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A microneutralisation test for the detection of rinderpest virus antibodies

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RÉSUMÉ

TAYLOR (W. P.), ROWE (L. W.). — Essai de microneutralisation pour la détection des anticorps du virus de la peste bovine. *Rev. Elev. Méd. vét. Pays trop.*, 1984, **37** (2) : 155-159.

Description d'une méthode pour la détection des anticorps neutralisants de la peste bovine par un système de microplats, méthode qui est aussi sensible que le rouleau classique mais qui l'est plus aux effets défavorables des inhibiteurs viraux non-spécifiques et des sérums cytotoxiques. Ces problèmes ont été résolus en diluant les sérums par 1 : 10 avant expérimentation.

Mots clés : Peste bovine - microneutralisation.

SUMMARY

TAYLOR (W. P.), ROWE (L. W.). — A microneutralisation test for the detection of rinderpest virus antibodies. *Rev. Elev. Méd. vét. Pays trop.*, 1984, **37** (2) : 155-159.

A microplate system is described for the measurement of rinderpest neutralising antibodies. The method is as sensitive as the conventional roller tube but is more adversely affected by non-specific viral inhibitors and by cytotoxic sera. These problems were overcome by diluting the sera 1 : 10 prior to testing.

Key words : Rinderpest - neutralisation.

INTRODUCTION

Rinderpest virus (RV) neutralising antibodies are generally measured by the inhibition of virus specific cytopathic effects (CPE) in roller tube cultures of calf kidney cells (6). By present standards this method is time-consuming and expensive, and a micro-adaptation is long overdue. Although alternative methods of estimating rinderpest antibody levels have been sought (1, 2, 3), we consider there to be a continuing need for an economical method of screening or titrating RV neutralising antibody. This report outlines the development of such a test and parallels the recent description by ROSSITER and JESSET (8).

MATERIALS AND METHODS

Cells

Primary calf kidney (BK) cells were grown in Glasgow modified Eagle's medium (GMEM) with ten p. 100 tryptose phosphate broth (TPB) and ten p. 100 ox serum. Cells were maintained in Earle's salts supplemented with 0.5 p. 100 w/v lactalbumin hydrolysate (LAH), 0.1 p. 100 w/v yeast extract and five p. 100 ox serum (EYL). Tube cultures of secondary BK cells were grown and maintained as above, while in microplates these cells were seeded in GMEM with 20 p. 100 ox serum.

Primary sheep kidney (SK) cells were grown with kidneys taken from adult animals.

Growth was initiated in Medium 199 in Hanks' salts with ten p. 100 ox serum but changed to BK growth medium after five days. Monolayers were confluent by day eight or nine and were maintained in Medium 199 in Earle's salts with five p. 100 ox serum (E/199). Tube cultures of secondary SK cells were grown in BK growth medium and maintained in E/199, while in microplates SK cells were grown in GMEM with 20 p. 100 ox serum.

Virus and virus titration

RV, strain RBOK, was grown to between 95 and 100 passages in secondary BK cells. Fluid harvests were clarified, mixed with an equal volume of an aqueous solution of LAH (5 p. 100 w/v) and sucrose (10 p. 100 w/v) and frozen at minus 70 °C in small volumes. A fresh aliquot of virus was used for each experiment.

Virus was diluted in 0.5 Log_{10} steps in TPB and titrated in roller tubes or microplates in which virus and cells were added together. At each dilution five tubes were inoculated with 0.2 ml virus and 1.0 ml cell suspension or four microwells were inoculated with 0.05 ml virus and an equal volume of cells. For tube cultures cell counts were adjusted to 1.0×10^5 (SK) or 2.0×10^5 (BK) per ml ; in microplates cell concentrations were doubled.

