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mediately into thioglycollate broth. From respiratory cases, a swab was taken from each nostril. Acute and convalescent (2–4 weeks) serum specimens were taken from respiratory-case calves. Specimens were kept on ice until arrival at the laboratory the same afternoon.

Similar specimens were collected from control calves. For each case of diarrhea or respiratory disease, a calf without disease signs was selected. Control calves were identified as calves without signs of disease, of the same age ( $\pm 10$  days) and from the same farm (or from the next farm visited if none were available on the same farm). If two or more calves were eligible to be controls, then the calf nearest in age to the ill calf was chosen.

The minimum number of calves to be studied was 100 with diarrhea and 100 controls. These were the numbers calculated as necessary to detect the difference between 40% (estimated as a possible prevalence rate of rotavirus in feces of calves with diarrhea) and 20% (estimated as a possible prevalence rate of rotavirus in feces of calves without diarrhea) (Reynolds et al., 1986; Snodgrass et al., 1986) at a level of significance of 5% and a power of 80%.

### *Agent identification*

#### *Bacteriologic examination*

One rectal swab from each case and control animal was incubated in thioglycollate broth for 24 h at 37°C; aliquots of this culture were streaked subsequently onto MacConkey, Eosin Methylene Blue (EMB) and Salmonella–Shigella agars. Identification of all different colonies was accomplished using standard biochemical methods for Gram-negative organisms (Kelly et al., 1985).

One to 2 g of feces were inoculated into tetrathionate broth and incubated at 37°C for 24 h and aliquots of this culture were streaked onto MacConkey, EMB and Salmonella–Shigella agars. Colonies were identified as *Salmonella* by standard biochemical and serologic methods (Edwards and Ewing, 1972).

For identification of K99+ *E. coli*, rectal swabs were streaked onto MacConkey agar, and after 24 h of incubation at 37°C, lactose-positive colonies were selected and inoculated into Minca medium (Guinee et al., 1977) and incubated at 37°C for 24 h, then centrifuged and resuspended in buffer diluent of a commercial K-99 detection kit (Coli-Tect 99 Antigen Test Kit, Molecular Genetics, Inc., Minnetonka, MN). Aliquots of 100  $\mu$ l of this culture were tested in triplicate by the ELISA procedure for K-99 antigen in plates previously coated with monoclonal antibody specific to the pilus of K99+ *E. coli* (kindly provided by Dr. D. Reed, Molecular Genetics, Inc., Minnetonka, MN).

### *Virologic examination*

A rectal swab from each diarrhea case-control pair was tested for rotavirus using a commercially available ELISA kit (Rotazyme II Diagnostic Kit, Abbott Laboratories, North Chicago, IL). Reactivity of specimens was scored, with one considered nonreactive and four being most reactive.

Suspensions of nasal swabs from respiratory cases and respective controls were inoculated onto bovine turbinate (BT) cells (ATCC No. CRL-1390), incubated at 37°C under 5% CO<sub>2</sub> atmosphere and observed for cytopathic effect daily for 72 h.

Paired acute and convalescent sera from respiratory cases and their respective controls were tested by microtiter serum neutralization test for antibodies against bovine herpesvirus 1 (BVH 1) (infectious bovine rhinotracheitis, IBR), and the hemagglutination inhibition test for antibodies against bovine parainfluenza-3 virus using standard methodology (Revozzo and Burke, 1973).

### *Parasitologic examination*

The method of Willis was employed for identification of gastrointestinal helminths and coccidia (Pessoa, 1963). The Baermann procedure (Bock and Supperer, 1982) was employed to identify eggs of *Dictyocaulus viviparus* in feces of respiratory cases and their controls.

### *Statistical analysis*

Statistical comparisons between isolation rates were made using the  $\chi^2$  test for independence, with 1 degree of freedom.

## RESULTS AND DISCUSSION

From March to August 1987, specimens were collected from 300 calves on 30 of the 42 dairy farms visited. Of the 300 calves, 131 had diarrhea, 16 had signs of respiratory disease, and three had both diarrhea and respiratory disease signs; 150 calves served as controls. Two hundred and four were Holstein, 81 Jersey, 1 Guernsey, 10 crosses of the preceding breeds, 2 Zebu cross, 1 Brown Swiss and 1 unknown. Nine were male calves, and 291 female. Twenty-six of 273 serum specimens (9.5%) were negative for immunoglobulins by the zinc sulfate turbidity test. (Serum specimens were not available from all calves.)

