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Comparison of techniques for demonstrating antibodies to Rift Valley fever virus

By R. SWANEPOEL, J. K. STRUTHERS, M. J. ERASMUS, S. P. SHEPHERD, G. M. McGILLIVRAY

Department of Virology, University of the Witwatersrand and National Institute for Virology, Sandringham 2131, Republic of South Africa

B. J. ERASMUS and B. J. H. BARNARD

Veterinary Research Institute, Onderstepoort 0110, Republic of South Africa

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SUMMARY

Nine serological techniques were compared by monitoring the response to infection with Rift Valley fever (RVF) virus in three sheep. Antibodies were monitored daily for the first 14 days after infection, then weekly and later fortnightly up to week 24. The earliest antibody response was detected in one sheep on day 3 by a plaque reduction neutralization test, and by day 6 antibodies were demonstrable in all three sheep by haemagglutination-inhibition, reversed passive haemagglutination-inhibition, immunodiffusion, indirect immunofluorescence (IF), enzyme-linked immunosorbent assay and neutralization of cytopathic effect in cell cultures. Antibodies were demonstrable by complement fixation on day 8 at the earliest. IF and the two neutralization techniques produced the highest titres, but all tests could be used satisfactorily for the serological diagnosis of RVF. Inactivated antigen could be used for all except the neutralization tests. A radioimmunoassay technique using ^{125}I-labelled staphylococcal protein A detected antibodies on day 8 at the earliest and produced lower mean titres than some of the other techniques. This was probably because sheep immunoglobulins bind protein A poorly.

INTRODUCTION

Rift Valley fever (RVF) has long been known as a viral disease of man and livestock in Africa, but interest in the disease was heightened in 1977 by the occurrence of the first outbreak of RVF to be recognized in Egypt. This outbreak was associated with human deaths on an unprecedented scale (Imam, Darwish & El Karamany, 1979) and raised fears of the spread of the virus beyond the continent of Africa (Niklasson, Meegan & Bengtsson, 1979; Brès, 1981). There was international concern to standardize diagnostic procedures (Johnson, 1981) and we were prompted to investigate serological techniques. Tests in common use for the diagnosis of RVF and tests adapted from those in use for other diseases were compared by monitoring antibody responses over a period of 6 months in experimentally infected sheep.

MATERIALS AND METHODS

Sheep experiment

Three cross-bred Dorpers, two ewes and a ram aged 18 months, were infected subcutaneously with 10^6 plaque forming units (pfu) of RVF virus 1678/78 which had been isolated from bovine organs during the 1978 epizootic in Zimbabwe (Swanepoel, 1981). Rectal temperatures were recorded twice daily for 2 weeks. Blood was taken daily for the first 14 days, then at weekly and later at fortnightly intervals during the 6 months following infection as indicated in the results. A final sample was taken at 48 weeks.

Viraemia was tested during the first 14 days by inoculating heparinized whole blood intracerebrally into litters of day-old mice. Samples of blood were held at −70 °C while the mice were observed and the levels of virus present in bloods which killed mice were determined by plaque titration.

Replicate serum samples were stored at −70 °C at each bleeding to obviate repeated freezing and thawing for carrying out serological tests.

Haemagglutination-inhibition (HAI) tests

Techniques used for preparing haemagglutinating antigen and for performing HAI tests were modifications of the procedures of Clarke & Casals (1958). HA antigen was prepared by sucrose–acetone extraction of RVF-infected mouse liver, but following extraction the antigen was reconstituted in tris buffered saline, pH 9·0, rather than borate-saline, to allow infectivity to be inactivated with beta-propriolactone (BPL) (Shope & Sather, 1979) without loss of haemagglutinating activity. The reconstituted antigen was subjected to ultrasonic disruption (Ardoin, Clarke & Hannoun, 1969), clarified by centrifugation for 1 h at 11000 ***g*** and 4 °C, inactivated with BPL overnight and freeze-dried in 0·5 ml amounts.

The HAI test was adapted to a micro-technique. Non-specific inhibitors of haemagglutinin were removed by acetone extraction of 0·05 ml volumes of sera. Doubling dilutions of extracted sera from 1 in 20 upwards were prepared in 0·025 ml volumes of diluent in 'V' bottom, 96–well microplates (Linbro Division, Flow Laboratories, Inc., Hamden, Conn., U.S.A.) and tested against equal volumes of antigen containing 4–8 HA units. Serum–antigen mixtures were held at 4 °C overnight before addition of 0·05 ml goose erythrocytes. Endpoints were recorded as the reciprocals of the highest serum dilutions producing complete inhibition of haemagglutination.