Neutralisation tests

Following overnight neutralisation at 4 °C, residual infectivity was estimated in roller tube cultures (6) or microplates. Micro virus-neutralisation (MVN) tests were carried out in 96-well flat bottomed tissue culture grade microplates. Doubling serum dilutions were made in GMEM using 0.05 ml volumes and transferring with a Titertek multichannel pipetter. Each sample was diluted in duplicate and tips were only changed between samples. Virus was diluted in TPB to contain between $10^{1.8}$ and $10^{2.8}$ $TCID_{50}$ per 0.05 ml and this volume was added to each well. Plates were agitated briefly on a Dynatech microshaker, loosely covered and incubated. Following the addition of 0.05 ml cell suspension, plates were covered with pressure-sensitive adhesive tape and incubated at 37 °C. Each test included a positive serum control and a verification of the virus dose. Final microscopic readings were made after six days for BK cells or seven days for SK cells. For calculating end points, serum dilutions were considered to double following the addition of virus.

Sera

Cattle sera were taken from four British steers experimentally infected with the RBOK strain of rinderpest virus after 95 BK passages (2) or from a rinderpest vaccinated field population collected in Bangladesh. Unvaccinated field sera were collected in Britain or the West Indies, while the positive control serum was taken from a British steer infected with the RBT/1 strain (5) and hyperimmunised as previously described by EL HAG ALI and TAYLOR (4). Sera were stored at minus 20 °C and inactivated at 56 °C for 30 min immediately before use ; in some tests they were diluted in GMEM prior to inactivation.

RESULTS

Rinderpest virus assay in microplates

In microplates BK cells developed a CPE as early as day three with the appearance of small focal groups of darkly refractile cells. Both the number and size of cytopathic foci increased in the following 24 h but thereafter foci increased in size only. At low virus dilutions affected areas tended to coalesce but syncytial formations were seldom seen. At high dilutions, foci remained small and discrete. Final results were obtained on day six when the pH remained slightly above neutral (7.1) and control cells were still healthy and ungranular. Beyond this stage maintenance media became more acid, monolayers became densely granular and non-specific retraction occurred in some. No efforts were made to investigate the effects of medium changes on the sensitivity of our microsystem.

With LK cells the cytopathic effect was characterised by the appearance of large refractile stellate cells and a lack of syncytial development. LK cells could be maintained one to two days longer than BK cells before acidity and non-specific retraction interfered with microscopic observations.

Using BK cells, parallel titrations were undertaken in roller tubes and microplates (Table 1). The results from eight different

TABLE N°I-Comparative titrations of RV in BK roller tube cultures and microplates

Experiment number	BK number	$TCID_{50}$/ml in		Difference in titre (a-b)
		(a) roller tube	(b) microplate	
1	39	6.9	6.4	0.5
2	40	7.0	6.9	0.1
3	40	6.8	6.6	0.2
4	42	6.9	6.5	0.4
5	43	7.0	6.5	0.5
6	43	6.9	6.6	0.3
7	43	6.8	6.6	0.2
8	44	7.2	7.0	0.2
Mean		6.94	6.64	0.3

assays with the same batch of virus in secondary cells from five different calves clearly showed that microplates were approximately two-fold lower in sensitivity than roller tubes.

In seven further parallel assays, titres were compared in roller tubes only, using cells from each of four different batches of BK and LK cells. No difference could be detected in the sensitivity of the two cell types. Finally, comparative titrations with LK cells in roller tubes and microplates again demonstrated a two-fold reduction in sensitivity in the microsystem.

Variation of neutralising antibody titre with virus dose

Using a hyperimmune ox serum, LW32, box titrations were carried out to estimate neutralising antibody levels against increases in virus dose. Two-fold serum dilutions were tested against 0.5 Log_{10} increments in virus level and the regression line of the serum titre calculated (Table II). In the first experiment, a master set of virus-serum mixtures were tested simultaneously in roller tube and microplate cultures of BK cells. The results indicate that there was no significant difference in the two regression equations. Similar results were obtained using a different batch of virus in two separate experiments in microplates of BK or SK cells. It was concluded from these results that 10-fold differences in virus titre would not result in a greater than three-fold variation in serum titre.