### *Calves with diarrhea*

Mean and median ages of diarrhea calves at time of sampling were 21.4 and 13 days, respectively, and for their control calves, 22.3 and 17 days. One-quarter of calves with diarrhea were aged 1-7 days, one-quarter 8-14, one-

quarter 15–23 days and on one-quarter 24–90 days (quartiles). Of calves with diarrhea, 16 of 127 (12.6%) were negative for immunoglobulins by the zinc sulfate turbidity test, as compared with 7 of 120 (5.8%) of control calves ( $\chi^2$  2.59,  $P=0.11$ ).

#### *Rotavirus identification*

Rotavirus was identified in 10.4% of rectal swabs from calves on 26.2% of the farms sampled (Table 1). In 12 of 28 calves, rotavirus was the only agent identified; the remainder harbored mixed infections (i.e. K99+ *E. coli* and/or *Salmonella* spp. and/or coccidia) with rotavirus. Prevalence reports of rotavirus in dairy calves in other countries include: New Zealand, 3.5–8.8% (Schroeder et al., 1985); U.S.A. (Idaho and Oregon), 6.7% (Bulgin et al., 1982) and (Washington), 9% (Evermann, 1979); southern Britain 42% in diarrheic calves and 13% in healthy controls (Reynolds et al., 1986); Britain, 51.5% of diarrhea cases and 18% of controls (Snodgrass et al., 1986); Netherlands, 54% (Moerman et al., 1982); Panama 56% (cattle ages not specified) (Ryder et al., 1986); Finland, 55.8% (Pohjola et al., 1986); and Canada (Quebec), 70% (rotavirus and coronavirus) (Morin et al., 1980). Waltner-Toews et al. (1986a) reported a farm prevalence rate of 19% for Ontario dairy farms. In a previous study in Costa Rica, an individual-calf prevalence rate of approximately 20% was reported (Hernandez et al., 1987; Oviedo et al., 1987).

Nineteen (14.2%) calves with positive results for rotavirus had diarrhea and 9 (6.7%) were control calves ( $\chi^2$  3.23,  $P=0.07$ , Table 1). In previous studies where case and control calves have been compared, the difference between identification rates between the two groups has been greater than that observed in this study (De Rycke et al., 1986; Reynolds et al., 1986; Snodgrass et al., 1986; Waltner-Toews et al., 1986a). Five of 27 (18.5%) calves

TABLE 1

Identification and calf age at identification of rotavirus, K99+ *E. coli*, and coccidia from calves on Costa Rican dairy farms

| Unit                    | Rotavirus                        | K99+ <i>E. coli</i>              | Coccidia                         |
|-------------------------|----------------------------------|----------------------------------|----------------------------------|
|                         | No. positive/<br>no. sampled (%) | No. positive/<br>no. sampled (%) | No. positive/<br>no. sampled (%) |
| Farms                   | 11/42 (26.2)                     | 15/42 (35.7)                     | 19/42 (45.2)                     |
| Calves                  | 28/268 (10.4)                    | 35/268 (13.1)                    | 85/229 (37.1)                    |
| Calves with diarrhea    | 19/134 (14.2)                    | 12/134 (9)                       | 32/100 (32)                      |
| Calves without diarrhea | 9/134 (6.7)                      | 23/134 (17.2)                    | 47/117 (40.2)                    |
| Age, mean (days)        | 22.9                             | 20.8                             | 36.4                             |
| Age, median (days)      | 14.5                             | 15                               | 33                               |

TABLE 2

Rotavirus identification by age, for dairy calves ill with diarrhea and healthy controls, Costa Rica, 1987<sup>1</sup>

| Disease status                   | Age (days)     |    |      |    |       |    |       |    |       |    |       |    |
|----------------------------------|----------------|----|------|----|-------|----|-------|----|-------|----|-------|----|
|                                  | 1-7            |    | 8-14 |    | 15-21 |    | 22-29 |    | 30-60 |    | 61-90 |    |
|                                  | + <sup>2</sup> | 0  | +    | 0  | +     | 0  | +     | 0  | +     | 0  | +     | 0  |
| Diarrhea, no respiratory disease | 3              | 14 | 7    | 40 | 2     | 15 | 3     | 17 | 3     | 22 | 1     | 4  |
| Control                          | 2              | 24 | 2    | 31 | 1     | 22 | 2     | 17 | 1     | 23 | 1     | 7  |
| Diarrhea and respiratory disease | 0              | 0  | 0    | 0  | 0     | 0  | 0     | 1  | 0     | 1  | 0     | 1  |
| Total                            | 5              | 38 | 9    | 71 | 3     | 37 | 5     | 35 | 4     | 46 | 2     | 12 |

<sup>1</sup>Age for 1 of 134 control calves was unknown.