Complement-fixation (CF) tests

The CF tests were performed by a micro-adaptation of the technique of Bradstreet & Taylor (1962) using 'U' bottom 96-well microplates and four 0·025 ml reagent volumes. Three 100 % lytic doses of complement were used. The antigen was the same as used in the HAI test and the optimal dilution was determined by chessboard titration against RVF-immune mouse ascitic fluid (Swanepoel, Struthers & McGillivray, 1983). The test sera were inactivated at 59 °C for 30 min and complement was fixed by overnight incubation at 4 °C. Endpoints were recorded as the reciprocals of the highest dilutions of serum producing complete fixation of complement.

Immunodiffusion (ID) tests

The micro-ID tests were performed on standard microscope slides covered with 3 ml of 1 % agarose in borate–saline buffer, pH 9·0 (Clarke, 1964). Patterns of six peripheral and a central well, all 5 mm in diameter and 3 mm apart, were cut with a cutter and template kit obtained from Miles Laboratories, Elkhart, Ind., U.S.A. Undiluted sera, and twofold serial dilutions in saline, were placed in the peripheral wells, and the central wells were filled with undiluted HAI antigen. Tests were incubated at room temperature (22 °C) in humid containers and read at 24 h and 48 h.

Reversed passive haemagglutination-inhibition (RPHI) tests

The RPHI tests were performed as described elsewhere (Swanepoel, Struthers & McGillivray, 1983), using HA antigen and glutaraldehyde-fixed sheep erythrocytes sensitized with immune mouse ascitic fluid.

Cytopathic effect neutralization (CPENT) tests

Vero cells were used with medium (Leibovitz, 1963) containing 5 % inactivated fetal calf serum (FCS) and 50 μg/ml gentamicin. Virus stocks consisted of the supernatant fluids from Vero cell cultures infected with RVF 1678/78 stored in small volumes at −70 °C. Sera were inactivated at 59 °C for 30 min and serial twofold dilutions from 1 in 2 upwards were prepared in 0·05 ml volumes of medium in flat bottomed 96–well cell-culture microplates. Equal volumes of virus suspension containing a calculated 100 $TCID_{50}$ were added to each well and the serum–virus mixtures incubated for 45 min at 22 °C before seeding with 2×10^4 cells per well in 0·025 ml medium. The plates, with loose lids, were incubated in sealed, humid chambers and the tests read after 6 days at 36 °C. Six replicates per dilution were used in control virus titrations and the end-points were estimated by the method of Kärber (1931). The sera were tested in duplicate and the end-points were recorded as the reciprocals of the highest serum dilutions at which neutralization occurred in both replicates.

Plaque reduction neutralization (PRNT) tests

The PRNT tests were based on the technique of Earley, Peralta & Johnson (1967). Monolayers were prepared by seeding 16 mm diameter wells in 24-well cluster plates (Costar, Cambridge, Mass., U.S.A.) with 2×10^5 Vero cells in 1 ml of Hanks'-based minimal essential amino acid medium (HMEM) (Eagle, 1959) with 10 % FCS and 50 μg/ml gentamicin. The plates, with loose lids, were incubated in sealed humid containers at 36 °C and the monolayers were used 24 and 48 h later. Sera were inactivated at 59 °C for 30 min and serial fourfold dilutions from 1 in 4 upwards were prepared in sterile 'U' or flat-bottomed microplates in the same medium as used in CPENT tests. Equal volumes of virus, containing a calculated 75 pfu per 0·05 ml, were added to each well and the serum-virus mixtures incubated for 45 min at 22 °C. Medium was drained from monolayers and 0·1 ml of each serum–virus mixture inoculated into duplicate wells. The inoculum was adsorbed for 30 min at 22 °C and cultures overlaid with HMEM containing agarose at a final concentration of 1 % and with the FCS

content reduced to 2%. The cultures were incubated in sealed humid containers at 36 °C for 3 days before being stained by the addition to each well of another 0·5 ml overlay medium containing 1 in 10000 neutral red. The results were recorded after a further 2 days incubation.

A control back-titration of the virus used in the test consisted of working-strength virus plus three serial fourfold dilutions incubated with equal volumes of a 1 in 4 dilution of fetal calf serum. Each control serum–virus mixture was inoculated into six replicate cultures and overlaid, incubated and stained as above. The average number of plaques was determined in the lowest control virus dilution which could be counted easily (usually a dilution yielding 10–20 plaques per well) and the actual number of pfu used in neutralization tests calculated. Serum titres were recorded as the reciprocals of the highest dilutions which produced an 80% reduction in this number of plaques and extrapolated to the nearest twofold dilution of serum.

