SEROLOGICAL STUDY OF TYPE A INDIAN FOOT-AND-MOUTH DISEASE VIRUS ISOLATES

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Summary. – The antigenic relationship of sixty type A foot-and-mouth disease (FMD) viruses isolated between 1968 and 1993 has been determined with reference to a post-vaccinal bovine serum produced against type A IND 17/82. A micro-neutralization test and ELISA were used to compare isolates. Analysis of the results indicated that there was a positive correlation between the data from the two methods. The study indicated that type A IND 17/82 had a broad immunogenic spectrum and could be considered as a candidate vaccine strain for incorporation in FMD vaccines in India.

Key words: type A foot-and-mouth disease virus; **r** value; antigenic relationship; candidate vaccine strain

Introduction

FMD is endemic in India and serotypes 0, A, C and Asia 1 are prevalent. The uncontrolled movement of animals, low percentage of vaccination coverage and antigenic variation within serotypes, are factors which cause problems in controlling FMD outbreaks. Antigenic variation in type A virus has been reported (Arrowsmith, 1975; Armstrong *et al.*, 1994; Belwal *et al.*, 1986, 1987). Extreme antigenic variation among type A FMD virus subtypes, especially among the subtype A_{22}, and its spread over wide areas have earned a special epidemiological significance for type A isolates. The present study describes the antigenic relationship of type A strains collected from outbreaks in India during 1968 to 1993, against A IND 17/82, a candidate vaccine strain.

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Abbreviations: BEI = binary ethyleneimine; CBCB = carbonate/bicarbonate buffer; FMD = foot-and-mouth disease; IGPS = immune guinea pig serum; ON = overnight; PBS = phosphate buffered saline; PBST = PBS containing 0.05% Tween 20; PBSBST = PBST containing 10% non-immune bovine serum; 5% normal rabbit serum and 0.05% Tween 20; RT = room temperature

Materials and Methods

FMD viruses obtained from different sources were adapted to BHK21 cells and stored at -20 °C after addition of equal volume of glycerine pH 7.4. Purified virus (146 S particles) was obtained using the method described by Brown and Cartwright (1963). The 146 S particles were acidified to prepare 12 S particles according to the method described by Abu Elzein and Crowther (1978).

Post-vaccination bovine sera. The antibody-free cattle aged 18 months, were injected subcutaneously with two doses of monovalent vaccine incorporating binary ethyleneimine (BEI)-inactivated type A IND 17/82 virus, at interval of 23 days and bled on 35th day. The serum was inactivated at 56 °C for 30 mins and stored at -20 °C.

Anti-146 S rabbit serum was produced against A IND 17/82 strain for use as capture antibody, as described by Rweyemamu *et al.* (1977). Briefly, viral antigen produced in infected tissue culture was inactivated with BEI. Virus was pelleted by centrifugation at 100,000 x g for 60 mins in the Hitachi RP 50/2 rotor. The pellet was resuspended in a small volume of phosphate buffered saline (PBS) and layered on to sucrose gradient (25 – 45% w/w in PBS). After 2 hrs centrifugation at 120,000 x g, gradient fractions were collected and virus-containing fractions identified after their examination in a spectrophotometer (reading A_{295} to detect virus RNA). The purified virus was emulsified with Freund's complete adjuvant and inoculated intramuscularly into rabbits. Final bleeding was carried out on the 35th day after a booster of the same material (in Freund's incomplete adjuvant) on the 21st day. The

serum was collected from individual animals and kept separate before titration and use, in ELISA described below.

Immune guinea pig serum (IGPS). Anti-146 S A IND 17/82 IGPS was prepared by the method described by Rweyemamu *et al.* (1977). The IGPS was used to detect captured viruses in ELISA described below.

Micro neutralization test (MNT). The strains were compared using a two-dimensional chessboard titration in microtiter plates as described by Rweyemamu *et al.* (1978). Briefly, serial two-fold dilutions of antiserum were mixed with a range of 0.5 log step dilutions of the respective test viruses in equal volumes of serum and virus (0.05 ml each). The mixtures were incubated for 1 hr at 37 °C, after which BHK21 cells (0.05 ml per well) were added at a seeding rate of 7.5 x 10^4 per well. The plates were incubated for 2 days at 37 °C (5% CO_2 atmosphere). Wells were examined under a microscope for cytopathic effect (CPE). At each virus dose examined, the corresponding level of neutralization was determined by the method of Karber (1931). A regression curve relating the serum titer against virus dose was plotted and the neutralizing titer determined as the final dilution of serum which neutralized 100 $TCID_{50}$ of each virus. The antigenic relationship was determined in terms of **r** value. The **r** values were calculated by dividing the mean titer of the serum against heterologous virus obtained from 3 replicate tests, by that against the homologous virus (A IND 17/82). The statistical significance of **r** values was tested by using an estimated pooled variance of 0.106. The strains were differentiated at a 99% level of significance, which requires a critical value of **r** ≥0.24, for a strain to be classified as being indistinguishable from the homologous isolate, in a three replicate test (Rweyemamu and Hingley, 1984).