TABLE N°II-Regression equations for neutralising antibody titres at different virus dose levels

Experiment	System	Regression equation
1	BK roller tubes	$y = 4.75 - 0.51x$
	BK microplates	$y = 4.65 - 0.49x$
2	BK microplates	$y = 4.50 - 0.4x$
3	SK microplates	$y = 4.95 - 0.46x$

Comparison of neutralising antibody levels determined in roller tubes and microplates

Sera were obtained from four British steers bled repeatedly during the first three months following infection. Neutralising antibody levels were estimated in BK roller tube and MVN tests with the results shown in Table III.

TABLE N°III-Comparative neutralising antibody estimations in roller tubes and microplates

Day number	Animal N°			
	NM 18	NM 19	NM 20	NM 21
0	0.0/0.0*	0.0/0.0	0.0/0.3	0.3/0.8
4	0.0/0.0	0.0/0.0	0.0/0.3	0.2/0.8
6	0.0/0.0	0.3/0.5	0.0/0.8	0.4/0.8
8	0.8/1.2	1.4/1.4	0.9/1.4	0.6/1.1
10	1.8/2.0	2.9/2.1	1.3/1.8	1.0/1.2
12	2.5/2.4	2.7/2.9	2.2/2.4	1.2/1.5
17	2.8/2.7	2.8/2.4	2.2/2.6	1.8/2.0
42	3.0/2.9	2.8/2.3	2.7/2.4	2.4/2.1
75	3.5/3.2	2.7/2.3	2.6/2.6	2.4/2.1
90	3.6/3.0	2.3/2.1	2.8/2.6	2.7/2.3

* Log_{10} SN_{50} antibody level in roller tube cultures/ antibody level in microplate cultures.

Early antibody could be detected between six and eight days post-inoculation and in general there was no obvious difference in the end points between the two methods. With steers NM19 and 20 antibody titres did not increase significantly beyond days 12 or 17 post-inoculation but with NM18 and 21 end points continued to rise slowly for the first 90 days.

Low levels of virus inhibition were found in pre-inoculation sera from steers NM20 and 21. As each animal developed a normal neutralising antibody response indicative of total virus susceptibility it follows that these inhibitors were of a non-specific nature. In both animals levels were always higher in MVN tests and in NM20 could only be detected by this method. This may be because of the prolonged period of virus-inhibitor contact inherent in the MVN test whereas in the roller tube method any such interaction would be terminated at the first fluid change.

Comparative tests with field sera

When the sera from 175 Caribbean cattle were examined in BK cells using both tests, some 47 were cytotoxic in the MVN system at final serum dilutions of 1 : 4 or 1 : 8 although no problems were encountered in tube tests with the same samples. In addition 44 samples which failed to neutralise virus in roller tube cultures inhibited the same virus dose in comparative MVN tests conducted at final dilutions of 1 : 4 or 1 : 8. The general nature of these problems was illustrated when a group of 109 British cattle sera was tested by the MVN method and shown to contain 15 samples that were cytotoxic and three that gave non-specific inhibition at a final dilution of 1 : 4. None of these samples inhibited virus or caused cytotoxicity in tube tests.

From these experiments it was apparent that in the MVN test undiluted field sera would produce false positive results with some samples and no result with others. However, it also appeared that these problems could be overcome by diluting the serum. It remained then to determine a satisfactory dilution level to adopt.

Using a group of 92 ox sera collected in Bangladesh three weeks after vaccination neutralising antibody titres were estimated by both methods. In tube assays 81.5 p. 100 (75/92) were positive with a mean titre of Log_{10} SN_{50} 1.24 ± 0.44 while in MVN tests 83.7 p. 100 (77/92) were positive with a mean titre of 1.49 ± 0.37. From this we have concluded that for survey purposes using the MVN tests, sera with values above Log_{10} SN_{50} 1.0 can reasonably be interpreted as coming from immune animals. We have therefore adopted a final serum dilution of 1 : 10 for all such tests.