Enzyme-linked immunosorbent assay (ELISA)

Antigen was prepared by growing virus 1678/78 in Vero cells with serum-free medium and harvesting the supernatant fluid when the cytopathic effect involved 50% of the cell sheets. The fluid was clarified by centrifugation at 11000 ***g*** for 30 min at 4 °C and virus was sedimented by centrifugation at 95000 ***g*** for 2 h at 4 °C. The pellet was resuspended in a carbonate–bicarbonate coating buffer, pH 9·0, using 1 ml for each 100 ml of starting volume, and stored in small volumes at −70 °C.

Anti-sheep immunoglobulin–peroxidase conjugate was obtained from Cappel Laboratories, Cochranville, PA., U.S.A., and the optimal dilutions of antigen and conjugate for use in the tests were determined in chessboard titrations of doubling dilutions of these reagents with high-titre immune sheep serum at a fixed dilution.

Wells in ELISA-microplates (A/S Nunc, Roskilde, Denmark) were coated with 0·1 ml volumes of antigen at the optimal dilution in coating buffer, pH 9·0. The antigen was adsorbed for 2 h at room temperature or overnight at 4 °C and plates washed once with phosphate-buffered saline, pH 7·2, containing 0·05% Tween 20 (PBST). Wells were post-coated with 0·2 ml volumes of 1% bovine albumin in PBST for 2 h at room temperature. The wells were then washed thrice with 0·2 ml volumes of PBST and 0·1 ml volumes of serum dilutions were added in diluent consisting of PBST containing 1% bovine albumin or fetal calf serum.

The sera were tested in doubling dilutions from 1 in 40 upwards and were allowed to react for 30 min at room temperature before the plates were washed as previously. The conjugate was added at the optimal dilution in 0·1 ml volumes of diluent and allowed to react for 30 min at room temperature before the plates were again washed. The substrate (0·04 g *o*-phenylenediamine, 0·7 g disodium tetraborate, 0·36 g succinic acid and 0·08 g urea hydrogen peroxide/dl distilled water) was prepared immediately prior to use. It was added as 0·2 ml quantities to each well and allowed to react for 30 min in the dark at room temperature before the reaction was stopped by addition of 0·025 ml of 3 M-HCl per well. Tests were read with a spectrophotometer at 492 nm against a blank of substrate plus HCl. The results were recorded in two ways: in the first method the optical density (OD) produced by the 1 in 40 dilution of each serum was merely recorded, while in the

second method those dilutions of sera which produced more than twice the OD of similar dilutions of known negative sera were scored as positive. For the interpretation of results in the second method, pre-infection serum of each sheep served as negative control serum for that sheep.

Radioimmunoassays (RIA)

Preparation of infected cells for use as antigen in the RIA test was based on the technique of Zaia & Oxman (1977). Vero cell monolayers were infected with RVF 1678/78 at a multiplicity of 10:1. The cells were harvested by trypsinization 16–18 h after infection and resuspended at a concentration of 2×10^6 cells/ml in ice-cold Earle's balanced salt solution (EBSS). They were fixed in 0·075% glutaraldehyde in ice-cold EBSS for 60 sec at 0 °C and fixation was stopped by the addition of an equal volume of cold EBSS containing sufficient glycine (0·15 M) to yield a final molar ratio of glycine:glutaraldehyde of 10:1. The cells were centrifuged at 600 *g* for 10 min at 4 °C and resuspended to 2×10^6 cells/ml in HMEM containing 30% FCS and 8% dimethyl sulphoxide and stored at −70 °C. Non-infected, control cells were fixed and stored in the same way.