<sup>2</sup>+, Rotavirus identified; 0, rotavirus not identified.

from which rotavirus was identified and for which zinc sulfate results were available were negative for immunoglobulins by the zinc sulfate test, and 18 of 220 (8.2%) calves from which rotavirus was not isolated were negative for immunoglobulins by the zinc sulfate test ( $\chi^2$  1.94,  $P=0.16$ ). Rotavirus identification by age of calf is shown in Table 2. During the dry season (March, April) rotavirus was identified in 9 of 100 (9%) specimens and during the rainy season (May–August) the agent was identified in 19 of 168 (11.3%) specimens ( $\chi^2$  0.15,  $P=0.7$ ). Rotavirus was identified in feces of 12% of Holsteins studied, and in 8.5% of Jerseys ( $\chi^2$  0.33,  $P=0.56$ ).

#### *K99+ Escherichia coli* identification

*Escherichia coli* was isolated from cultures from nearly all fecal swabs (250 of 268, 93%). The K99 antigen was demonstrated in 13.1% of 268 fecal swabs from 35.7% of farms (Table 1). In 15 of 35 calves, K99+ *E. coli* was the only agent isolated; the remainder harbored mixed infections. Acres (1985) summarized the results of seven prevalence studies in the U.S.A. and Canada for enterotoxigenic *E. coli* in diarrheic calves; prevalence ranged from 6 to 31% (mean 18.3%, median 18%). Prevalence reports from other countries include: France, 0% (De Rycke et al., 1986); Britain, 3% of calves with diarrhea (Reynolds et al., 1986); U.S.A. (Idaho and Oregon), 6.6% (Bulgin et al., 1982); Britain, 7.9% in diarrheic calves and 0% in healthy controls (Snodgrass et al., 1986); Netherlands, 11% (Moerman et al., 1982); and U.S.A.

TABLE 3

K99+ *Escherichia coli* identification by age, for dairy calves ill with diarrhea, and healthy controls, Costa Rica, 1987<sup>1</sup>

| Disease status                   | Age (days)     |    |      |    |       |    |       |    |       |    |       |    |
|----------------------------------|----------------|----|------|----|-------|----|-------|----|-------|----|-------|----|
|                                  | 1-7            |    | 8-14 |    | 15-21 |    | 22-29 |    | 30-60 |    | 61-90 |    |
|                                  | + <sup>2</sup> | 0  | +    | 0  | +     | 0  | +     | 0  | +     | 0  | +     | 0  |
| Diarrhea                         | 3              | 14 | 3    | 44 | 0     | 17 | 3     | 17 | 2     | 23 | 1     | 4  |
| Control                          | 3              | 23 | 7    | 26 | 4     | 19 | 5     | 14 | 3     | 21 | 1     | 7  |
| Diarrhea and respiratory disease | 0              | 0  | 0    | 0  | 0     | 0  | 0     | 1  | 0     | 1  | 0     | 1  |
| Total                            | 6              | 37 | 10   | 70 | 4     | 36 | 8     | 32 | 5     | 45 | 2     | 12 |

<sup>1</sup>Age for 1 of 134 control calves was unknown.

<sup>2</sup>+, K-99 antigen identified; 0, K-99 antigen not identified.

(Utah), 10-35% (Allen and White, 1985). Waltner-Toews et al. (1986a) reported a 41% farm prevalence on Ontario dairy farms.

The isolation rate of K99+ *E. coli* for calves with diarrhea was 12 of 134 (9%) and from control calves 23 of 134 (17.2%) ( $\chi^2$  3.29,  $P=0.07$ , Table 1). This differs from other reports, where isolation rates were higher from diarrhea calves than from controls (Sihvonen and Miettinen, 1985; Snodgrass et al., 1986). Three of 32 (9.4%) calves from which K-99 antigen was detected were negative for immunoglobulins by the zinc sulfate test, and 20 of 215 (9.3%) calves from which K-99 was not identified were negative to the zinc sulfate test ( $\chi^2$  0.11,  $P=0.75$ ). K99+ *E. coli* identification by age of calf is shown in Table 3. During the dry season (March and April), K99+ *E. coli* antigen was detected in 15 of 100 (15%) fecal swabs, and during the rainy season (May-August) it was detected in 20 of 168 fecal swabs (11.3%) ( $\chi^2$  0.29,  $P=0.59$ ). Isolation rates were similar for Holstein and Jersey calves.