Titration of rabbit sera in ELISA. The rabbit sera were used to coat microtiter plate wells to act as capture reagent for the tests described below. Briefly, 50 µl of rabbit anti-A IND 17/82 diluted in 0.05 mol/l carbonate/bicarbonate buffer pH 9.6, (CBCB) was added as a dilution range (in triplicate) across 11 wells of a row. The sera were diluted from 1/200 in a 2-fold series. After overnight (ON) incubation at room temperature (RT), the plates were washed by flooding and empying 5 times with PBS containing 0.05% Tween 20, (PBST) and blotted almost dry. A constant dilution of A IND 17/82 purified virus at 1 µg/ml was then added across the wells, diluted in blocking buffer. This consisted of PBST containing 10% non-immune bovine serum, 5% normal rabbit serum and 0.05% Tween 20 (PBSBST). Plates were incubated at 37 °C for 1 hr, then washed. Captured virus was then detected by the addition of 50 µl of anti/A IND 17/82 guinea pig serum diluted to 1/4000 in PBSBST. Plates were incubated at 37 °C for 1 hr, and washed. Bound guinea pig antibodies were detected by the addition of 50 µl per well of horse radish peroxidase-labelled anti-guinea pig IgG conjugate (Sigma), followed by incubation at 37 °C for 1 hr. Wells were washed and the test developed after the addition of 50 µl per well of ortho-phenylenediamine (Sigma)/ hydrogen peroxide; the colour was allowed to develop for 10 mins in the dark. The reaction was stopped at this time by the addition of 50 µl per well of 1 mol/l H_2SO_4. A_{492} was read using a multichannel spectrophotometer (Biotek). The dilution of rabbit serum used in liquid phase blocking ELISA described below was taken as the dilution of the last well showing maximum colour.

Titration of IGPS in ELISA. The individual IGPS were titrated and tested for their specificity against A IND 17/82, 146 S and 12 S particles in the sandwich-ELISA. Wells of microtiter ELISA plates (Maxisorb, Nunc) were coated with 50 µl of the pre-titrated dilution of rabbit sera in CBCB by incubation ON at RT in a humid chamber. The plates were then washed as described above, and blotted almost dry. To one plate, 50 µl of 146 S antigen at 1 µg/ml was added to wells, diluted in PBST. To another plate, 50 µl of 12 S antigen at 1 µg/ml was added to wells in the same buffer. Plates were then incubated at 37 °C for 1 hr. The plates were washed and two-fold dilutions of individual IGPS starting from 1:200 were added, diluted in the same blocking buffer as described above. Plates were incubated at 37 °C for 1 hr, and washed. Bound guinea pig antibodies were detected by the addition of anti-guinea pig conjugate, incubation, washing and addition of substrate/chromophore, as already described. The mean absorbances for the reactions with 146 S and 12 S particles were plotted and the optimal dilution to be used in the blocking assay was taken from the penultimate dilution where maximum colour was observed (plateau region for antigen detection). A pool of the successful guinea pig anti-A IND 17/82 sera was used as detecting serum in the liquid phase blocking ELISA. This pool was re-titrated as described above to arrive at the pre-titrated dilution quoted below.

Liquid phase blocking ELISA. This was based on the method described by Hamblin *et al.* (1986). Briefly, microtiter ELISA plates were coated with 50 µl of a pre-titrated dilution (1/5000) of the rabbit anti-A IND 17/82 serum in CBCB per well, and incubated ON at RT in a humidified chamber. Meanwhile, two-fold dilutions of post-vaccination bovine sera were reacted with a constant dilution (in PBST) of each of the viruses described, the mixture was kept at 4 °C ON a carrier plate (Laxbro). Each of the viruses was previously titrated in the sandwich ELISA, using the same rabbit and guinea pig sera, under the conditions described in this test except that no bovine serum was added. The dilutions of each of the viruses was adjusted so that similar A_{492} values (1 to 1.2) were used in the assay.