The tests were based on procedures developed by Cleveland *et al.* (1979, 1982) and Richman *et al.* (1981). They were performed in a 'Millititer' immunofiltration apparatus (Millipore Corp., Bedford, Mass., U.S.A.) in 96-well microplates in which the bottoms of the wells are formed by polyvinylidene fluoride membrane (Millititer SV plates). The plates served as incubation chambers during reactions and as filtration manifolds during washing procedures. Wells were conditioned by adding 0·1 ml of phosphate-buffered saline (PBS), pH 7·4, containing 10% FCS and 1% bovine albumin. The wells were flushed after 10 min and $2{\cdot}5 \times 10^4$ infected or control cells were added per well in 0·05 ml of the same buffer. The diluent was flushed through and the cells washed once with 0·2 ml of 0·3% gelatin in PBS (PBSG). Twofold dilutions of test sera from 1 in 8 upwards were added as 0·05 ml volumes per well in PBSG and allowed to react for 60 min at 37 °C. Two replicates of each serum dilution were tested with both infected and control cells. Wells were washed thrice with PBSG, and 50000 c.p.m. of [^{125}I]*Staphylococcus aureus* protein A ([^{125}I]SPA) conjugate (Amersham International, Amersham, Bucks, U.K.) were added per well in 0·05 ml PBSG. The plates were again held at 37 °C for 60 min and washed as before. The wells were dried by allowing the vacuum line to draw air through them for 3 min. The filter disks were punched from the wells into vials and the bound [^{125}I]SPA determined with a gamma counter. The results were obtained by subtracting the mean count for the two control wells from the mean count for the test wells at each serum dilution, and the serum titres were recorded as the reciprocal of the highest serum dilution at which readings for test wells exceeded those for control wells by 1000 c.p.m.

Indirect immunofluorescence (IF) tests

Antigen slides were prepared by a modification of the method of Johnson, Elliott & Heymann (1981). Flask cultures of Vero cells were infected with RVF 1678/78 as described for preparation of RIA antigen and the cells were harvested by trypsinization 16–20 h post-infection at an early stage of cytopathic effect. The cells were centrifuged at 800 *g* for 5 min and resuspended at 10^6/ml in tris-buffered

saline, pH 7·4, containing 5% FCS and with 0·1% BPL added to inactivate virus. The cells were held at 4 °C overnight, washed, resuspended at 10^6/ml in isotonic saline with 5% FCS and mixed in a ratio of 1:5 with a similar suspension of non-infected, control cells. Spots were prepared from 0·01 ml volumes of cell suspension on 8-well Teflon-templated microscope slides (Flow Laboratories Ltd, Irvine, Scotland). The slides were air-dried, fixed in ice-cold acetone for 10 min and stored at −70 °C until used. Sheep sera were tested in 0·01 ml volumes at doubling dilutions from 1 in 2 upwards and were allowed to react for 30 min at 37 °C before the slides were washed for 10 min in stirred PBS, pH 7·2. Anti-sheep immunoglobulin–fluorescein conjugate (Cappel Laboratories, Cochranville, PA, U.S.A.) was allowed to react for a further 30 min at 37 °C before the slides were again washed, dried and prepared with glycerol mounting medium and cover-slips for examination on an incident-light fluorescence microscope.

Attempts were made to demonstrate IgM antibody activity in IF and ELISA tests by using specific anti-IgM conjugates from various sources.

Immunoprecipitation of viral proteins

The appearance of antibody activity directed against specific viral proteins was monitored by immunoprecipitating ^{35}S-labelled viral proteins from infected cell-culture lysates with sheep sera, and analysing the precipitated proteins by electrophoresis on polyacrylamide gels. Labelling of virus, immunoprecipitation of culture lysates, electrophoresis and fluorography of gels were performed as described elsewhere (Struthers, Swanepoel & Shepherd, 1984).

RESULTS

The results are presented graphically in Fig. 1. The sheep exhibited transient fever and viraemia with mild hyperpnoea, depression and anorexia during fever. Antibody response was demonstrated with facility by all the methods tested.

The earliest antibody activity was demonstrated by PRNT on day 3 following infection. By day 4, antibodies were demonstrable in one or more sheep by HAI, PRNT, CPENT, IF and both ELISA scoring methods. On the following day the ID test became positive in one sheep, and on day 6 all sheep were positive by all tests except the RIA and CF tests. Antibody activity was first demonstrated by RIA test on day 8 and by CF test on day 9.

Peak CF titres of 64 occurred in all three sheep at the third weekly bleeding, and by week 24 titres had fallen to 16 or 8. Peak ID titres in individual sheep ranged from 16 to 64 and occurred between day 13 and week 6. Antibody was no longer demonstrable by the ID test in one sheep from week 20 onwards and titres had declined to 4 and 8 in the remaining two sheep by week 24.

The RPHI test produced similar results to the HAI test. The highest RPHI titres in individual sheep were 512–1024 and occurred between day 7 and week 14. Peak HAI titres of 1280–2560 occurred between day 11 and week 14. At week 24 HAI titres ranged from 320–1280 and RPHI titres from 256–512.

The mean PRNT titres at each bleeding exceeded CPENT titres 2·8-fold with a standard error of 1·1. Peak CPENT titres of 4096–32768 occurred between day 9 and week 22. At week 24 CPENT titres ranged from 512 to 4096 and PRNT titres from 4096 to 8192.