#### *Salmonella and Arizona identification*

*Salmonella typhimurium* was isolated from two calves, one case and one control. *Arizona* spp. was also isolated from one case and one control calf. Results of *Salmonella* prevalence reports in dairy calves in other countries include: Finland, 0% (Pohjola et al., 1986); Britain, 2% (Snodgrass et al., 1986); France, 2% (De Rycke et al., 1986); Britain 12% (Reynolds et al., 1986); U.S.A. (Utah), 17% (Allen and White, 1985) and (Idaho and Oregon) 34.3% (Bulgin et al., 1982). Farm prevalence in Ontario dairy farms was 22% (Waltner-Toews et al., 1986a).

### Intestinal parasite examination

Coccidia were identified in 85 of 229 (37%) calves sampled on 19 (45%) farms (Table 1). All calves for which feces could be obtained, calves with diarrhea, calves with respiratory signs, and calves with both; and their respective controls, were tested. In 67 of 85 (78.8) coccidia were the only agent isolated; the rest were mixed infections. Results of other prevalence studies in dairy calves include: Idaho and Oregon, 0% (Bulgin et al., 1982); Costa Rica, 25% (Oviedo et al., 1987); and Brazil, 70% or higher (Cerqueira-Leite, 1984).

Thirty-one of 97 (32%) calves with diarrhea, 6 of 30 (20%) respiratory cases, 1 of 3 of calves with both diarrhea and respiratory signs, and 40.2% of controls yielded coccidia ( $\chi^2$  1.22,  $P=0.27$ , Table 1). Table 4 shows coccidia identification by calf age. The organism was observed in feces of 26.3% of calves negative for immunoglobulins by the zinc sulfate test, and in 35.3% of positive calves ( $\chi^2$  0.28,  $P=0.65$ ). Identification rates were nearly identical in dry vs. rainy season and for Holstein vs. Jersey calves. *Strongyloides* spp. were identified in the feces of 2.1% calves with diarrhea and from 2% of their controls. An identification rate of 11% was reported in another study from Costa Rica (Oviedo et al., 1987).

No disease agents (rotavirus, K99+*E. coli*, *Salmonella*, coccidia) were identified in 61.2% of diarrhea case calves. Others have reported rates approximately one-half as large (Reynolds et al., 1986). However, the purpose of this study was not to assign causes to cases of diarrhea but rather to determine agent prevalence. In addition, the diarrhea rate in calves in the prospective study of which this study was a part was 36% for 1152 calves (D. Hird et al., unpublished data), higher than that reported elsewhere (Waltner-Toews

TABLE 4

Coccidia identification by age, for dairy calves ill with diarrhea, and healthy controls, (calves with signs of respiratory disease and their respective controls not considered), Costa Rica, 1987

| Disease status                         | Age (days)     |    |      |    |       |    |       |    |       |   |       |   |
|----------------------------------------|----------------|----|------|----|-------|----|-------|----|-------|---|-------|---|
|                                        | 1-7            |    | 8-14 |    | 15-21 |    | 22-29 |    | 30-60 |   | 61-90 |   |
|                                        | + <sup>1</sup> | 0  | +    | 0  | +     | 0  | +     | 0  | +     | 0 | +     | 0 |
| Diarrhea                               | 0              | 13 | 3    | 30 | 1     | 11 | 10    | 4  | 15    | 5 | 2     | 3 |
| Control                                | 1              | 21 | 2    | 20 | 4     | 12 | 10    | 5  | 16    | 2 | 5     | 3 |
| Diarrhea and<br>respiratory<br>disease | 0              | 0  | 0    | 0  | 0     | 0  | 0     | 1  | 1     | 0 | 0     | 1 |
| Total                                  | 1              | 34 | 5    | 50 | 5     | 23 | 20    | 10 | 32    | 7 | 7     | 7 |

<sup>1</sup> +, Coccidia identified; 0, coccidia not identified.

et al., 1986b); this may indicate that many cases of diarrhea are not associated with infectious disease agents.