The following day, 50 µl of each of the respective mixtures were transferred to the washed plates coated with anti-type A antibodies. Control wells containing mixtures from wells containing (a) PBST and virus only (representing 0% inhibition controls where maximum colour is obtained in wells) and, (b) blocking buffer only (representing 100% competition since no virus is available for capture), were made. The plates were incubated for 1 hr at 37 °C. The plates were then washed and 50 µl of a pre-titrated (1/1000) quinea pig antiserum was added to each well. After incubation for 1 hr at 37 °C the wells were washed and 50 µl of anti-guinea pig IgG horse radish peroxidase conjugate was added to each well. Following incubation for 1 hr at 37 °C, the plates were washed and ortho-phenylenediamine solution containing hydrogen peroxide substrate was added. Colour was allowed to develop for 10 mins in the dark and the reaction was stopped by the addition of 50 µl 1 mol/l sulphuric acid. A_{492} was read in a multichannel spectrophotometer. The titers of the sera were read as the log dilution where a 50% inhibition of the difference between the maximum colour (with respect to the values obtained for the 0% inhibition control) and minimum colour (100% inhibition control) was observed. The titers were estimated after regression of serum dilution against the absorbances.

Table 1. The r values obtained using anti-A IND 17/82 bovine serum in MNT and ELISA

Virus	r		Virus	r	
	MNT	ELISA		MNT	ELISA
A IND 17/82	1.00	1.00	A IND 25/82	>1.00	>1.00
A IND 7/82	>1.00	1.00	A IND 26/82	>1.00	>1.00
A5 ALLIER 1/68	0.18[a]	0.35[b]	A IND 29/82	>1.00	0.62
A10 KEMRON	0.07[a]	0.18[a]	A IND 34/82	>1.00	0.35[b]
A22 IRAQ 24/24	0.52	0.33[b]	A IND 36/82	0.51	0.30[b]
A24 CRUZEIRO	0.05[a]	0.32[b]	A IND 37/82	>1.00	0.69
A IVRI	0.26	0.22[b]	A IND 25/84	0.78	0.72
A22 IVRI	0.10[a]	0.22[b]	A GUM 34/84	>1.00	0.71
A IND 2/68	0.42	0.35[b]	A APH 41/84	0.76	0.38[b]
A IND 5/68	0.20[a]	0.20[b]	A ORS 64/84	0.13[a]	0.46
A IND 1/70	0.20[a]	0.62	A KAB 66/84	>1.00	0.60
A IND 3/71	0.17[a]	0.32[b]	A KEM 83/84	0.63	0.30[b]
A IND 3/73	0.10[a]	0.20[b]	A KEW 25/85	0.50	0.21[b]
A IND 8/76	0.21[a]	0.37[b]	A KAM 59/85	0.40	0.50
A IND 19/76	0.19[a]	0.24[b]	A RAA 68/85	0.37	0.42
A IND 27/76	0.15[a]	0.35[b]	A TNN 107/85	0.72	0.25[b]
A IND 3/77	0.21[a]	0.31[b]	A APH 174/85	0.40	0.62
A IND 54/79	0.19[a]	0.23[b]	A GUK 47/86	0.50	0.17[a]
A IND 57/79	0.18[a]	0.42	A AP 72/86	0.26	0.23
A IND 72/79	0.14[a]	0.24[b]	A GUJ 12/87	0.68	0.40
A IND 73/79	0.08[a]	0.38[b]	A GUB 13/87	0.39	0.54
A IND 86/79	>1.00	0.32[b]	A ORS 75/88	0.20[a]	0.21[b]
A IND 25/81	>1.00	0.28[b]	A GUS 91/88	0.45	0.52
A IND 13/82	0.69	0.25[b]	A MAB 130/88	0.27	0.35[b]
A IND 14/82	0.54	0.20[b]	A WBC 11/89	0.63	0.12[a]
A IND 16/82	0.48	0.18[a]	A KAM 21/89	0.41	0.30[b]
A IND 19/82	>1.00	0.76	A GUA 24/91	>1.00	0.40
A IND 20/82	>1.00	0.56	A GUA 27/91	0.93	0.39[b]
A IND 22/82	>1.00	0.95	A GUK 11/92	0.49	0.36[b]
A IND 23/82	>1.00	>1.00	A MAT 9/93	0.74	0.20[b]

[a] Significantly different at $p \leq 0.01$.

[b] Intermediary relationship.

The **r** values were calculated by dividing the mean titer of the serum against heterologous virus by that against the homologous virus A IND 17/82.

Results

The results of the comparison of sixty type A isolates using both MNT and ELISA are shown in Table 1. A comparison of the results obtained by MNT and ELISA by linear regression analysis showed a significant positive correlation ($p < 0.01$). The criteria used to establish the significance of the values obtained for the MNT and ELISA are different. The ELISA criteria were based on those of Samuel *et al.* (1990), where **r** values of 0 to 0.19 indicated a highly significant antigenic difference between isolates; **r** values of 0.20 to 0.39 indicated intermediary differences and **r** values of 0.40 to 1.00 indicated no significant differences. The MNT criteria were those used by Rweyamamu *et al.* (1984), where only values of **r** below 0.24 indicated significant antigenic difference between isolates.