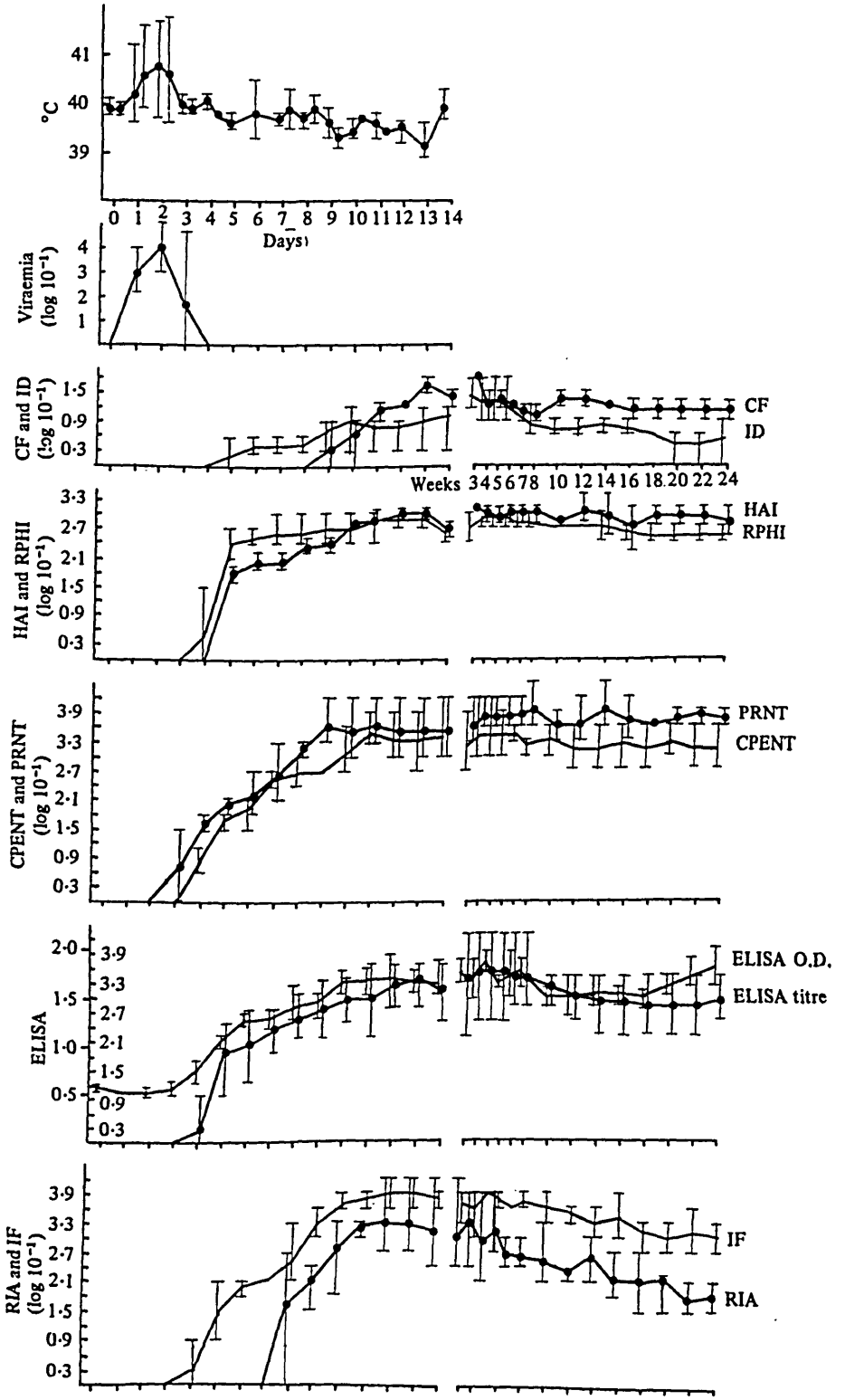

Fig. 1. Fever, viraemia and antibody response to subcutaneous infection with Rift Valley fever virus in three sheep. Curves for temperature and viraemia show mean values and range. Antibody curves show geometric mean values and range. Abbreviations for antibody techniques are as indicated in the text.