Using data recorded for the continuing study, it was ascertained that for 96 of this study's control calves for which complete data were available, 22 had episodes of diarrhea recorded before selection as controls, 19 developed diarrhea after having been selected as controls, and 53 never developed diarrhea. For the 74 calves with no recorded diarrhea before selection as controls, the following percentages of control calves, grouped by agent first isolated, subsequently developed diarrhea: rotavirus, 4 of 6 (67%); K99+ *E. coli*, 5 of 12 (41.7%); coccidia, 2 of 17 (11.8%); and no agent isolated, 10 of 39 (25.6%). Comparison of agents isolated between control calves which subsequently developed diarrhea and their respective cases revealed that in only two instances was the same agent (rotavirus, K99+ *E. coli*) isolated from a case-control pair, indicating that the observed prevalence of disease agents in feces of control calves was not exaggerated by the proximity of diarrhea cases.

#### *Calves with respiratory disease signs*

Mean and median ages of calves with respiratory disease signs were 45 and 43 days at sampling, respectively, and 46.8 and 46 days for their controls. Two of 29 (6.9%) acute sera were totally inhibitory against BHV 1 at a dilution of 1:10, probably indicating passive transfer of natural maternal immunity to the calf. None of 16 acute-convalescent sera pairs showed seroconversion at > 1:10 dilution. Cattle on the study farms were not vaccinated against viral diseases. Isolation of BHV 1 has been reported in Costa Rica (Rodriguez and Fernandez, 1987), and a 16% seroprevalence in Costa Rican dairy cattle has been demonstrated (Donato, 1984). In a study of Oregon and Idaho calves, a rate of 2.4% was reported (Bulgin et al., 1982), and in Washington, an isolation rate of 10% in calves with signs of upper respiratory disease was reported (Evermann, 1979). Antibodies to parainfluenza-3 virus were demonstrated in 23 of 28 (82.1%) acute sera and 1 of 16 (6.3%) acute-convalescent pairs showed seroconversion. In a Washington study, this virus was implicated in 10% of upper respiratory disease cases in calves, and in 8% of pneumonia cases (Evermann, 1979). No cytopathic effect was observed in nasal swab suspension tested on BT cells; BT cells are susceptible to viruses of bovine virus diarrhea, IBR, PI-3, as well as bovine adenoviruses and enteroviruses (McClurkin et al., 1974). In summary, our laboratory results did not indicate association of respiratory illness with presence of viral agents or antibodies against the viral agents tested. However, this conclusion was based on the relatively few specimens tested.

*Dictyocaulus viviparus* was identified in feces of one of five respiratory case calves and none of five controls.



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# Detection of foot-and-mouth disease virus infection-associated antigen antibodies: comparison of the enzyme-linked immunosorbent assay and agar gel immunodiffusion tests

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## ABSTRACT

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A liquid-phase enzyme-linked immunosorbent assay (ELISA) was compared with the standard agar gel immunodiffusion test (AGID) to identify and quantify antibodies against foot-and-mouth disease (FMD) virus infection-associated (VIA) antigen. A total of 3181 cattle sera were tested. Of these sera, 1885 were from cattle which had not been exposed to FMD. A total of 1296 sera were either from cattle which were experimentally exposed to FMD virus or from cattle involved in field outbreaks. The results indicate that the ELISA has the same specificity as the AGID test, but is more efficient in detecting cattle exposed to FMD virus. The ELISA technique will probably prove to be a more satisfactory test in support of the prevention, control and eradication programs for the disease.

## INTRODUCTION

Virus infection-associated (VIA) antigen of foot-and-mouth disease (FMD) virus was identified by Cowan and Graves (1966). The antigen is common for all seven types of FMD. Polatnick et al. (1967) established that VIA antigen is the viral RNA polymerase. McVicar and Suttmoller (1970) utilized the agar gel immunodiffusion test (AGID) to demonstrate that the presence of VIA antibody was indicative of previous FMD infection. However, Pinto and Garland (1979) and Ahl and Wittman (1986) showed that cattle vaccinated with formalin- or acetyleneimine-inactivated vaccines would occasionally react positively to the VIA antibody test.

Villinger et al. (1989) developed an indirect enzyme-linked immunosorbent assay (ELISA) for the identification of VIA antibodies in animal sera.

The VIA antigen for this test was produced in *Escherichia coli* using recombinant DNA techniques. Because of its low specificity, the test could only be used in periodic surveys to determine the prevalence and incidence of VIA antibodies.

At present, the bulk of field samples are tested for VIA antibodies by the AGID test. However, this test has a low sensitivity, since a considerable proportion of exposed animals remain negative.

This paper presents the results obtained with the standard AGID test and those of a liquid-phase competition indirect sandwich ELISA test.