DISCUSSION

An earlier MVN test developed by RIOCHE (7) did not gain widespread acceptance and until recently most laboratories have favoured the roller-tube method of PLOWRIGHT and FERRIS (6) for the estimation of RV neutralising antibodies. More recently ROSSITER and JESSETT (8) have outlined an MVN method developed in Kenya and the present report confirms and extends the practicability and usefulness of such a test.

Our secondary BK and LK cells grown in GMEM medium did not become acid until the seventh day of incubation. The presence of phosphate buffer in the TPB used to dispense virus in the tests may have contributed to this increased period of acid-free maintenance and to the ease with which MVN end-points could be determined. Even so at high virus dilutions CPE tended to remain focal throughout the test period. It was also noted that SK cells obtained from adult sheep's kidneys were less prone to acidification than BK cells and as the sensitivity of the two systems is identical SK cells appear particularly suited for MVN work.

ANDERSON and ROWE (1) reported a colour change produced by an indirect immunoperoxidase test that may be used as an alternative to microscopy for determing those wells in the MVN test where virus growth has occurred. Although such tests can be conducted with unfixed cultures a reduction in background colour is achieved by fixation in 10 p. 100 formalin in PBS. By a combination of the removal of the sealing tape, washing and drying the plate, and increasing the refractility of infected cells microscopic determination of foci of CPE is also made easier so that irrespective of the method of reading end-points this fixation step may be usefully added to the basic technique.

The MVN test measures antibody levels with the same sensitivity as the roller tube test and

carries the same relationship between virus dose and neutralising antibody level as that test (6). In these respects our test is in agreement with that of ROSSITER and JESSETT (8) although in minor detail the two test systems differ slightly. Due to the slight reduction in sensitivity of microplates compared to roller tubes the virus dose for MVN work should be calculated in microplates.

ANDERSON *et al.* (3) compared sera from steers NM18, 19, 20, and 21 using an ELISA test for virus specific IgM, IgG and IgA antibodies. Pre-inoculation serum from NM21 contained high levels of antiviral IgM. However, as steer NM20 also showed some non-specific neutralisation but did not contain pre-inoculation virus specific IgM it was concluded that non-specific activity was not always associated with this serum fraction. Further evidence for the widespread distribution of non-specific rinderpest inhibitors was found in tests on caribbean and British field sera. No attempts were made to remove these inhibitors.

In addition certain sera were found to be cytotoxic and it became clear that the MVNT could not be used with undiluted serum unless either the serum was treated to remove inhibitors and toxic substances or a fluid change was carried out in the wells. ROSSITER and JESSETT (8) adopted this latter approach but we believe that this is an undesirable step and incurs a considerable risk of cross-contamination. Rather than employing pre-test treatments we chose to dilute out the unwanted constituents. Using a 1 : 5 initial serum dilution neither non-specific inhibitors or cell cytotoxicity could be found in the final 1 : 10 virus-serum dilution to which cells were added. While this may result in a failure to detect the occasional sample with low levels of antibody, for routine screening of field sera to obtain qualitative or quantitative results this loss is compensated by the cheaper and more rapid production of results. In cases where complete rinderpest susceptibility is of unusual importance, such as in the selection of cattle for vaccine testing, it is suggested that the classic roller tube method be retained (6).

RESUMEN

TAYLOR (W. P.), ROWE (L. W.). — Una prueba de microneutralización para la estimación de anticuerpos neutralizantes en peste bovina. *Rev. Elev. Méd. vét. Pays trop.*, 1984, **37** (2) : 155-159.

Se describe un método para estimar el título de anticuerpos neutralizantes de peste bovina usando un sistema de microplatas. El método es tan sensible como lo de tabos girantes, pero es más susceptible a inhibidores non específicos y sueros tóxicos. Se han resuelto estos problemas al diluir los sueros antes de la prueba.

Palabras claves : Peste bovina - Microneutralizacion.

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