It can be seen in Fig. 1 that 1 in 40 dilutions of the pre-infection and early post-infection sera of the sheep produced OD readings of 0·5 to 0·6 in the ELISA test. Despite these high background readings, antibody response was clearly discernible by day 4 and the OD curve followed a pattern consistent with the antibody response as determined by the other serological methods. As noted above, the antibody response was also discernible on day 4 by the alternative method of scoring ELISA results, whereby serum dilutions producing more than twice the OD produced by similar dilutions of pre-infection sera were recorded as positive. Maximum titres recorded in individual sheep by this second method ranged from 640 to 20480, and such maxima occurred between day 12 and week 10. At week 24 the titres ranged from 320 to 2560.

Although the high background readings did not invalidate the ELISA results presented here, attempts were made to reduce them. More elaborate purification of antigen, including centrifugation on sucrose density gradients, made no difference in tests with sheep serum and was deemed unnecessary, since it had been found that no problem with high background readings was encountered in tests with mouse or human sera (data not presented). Post-coating with increased concentrations of bovine albumin or with gelatin failed to solve the problem. Instead, it was found that merely replacing substrate chemicals with similar reagent-grade chemicals from a second manufacturer reduced background OD readings with sheep sera to commonly accepted levels of 0·1–0·2.

IF titres, first detected on day 5, rose to maximum levels of 8192–16384 in individual sheep, and such levels occurred between day 11 and week 8. By week 24, titres ranged from 512 to 2048.

The RIA test, using ^{125}I-labelled *Staphylococcus aureus* protein A, detected onset of antibody response relatively late compared to the other techniques and produced maximum titres of 1024–16384 in individual sheep between day 11 and week 4. Titres fell to 32–128 by week 24.

Only HAI and ID tests were performed on sera collected at week 48. HAI titres were 640 in two sheep and 320 in the third, and while two sheep had ID titres of 2 and 4, the third lacked ID antibody activity.

Inconsistent results were obtained in IF and ELISA tests with anti-IgM conjugates from several sources, and it was concluded that the available reagents were unsatisfactory.

The immunoprecipitation of specific viral proteins by serum collected at various intervals after infection was monitored in two sheep, and the results are presented in Fig. 2. Of the five known RVF virus-associated proteins (Struthers, Swanepoel & Shepherd, 1984), four were detected in immunoprecipitates. Strong precipitation of the nucleocapsid protein (molecular weight 25 Kdaltons), occurred from day 6 post-infection onwards. Immunoprecipitation of the two envelope glycoproteins, G1 and G2 with molecular weights of 58 and 60 Kdaltons, could be discerned from day 10 onwards and the non-structural protein NS1, with a molecular weight of 35 Kdaltons, was demonstrable in immunoprecipitates from day 13 onwards. In addition, there was non-specific precipitation of a 45 Kdalton host (Vero cell) protein, a phenomenon observed previously with immune mouse serum (Struthers, Swanepoel & Shepherd, 1984).