## MATERIALS AND METHODS

### *Test samples*

A total of 615 sera were obtained from FMD-free countries (Chile, Costa Rica, Curaçao, Guyana, Trinidad and the U.S.A.). Also tested were 1270 sera which originated from FMD-infected countries, but from cattle to be used for vaccine potency tests. These cattle had no history of exposure to FMD and were not vaccinated against FMD. Furthermore, 398 likely positive sera were obtained from cattle 14–60 days after experimental infection, and 898 sera were collected from cattle 20–40 days after being involved in field outbreaks in Brazil, Ecuador, Paraguay and Venezuela. Sera were collected from cattle vaccinated or revaccinated with inactivated vaccines with antigens adsorbed to aluminum gel or emulsified in Freund's incomplete adjuvant (Auge de Mello et al., 1975). Finally, 16 cattle were included which had been vaccinated with attenuated live virus (ALV) (Palacios, 1968). These cattle were later exposed to virulent FMD virus O<sub>1</sub> Campos-Br/58.

### *VIA antigen*

Suspensions of FMD strain A<sub>24</sub> Cruzeiro-Br/55 were produced in BHK-21 cell cultures grown in roller bottles. The harvests were clarified by low-speed centrifugation. Next, the VIA antigen was removed by the addition of 0.05% (w/v) DEAE-Sephadex A50 (Pharmacia Laboratory separation, Piscataway, NJ, U.S.A.). The VIA antigen was eluted from the Sephadex by the addition of 1 M NaCl and 0.02 M Tris buffer (pH 7.6) and precipitated with ammonium sulfate. The final suspension was inactivated with binary ethyleneimine (Bahnmann, 1975) and stored with 0.02% sodium azide, at 4°C. The resulting VIA antigen preparation did not contain detectable amounts of other FMD antigens when tested by the ELISA technique (Roeder and Le Blanc Smith, 1987).

### *Antisera*

Capture bovine IgG was prepared as described by Hudson and Hay (1976) from sera of cattle recovered from an experimental exposure to FMD virus C<sub>3</sub> Resende-Br/55. Detector antisera were collected from guinea pigs inoculated and hyperimmunized with infectious FMD virus O<sub>1</sub> Campos-Br/58 (Alonso Fernandez et al., 1983).

### *ELISA procedure*

The competition liquid-phase indirect sandwich ELISA test for the identification of FMD epitopes (McCullough et al., 1985) was adapted for the detection of VIA antibodies. All reagents were used in optimum concentrations as determined by titration. Antigens, test sera, guinea pig antiserum and conjugate were diluted in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST), 1% ovalbumin and 2% normal bovine serum (PBSTB). Plates were washed three times between each step with PBST. Solid-phase ELISA plates (Falcon, Division of Becton Dickinson Co., Oxnard, CA, U.S.A.) were coated overnight at 4°C with 100 µl of the appropriate dilution of bovine IgG in carbonate buffer (pH 9.6). Subsequently, the plates were blocked with 1% ovalbumin for 1 h at 25°C. The liquid phase, consisting of mixtures of a two-fold dilution series of test sera, starting at 1:1, and constant VIA antigen dilutions, was incubated for 1 h at 37°C. Of the liquid phase, 50 µl were transferred onto the solid phase. The guinea pig antiserum and the conjugate were added successively in 50-µl volumes and incubated for 30 min at 37°C on a plate shaker (Flow laboratories Inc., McLean, VA, U.S.A.).

The *o*-phenylenediamine substrate was added in 50-µl volumes. The reaction was allowed to proceed for 15 min at 25°C and stopped by acidification.

The optical densities were read with a Flow Titertek Multiskan photometer at 492 nm. End-point titers were expressed as the reciprocal log<sub>10</sub> dilution giving 50% of the absorbance estimated according to the method of Spearman-Kärber.

### *AGID*

The AGID test was performed in disposable plastic plates with a 90-mm diameter (McVicar and Suttmoller, 1970). For the AGID test and the ELISA, the same VIA antigen and capture bovine IgG were used.

## RESULTS

*Variability*

Five replicates were performed with the ELISA test using seven mixtures of sera from cattle recovered from FMD infections of the three different serotypes. The mean coefficient of variation was 18.5%, with individual values of 16, 16, 15, 11, 19, 4 and 38.

*Sensitivity*

The same sera used to determine the variability of the ELISA test results were titrated with the AGID technique. The mean titers of five replicates with each test showed a similar discrimination for different levels of VIA antibodies. In general, the titers obtained with the ELISA test were about 10 times higher than with AGID.

