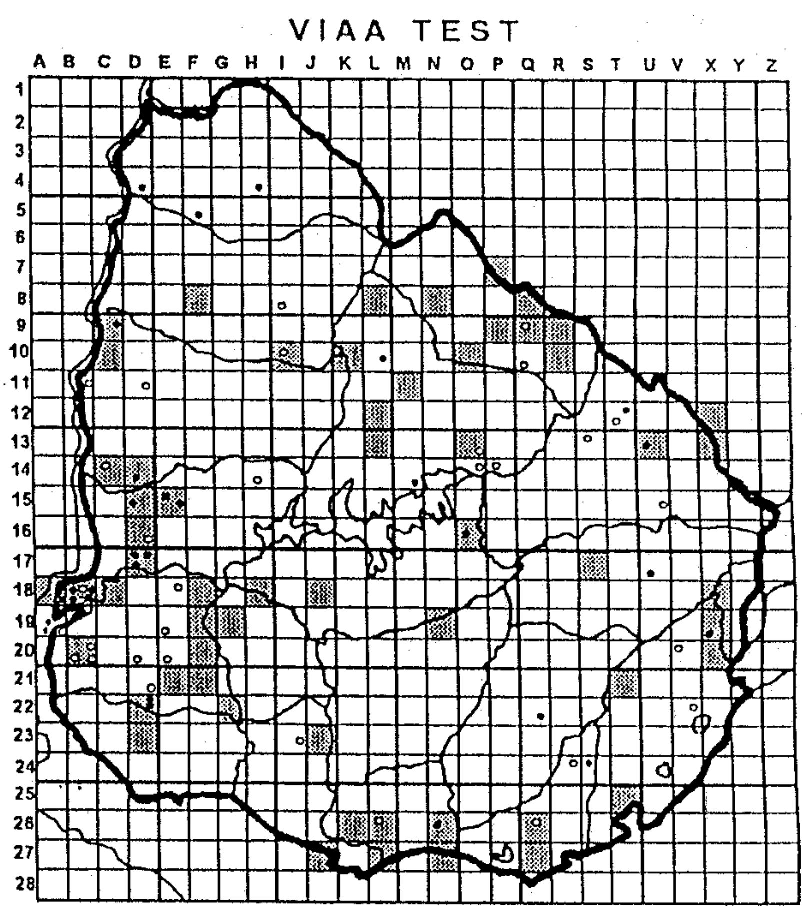


Figure 3. Distribution of FMD seropositive herds in Uruguay as assessed by EITB and IDAG test.
Farms included in the survey, represented by dots, were located on a grid square superimposed on the map of Uruguay. The shaded squares indicate that FMD has been confirmed in 1989-1990. The dark dots represent farms with at least one positive serum in the EITB or IDAG (VIAA test) assays. Empty dots correspond to farms where no positive-determinations were recorded. (Reference: Bergmann et al, 1995)

A paired comparison of the results obtained by the EITB and IDAG tests for the individual serum samples with regard to age categories and geographical distribution is shown in Table 4.

Table 4: **Number of positive and negative EITB and IDAG determinations according to age and past FMD clinical experience:**

SQUARES WITH NO OUTBREAKS SINCE 1989

Animals under two years

	IDAG +	IDAG -	TOTAL EITB
EITB +	0	0	0
EITB -	21	496	517
TOTAL IDAG	21	496	517

Animals over two years

	IDAG +	IDAG -	TOTAL EITB
EITB +	0	0	0
EITB -	36	505	541
TOTAL IDAG	36	505	541

SQUARES WITH LAST EPISODES IN 1989 OR 1990

Animals under two years

	IDAG +	IDAG -	TOTAL EITB
EITB +	0	0	0
EITB -	29	533	562
TOTAL IDAG	29	533	562

Animals over two years

	IDAG +	IDAG -	TOTAL EITB
EITB +	9	23	32
EITB -	39	503	542
TOTAL IDAG	48	526	574

Reference: Bergmann et al, 1995.

Elimination of false-positive IDAG results in field samples

A serologic survey was made in Ayacucho, Buenos Aires, Argentina, two years after the last FMD recorded episodes. Results had shown an unusually high number of IDAG positive determinations (10% and 60% in animals under and over 2 years of age, respectively). All IDAG positive results obtained with sera from animals born after the last outbreak, became negative by EITB. In addition, the EITB reduced the number of IDAG-positive results from 60% to 16% in animals over 2 years of age. **(Table 5).**

Table 5: Comparison of the results obtained by EITB and IDAG

Animals under two years

		IDAG +	IDAG -	TOTAL EITB
EITB	+	0	0	0
EITB	-	22	185	207
TOTAL IDAG		22	185	207

Animals over two years

		IDAG +	IDAG -	TOTAL EITB
EITB	+	30	9	39
EITB	-	117	86	203
TOTAL IDAG		147	95	242

In most of the IDAG-false positive sera, 3D (viral polymerase) was the only antigen reacting in the EITB test.