It was noticed that up to three lines of precipitation occurred in the ID tests

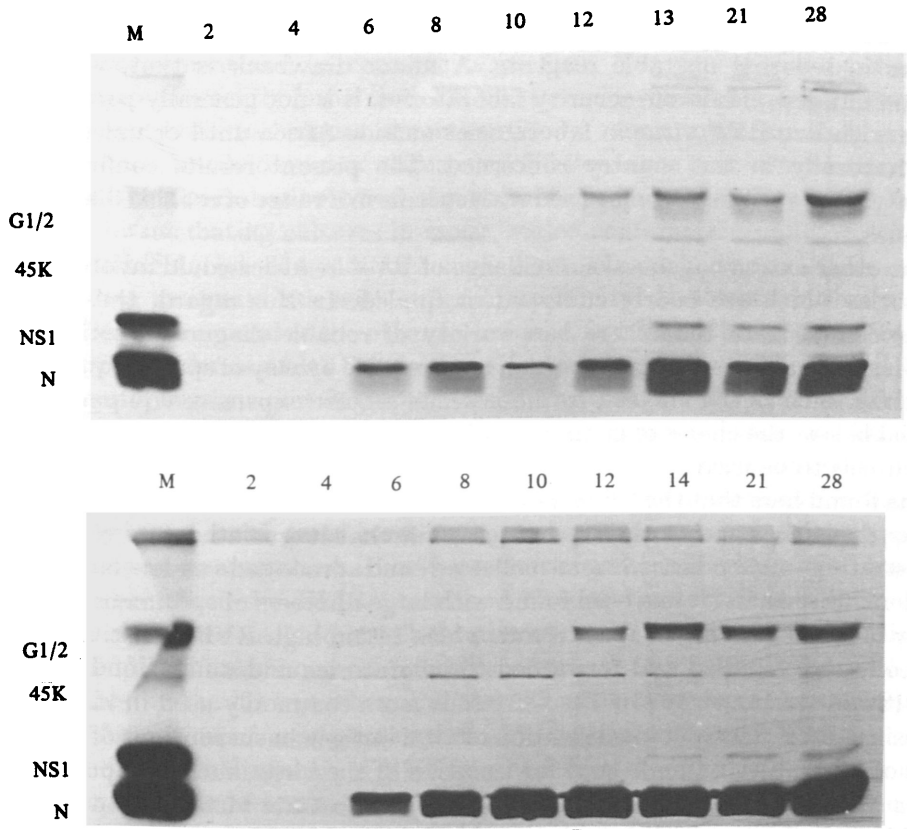

Fig. 2*a*, *b*. Immunoprecipitation of RVF virus-associated proteins from infected cell-culture lysates by antibodies in sera collected at intervals after infection from two sheep. The fluorographs shown here were prepared from polyacrylamide gels on which the immunoprecipitated, ^{35}S-labelled proteins were resolved by electrophoresis. Track M, proteins immunoprecipitated by immune mouse ascitic fluid; other tracks, proteins immunoprecipitated by sheep sera collected on the indicated days after infection. G1/2, envelope glycoproteins G1 and G2; NS1, non-structural protein; N, nucleocapsid protein; 45K, host (Vero cell) protein.

on microscope slides, and that the timing of the appearance in serum of antibodies producing the three lines of precipitation coincided with the appearance of antibodies which immunoprecipitated the nucleocapsid, envelope and non-structural proteins of RVF virus respectively, as demonstrated in the polyacrylamide gels described above. The two envelope glycoproteins are close in molecular weight and frequently migrate as a single band in electrophoresis (Struthers, Swanepoel & Shepherd, 1984), and it is probable that they appear as a single line of precipitation in ID tests.

DISCUSSION

Following the Egyptian outbreak of 1977, there was a need for laboratories to be able to diagnose RVF outside Africa, as well as for improved monitoring within the continent as part of international vigilance. At one extreme, this would involve

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well-equipped laboratories in developed countries, where it is relatively easy to acquire and maintain expensive equipment and where there are no logistic problems in securing unstable reagents. A minor drawback is that, with the exception of a few maximum-security laboratories, it is not generally permissible to work with live RVF virus in laboratories outside Africa until or unless RVF occurs naturally in the country concerned. The present results confirm that inactivated virus antigens can be used with success in a range of reliable diagnostic techniques.

At the other extreme, general surveillance of RVF in Africa could involve some laboratories which are poorly equipped or funded. In this regard, the present findings demonstrate that there is a variety of reliable diagnostic techniques available, ranging from those tests which require little by way of special equipment or reagents, such as the ID test, to those which require expensive equipment. As discussed below, the choice of an individual test is influenced by the circumstances in which it is to be used.

It was found here that the CF test detected antibody response slightly later and at lower titres than most other techniques, but that it was otherwise reliable in demonstrating antibodies in sera collected and processed under laboratory conditions. Elsewhere, it has been found with large numbers of specimens that CF activity is labile and may be lost in sera which retain high RVF HAI titres after being collected, handled and forwarded to laboratories under field conditions in Africa (Swanepoel *et al.* 1975). The CF test is more commonly used in virological diagnosis of RVF for rapid identification of viral antigen in suspensions of infected tissues of laboratory animals used for isolation of the virus, such as mouse brain or liver (Casals, 1978). It can also be used to demonstrate viral antigen directly in the tissues of naturally infected animals (Swanepoel, 1976).

The ID test is another technique which has proved to be useful for demonstrating viral antigen directly in tissues of diseased livestock or laboratory mice (Swanepoel, 1976, 1981). The present results indicate that it detects antibodies in serum at an early stage after infection and, despite the relatively low titres which are attained, it could find wide application as a serological technique which is inexpensive and simple to perform, and which requires no elaborate equipment.

The HAI technique has been used extensively as a diagnostic test for RVF (Swanepoel *et al.* 1975; Swanepoel, 1981). It requires pre-treatment of serum and remains somewhat laborious despite miniaturization and a degree of automation of the original technique, but has proved to be very reliable. The RPHI technique produces similar antibody titres to the HAI test and has certain potential advantages. Pre-treatment of serum is simpler and the fixed, sensitized cells can be stored or transported in the frozen state, so tests can be undertaken at short notice without the need for a supply of fresh erythrocytes. Moreover, the erythrocytes sensitized with antibody to the virus may prove to be useful for making a rapid virological diagnosis through demonstrating the presence of viral antigen (RPHA test) in viraemic serum (Swanepoel, Struthers & McGillivray, 1983).