From cattle involved in FMD field outbreaks, 898 sera were collected 20–40 days after detection of the disease. Of those sera 825 were positive by the ELISA test (titers  $> 0.3 \log_{10}$ ), but only 653 sera were positive by AGID (Table 1). Under field conditions, a proportion of the cattle will not become infected, but even so, the sensitivity of the ELISA test is at least 0.919. Of the 398 sera from experimentally infected cattle, 389 were positive by the ELISA test and 318 by AGID. Thus, assuming a 100% infection rate, the sensitivity of the ELISA test was 0.977.

TABLE 1

Identification of VIA antibodies by ELISA and AGID in cattle sera of different origins

| Origin of cattle                 | Number of sera | ELISA 50% titer $\log_{10}$ |         | AGID |      |
|----------------------------------|----------------|-----------------------------|---------|------|------|
|                                  |                | $\geq 0.3$                  | $< 0.3$ | +    | -    |
| Free areas <sup>1</sup>          | 615            | 0                           | 615     | 0    | 615  |
| Potency test <sup>2</sup>        | 1270           | 0                           | 1270    | 0    | 1270 |
| Field infected <sup>3</sup>      | 898            | 825                         | 73      | 653  | 245  |
| Laboratory infected <sup>4</sup> | 398            | 389                         | 9       | 318  | 80   |
| Total                            | 3181           | 1214                        | 1967    | 971  | 2210 |

<sup>1</sup>Cattle from FMD-free areas.

<sup>2</sup>Cattle used in Argentina, Brazil and Uruguay for FMD vaccine potency control. Serum was collected prior to vaccination.

<sup>3</sup>Cattle involved in FMD field outbreaks, bled 20–40 days after detection of the disease.

<sup>4</sup>Experimentally infected cattle, bled 14–60 days post-infection.

### *Specificity*

All 1885 cattle sera from FMD-free areas as well as the sera from cattle designated for vaccine potency tests gave negative results in both the ELISA test (titers  $< 0.3 \log_{10}$ ) and in AGID (Table 1). Thus, the specificity is 1 for both tests.

### *VIA antibodies in vaccinated and revaccinated cattle*

In the majority of the cattle, vaccination with ALV induced VIA antibodies detectable by both tests. It must be noted that most of those cattle did not show clinical signs following the application of the ALV. All 16 cattle were positive to both tests after exposure to virulent FMD virus (Fig. 1). Inactivated aluminum-gel vaccines with high concentrations of non-purified antigen (18 and 54  $\mu\text{g}$  per dose of 5 ml) induced VIA antibodies detectable only by the ELISA test in 2 of the 20 cattle vaccinated with the vaccine containing the highest concentration of antigen. However, the majority of the cattle became positive to both tests between 15 and 30 days post-revaccination (DPR). Some of the sera continued to be positive for as long as 90 DPR (Fig. 2).

### *Persistence of VIA antibody in revaccinated and recovered cattle*

Eleven of 12 cattle infected with FMD virus O<sub>1</sub> Campos-Br/58 remained positive with the ELISA test for 15 months. With AGID, only 5 of the 12 sera continued to be positive for this long. Results of the ELISA test presented in Fig. 3 show that VIA antibodies persist in infected cattle at high levels for at least 18 months. However, the groups of revaccinated cattle displayed a sharp titer drop in contrast to the convalescent cattle.

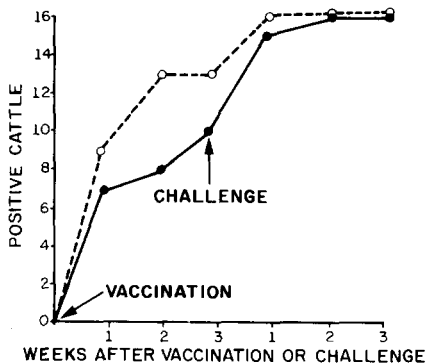


Fig. 1. VIA antibodies identified by ELISA (○) and AGID (●) in 16 cattle vaccinated with ALV vaccine and challenged with infectious FMD O<sub>1</sub> Campos-Br/58 virus.