The ELISA results showed that only 4 virus isolates, including a reference strain , were heterologous to A IND 17/82 and the rest showed complete or partial homology (intermediary). The reference strains A_5 Allier, A_{24} Cruzerio and A_{22} vaccine strain of Indian Veterinary Research Insttitute and 11 other field isolates shared intermediary relationship with A IND 17/82 by ELISA, while none of these 14 strains were found to be related to A IND 17/82 when tested by MNT. A_{22} Iraq 24/84 and 17 field isolates were found to be related to A IND 17/82 by MNT while the ELISA indicated that these strains had intermediary relationship with A IND 17/82.

Discussion

There is an absolute necessity for the constant monitoring of the performance of FMD vaccines in FMD endemic country like India. This can be made through the serological comparison of the antigenicity of field strains with the virus used in the vaccines. Decisions regarding incorporation of additional virus strains or the need to change a vaccine strain, may be based on the results of virus differentiation tests. Virus neutralization tests in cell culture have long been regarded as the tests of choice for this purpose. However, the ELISA system offers additional advantages such as sensitivity, specificity, reproducibility and also enables a quicker comparison of a field strain with standard reference strains (Hamblin *et al.*, 1986; Kitching *et al.*, 1988).

Variation in results of MNT and ELISA were observed in the present study. Samuel *et al.* (1990) made similar observation and attributed the variation to the sensitivity of the assay methods to measure the antibody populations. Armstrong *et al.* (1994) encountered similar variations between virus neutralization test and ELISA. Generally, the ELISA showed isolates to be more closely related than the MNT. Thus, 70% of the isolates tested could be regarded as similar to A IND 17/82 by MNT whereas 96% of the isolates showed various degrees of homology by the ELISA, taking into consideration the criteria used for comparing the critical **r** value with type 1 error as shown by Rweyemamu *et al.* (1984), using the MNT and three types of relationship viz. homologous, intermediary and heterologous as described by Samuel *et al.* (1990). A re-evaluation of the criteria used to assess antigenic relatedness might be useful when more data are available from both types of assay. It must also be noted that these results compare isolates using a pooled bovine antiserum against A IND 17/82.

Antigenic variation among Indian type A viruses has been reported by Belwal *et al.* (1968), and the present study of 60 Indian isolates by MNT and ELISA also indicated antigenic variation. This is not surprising since the isolates

originated from various parts of the country at different times between 1968 and 1993. The isolates showed varying degrees of serological relationship to the reference strains used in the comparison. Reference strains A_{22} Iraq 24/64, A_5 Allier 1/68 and A_{24} Cruzeiro exhibited intermediary relationship to A_{22} India 17/84 in ELISA. Only 4% of the Indian field strains showed antigenic variation when compared with A_{22} India 17/84 in ELISA. The study thus indicates that A_{22} India 17/82 has a broad serological spectrum and hence can be considered a candidate vaccine strain for incorporation in FMD vaccine. Since the present study showed a positive correlation between this form of ELISA and the MNT, the ELISA can be regarded as a good diagnostic tool for comparison of vaccine and field FMD viruses. In attempting to correlate the results of ELISA and MNT the different nature of antibodies participating in these two assays should be taken into account: whereas in ELISA the antigen binds all antibodies, in MNT the virus binds the neutralizing antibodies only.

The two systems described, relating the reactions of a single polyclonal bovine antiserum with a variety of viruses, should be examined. The kinetics of the two assays will be different since the concentration of virus in the neutralization test is approximately 10,000 times less than that used in the ELISA. Such a figure comes from considering that the 100 $TCID_{50}$ units of virus represent approximately 10^5 virus particles (assuming a particle to infectivity ratio of 1000:1) capable of binding neutralizing antibodies. In ELISA the amount of captured virus available corresponds to aproximately 25 – 50 ng/well ($1.25 - 2.5 \times 10^{10}$ particles). Each system received a similar amount of antibody for titration, thus the increased concentration of virus in the ELISA tends to increase reactivity considering the law of mass action. Thus is might be expected that cross-reactions will be enhanced in the ELISA through increasing the ability of lower affinity antibodies to bind with the considerably higher amounts of virus, which generally was the case. Care is also needed in interpreting the results since only a single, pooled bovine serum against the vaccine strain was used. This cannot show the variation in response of cattle given the same vaccine in terms of the quality and quantity of specific antibodies produced in individuals. The positive correlation of the data indicates that there is a relationship between two assays as was also indicated by Hamblin *et al.* (1986); this was not true when these authors compared neutralization data with those from other forms of ELISA.

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