Neutralization techniques utilizing cell cultures have been used in research (Swanepoel *et al.* 1978), but more costly and laborious mouse tests have been used more commonly in surveys and diagnosis, particularly in early investigations.

Since RVF virus grows well and is cytopathic in a wide variety of cells, cell culture techniques should supplant mouse tests. The CPENT technique described here is highly convenient and inexpensive to perform. The fact that it produces titres which are consistently lower than PRNT titres does not detract from its potential usefulness: both techniques detect antibody response at an early stage and demonstrate antibodies at a plateau level following a post-convalescent decline. It should be noted that both techniques can be performed without a CO_2 incubator simply by incubating cultures in moist, sealed containers.

The ELISA test demonstrates high levels of antibody, but it is somewhat misleading to equate this with superior sensitivity. It detects antibody response no earlier than several of the other techniques, and high ELISA titres are no more conclusive evidence of RVF infection than antibody titres determined by other techniques. It is a rapid diagnostic technique, but the IF, RPHI, HAI and CF tests can all be used to produce results within hours. ELISA can be used in a double-antibody (sandwich) technique to detect RVF viral antigen (Niklasson *et al.* 1983) but, as is true of the RPHA technique, the detection of antigen would probably be done with greatest reliability in experimental work or where specimens are processed on a regular basis. As seen in the present investigation, non-specific factors can affect the ELISA test, and the interpretation of results could be difficult where the technique is held in reserve for occasional use in diagnosing suspected RVF in a non-endemic area. Antigen can be detected with greater confidence by the ID test where an internal check is incorporated by monitoring for the formation of precipitation lines of identity with control antigen. Nevertheless, definitive diagnosis of RVF in a new area should ideally rest on isolation and identification of virus rather than on mere demonstration of antigen or antibody. Isolation is commonly achieved by inoculation of mice or hamsters (Swanepoel, 1981; Davies, 1975), but results can be achieved overnight by growing virus in cell culture and identifying it by IF (Davies, 1975).

The IF test has certain advantages as a serological technique. It detects antibody response at an early stage and demonstrates antibody at high levels comparable to any other technique. Antigen slides can be stored frozen, ready for immediate use, and the incorporation of non-infected cells serves as an internal check on specificity of antibody activity. Results can be obtained in about an hour. Sheep and cattle sera have a tendency to produce non-specific fluorescence of control cells at low dilutions (Swanepoel, Struthers & McGillivray, 1983). IF can also be used to detect viral antigen directly in impression smears of tissues of RVF-infected animals, particularly liver or fetal brain (Swanepoel, unpublished information).

The RIA test is generally considered to be the most sensitive of serological techniques, and the relatively poor results obtained here can probably be ascribed to poor binding of protein A by sheep immunoglobulin (Richman *et al.* 1982). Better results might have been obtained with species-specific anti-immunoglobulin conjugate, but such reagents are not readily available for many species, including sheep, and the intention was to explore techniques with wide applicability.

Similarly, better results might have been obtained in anti-IgM IF and ELISA tests by preparing high-grade reagents, but the intention was to determine whether suitable reagents are commercially available. Satisfactory results have

been obtained in ELISA test with anti-human IgM conjugate (Niklasson *et al.* 1984).

There are occasions when it is important to distinguish antibodies induced by administration of inactivated vaccine from antibodies induced by infection with live virus (Niklasson, Meegan & Bengtsson, 1979). The present findings suggest that the demonstration of antibody activity to the non-structural protein NS1 may serve this purpose, since it is unlikely that vaccine consisting of an activated virion preparation would induce antibodies to a protein associated with virus replication.

In conclusion, it must be stressed that no diagnostic technique is a substitute for sustained awareness of RVF. Although it is not always explicit in the scientific literature, unnecessary delays in arriving at an etiological diagnosis in major RVF epizootics have more often been due to lack of awareness than to non-availability of diagnostic techniques (Alexander, 1951; Christie, 1969; Hoogstraal, 1978; Swanepoel, 1981). Once the disease is suspected, modern international communications make it possible to obtain rapid diagnosis at a laboratory which may be remote from the outbreak, even on a different continent (Casals, 1978).

The present investigation did not address the question of specificity of serological tests. The fact that RVF virus is antigenically related to the phlebotomus fever group of viruses raises the possibility that cross-reactive antibodies may cause confusion in serological diagnosis of the disease (Johnson, 1981). We are investigating this subject with respect to RVF and the other known phleboviruses of sub-Saharan Africa.

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