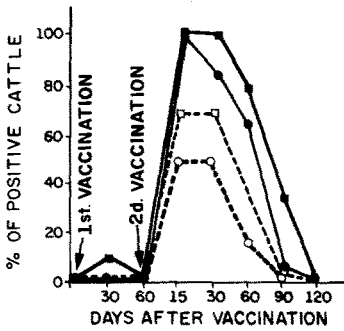


Fig. 2. VIA antibodies in two groups of 20 cattle vaccinated and revaccinated with inactivated vaccines containing 18 or 54  $\mu\text{g}$  of FMDV antigen respectively. ELISA 54  $\mu\text{g}$  (■), ELISA 18  $\mu\text{g}$  (□), AGID 54  $\mu\text{g}$  (●), AGID 18  $\mu\text{g}$  (○).

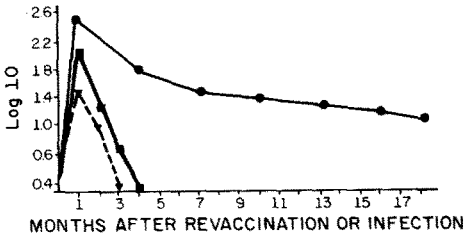


Fig. 3. Persistence of VIA antibodies by ELISA in three groups of five cattle: convalescent FMDV O<sub>1</sub> Campos-Br/58 infection (●) and revaccinated with vaccines containing 54  $\mu\text{g}$  (■) or 18  $\mu\text{g}$  (▼) of antigen respectively.

## DISCUSSION

The AGID test is an internationally accepted technique for the identification of VIA antibodies in animal sera. It is used for import/export testing and as an epidemiological tool to determine the spread of FMD virus in animal populations. The major drawback of the AGID assay is its low sensitivity, which motivated Morgan et al. (1978) to develop a purification procedure for VIA antigen (applicable in combination with other more sensitive techniques). These results stimulated us to examine the application of the ELISA technique for VIA antibody assay, as previously used by McCullough et al. (1985) and by Hamblin et al. (1986) to detect specific FMD epitopes and antibodies. We took advantage of the antigenic differences of the FMD virus types to make the assay system more specific. The capture IgG was prepared from FMD virus type C convalescent cattle serum. The VIA antigen was extracted from type A virus suspensions and the detector was produced by infecting and hyperimmunizing guinea pigs with virus type O. This procedure

also avoided immune reactions between BHK proteins and serum components, which previously caused problems when we attempted to apply the indirect sandwich ELISA tests for the assay of cattle sera. The variability of the ELISA test employed was evaluated in five replicate titrations, performed with seven mixtures of convalescent cattle sera. The low coefficient of variation (18.5%) indicates a high reproducibility of the technique. The specificity of the test was determined by the assay of 1885 cattle sera, originating from FMD-free areas or from cattle prior to their use in vaccine potency tests in Argentina, Brazil and Uruguay. All of these sera tested negative in the ELISA system and in AGID.

In the titration of seven mixtures of convalescent cattle sera, the ELISA test yielded titers which, on average, were 10 times higher than those of the AGID test. In an assay of 398 sera of experimentally infected cattle, the specificity of the ELISA test was 0.977. The assay of 898 sera from cattle involved in FMD outbreaks indicated a specificity of 0.919, but some of those cattle may not have been exposed to the virus. Vaccination and revaccination of cattle with vaccines containing high concentrations (18 and 54  $\mu\text{g}$  per 5-ml dose) of non-purified FMD antigen induced high titers of VIA antibodies in a high percentage of the cattle. Similar vaccines with a lower antigen concentration (6  $\mu\text{g}$  per 5 ml) did not induce the development of VIA antibodies (unpublished results), suggesting a dependence on the non-purified antigen concentration in the vaccine.

In Venezuela, 6–8 million doses of ALV FMD vaccines are used in cattle. The high percentage of cattle with VIA antibodies following ALV vaccination shows that VIA antibody surveys are not useful to evaluate the spread of virulent FMD strains in the cattle population under such conditions.

Vaccination campaigns based on revaccination of the cattle population at 4-month intervals can produce false positives with regard to infection. The collection of sera just prior to the next vaccination or of paired sera, 1 month apart, can partially avoid this obstacle. For the interpretation of the results, one should keep in mind the drastic drop which occurs in revaccinated cattle in comparison with the long persistence of VIA antibodies in convalescent cattle. However, we only presented data following one revaccination. More frequent revaccination may change this picture. It is suggested that a VIA antibody survey of the young-cattle population may be an excellent way to evaluate the presence or absence of FMD virus activity, principally in the population of areas under systematic FMD vaccination.

The persistence of high levels of VIA antibodies for at least 18 months may indicate that the FMD virus infection is maintained in those animals, probably for longer periods than the results of the probang test would suggest (Alonso Fernandez et al., 1975).

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