ASSAY PROCEDURE

A. Materials and reagents:

- Rocking platform shaker
- Precision pipettes (with disposable tips), capable of delivering 2-20 μl, 20-200 μl, and 200-1000 μl, respectively.
- Vortex mixer or equivalent
- Graduated cylinders 10, 25, 50, 100 and 1000 ml
- Forceps for strip handling
- Whatman paper
- Distilled water
- Incubation trays (with eight troughs each)
- FMDV-nonstructural antigen coated test strips (see appendix)
- Positive, weakly positive, cut-off, and negative control sera
- Saturation buffer
- Washing buffer
- Conjugate (rabbit anti-bovine IgG labeled with alkaline phosphatase)
- Chromogen substrate (NBT: Nitro blue tetrazolium/BCIP: bromochloro-indolylphosphate)
- Substrate buffer

B. Procedure

1. The required amount of test troughs should be assessed, taking into account that for each nitrocellulose sheet, which defines one transferred gel, a positive, a weakly positive, a cut-off, and a negative control serum should be assayed. In general gels resulted in 24 strips of 3 mm each. Each test strip and test trough for controls and test samples should be identified.

2. The required amount of test strips should be removed from their container, and placed into each of the test troughs, using forceps.

3. 0.8 ml of saturation buffer (50 mM Tris-HCl, Ph 7.5, 150 mM NaCl, 0.2% Tween 20, 5% nonfat dry milk and 0.05% of bacterial *E. coli* lysated) is added to each trough. The samples are incubated by placing the trays on a shaker, and agitating for 30 minutes at room temperature (20-22°C).

4. 4 μl of the appropriate sample, and of each of the controls is added to the appropriate trough. The strip must be completely submerged.

5. Strips are incubated for 60 minutes on shaker at room temperature.

6. Liquid is removed from the trays, and each test strip is washed 3 times with washing solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20) by agitating 5 minutes.

   Note: The alkaline phosphatase-conjugated rabbit anti-bovine serum dilution should be prepared 2 minutes prior to the end of the last wash step.

7. 0.6 ml of prepared conjugate solution is added to each test trough, and the strips are incubated with shaking for 60 minutes at room temperature.

8. The liquid is removed from the trays, and each test strip is washed 3 times with washing solution as above.

   Note: Substrate solution (0.015% bromochloroindolylphosphate/0.03% nitroblue tetrazolium) is prepared in substrate buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, Ph 9.3), 2 minutes prior to the end of the last wash.

9. 0.5 ml of prepared substrate solution is added to each test trough.

10. Strips are incubated by placing the test tray on the orbital mixer and agitating for 15 minutes at room temperature.

11. Strips are washed with running deionized water.

12. Strips are removed from the test troughs and placed on absorbant paper using forceps. As soon as the strips have dried completely, results can be interpreted. Developed strips will retain their color.

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* Papers enclosed.

APPENDIX

Preparation of the test strips containing the recombinant antigens

All bioengineered FMDV antigens were expressed in *E coli* C 600, and purified to electrophoretic homogeneity as described (Neitzert et al, 1991).

Construction of the vector containing the complete polymerase gene under the control of the inducible Pl promoter of bacteriophage lambda, located in front of a consensus Shine Dalgarno sequence, has been described (Neitzert et al, 1991). Plasmids used to express the other nonstructural polypeptides were described (Strebel et al, 1986). Proteins were expressed as fusions to the N-terminal part of the MS-2 polymerase gene, under the control of the inducible lambda PL promoter.

C-600 cells harboring the corresponding plasmids contained a temperature sensitive mutant of the lambda repressor gene cI on a kanamycin-resistant plasmid.

The protein products were obtained after thermoinduction of the bacterial cultures.

Purification of the expressed polymerase over phosphocellulose, followed by poly(U) Sepharose columns was described (Neitzert et al, 1991).

The fused polypeptides 3A, 3B, 2C and 3ABC were purified by sequential extraction of the bacterial extracts with increasing concentrations of urea. The 7M fractions containing the fusion proteins was further purified on a preparative 10% SDS-PAGE. The fusion protein band was excised from the gel and electroeluted.

20 ng/mm of a mixture of the purified recombinant polypeptides were separated on 12.5% SDS-PAGE, and electrophoretically transferred to nitrocellulose.

The method has been introduced through:

a) Publications

Neitzert, E.; Beck, E.; Augé de Mello, P.; Gomes, I.; and Bergmann, I.E.
"Expression of the Aphthovirus RNA Polymerase Gene in *Escherichia coli* and its use together with other Bioengineered Nonstructural Antigens in Detection of Late Persistent Infections." Virology 184: 799-804 (1991)

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b) Presentations to meetings

"The Use of Bioengineered Proteins as Antigens in Diagnostic Kits for Detection of Antibodies to Foot-and-Mouth Disease Virus Infection Associated Antigen".
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