DEVELOPMENT OF A THERMORESISTANT TISSUE CULTURE RINDERPEST VACCINE VIRUS

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Summary. – The currently used Plowright's tissue culture rinderpest vaccine (RBOK strain) gives full protection and lifelong immunity, but it is highly thermolabile and requires maintenance of cold chain from vaccine production till delivery. Keeping in view the need for a thermostabile vaccine in tropical developing countries with limited refrigeration facilities, we passaged serially the RBOK strain of rinderpestvirus (RPV) at gradually elevated temperature up to 40°C to obtain a thermoresistant RPV (TR-RPV) mutant. The thermoresistance (thermostability) and antigenicity of TR-RPV were compared with those of the vaccine virus by various methods, confirming the acquired properties. Thus, the infectivity titres of the TR-RPV mutant and vaccine virus were determined after incubation for various times at 37°C. Regression analysis indicated that TR-RPV had a half-life of 1.81 hr and a degradation constant of 0.1656, while the parent vaccine virus had a half-life of 1.11 hr and a degradation constant of 0.2686. In capture ELISA with four different monoclonal antibodies (MAbs) to the N protein of RPV, TR-RPV showed a 10-fold higher reactivity with one MAb as compared to the vaccine virus. Although TR-RPV did react also with the other three MAbs, its reactivity was only 4–5 times higher than that of the vaccine virus. A treatment of the virus with Triton X-100 resulted in 2–4 times higher reactivity with the MAbs. The ^{35}S-methionine-labeled vaccine virus-and TR-RPV-infected Vero cell lysates showed 6 polypeptide bands with identical pattern of migration in polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). Radioimmunoprecipitation assay (RIPA) of the TR-RPV and vaccine virus with a rabbit anti-RPV immune serum (RHIS) and bovine anti-RPV hyperimmune serum (BHIS) showed the presence of four identical antigenic proteins, namely H, N, F and M, for both viruses. It can be concluded that TR-RPV has indeed retained the antigenic properties of the parental vaccine virus besides acquiring thermoresistance.

Key words: rinderpest virus; RBOK strain; thermoresistant mutant; capture ELISA; SDS-PAGE; RIPA; vaccine

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Abbreviations: AGP = agar gel precipitation; BHIS = bovine anti-rinderpest hyperimmune serum; CIEP = counterimmunoelectrophoresis; CPE = cytopathic effect; p.i. = post infection; GMEM = Glasgow's Modified Eagle's Medium; IP = immunoperoxidase; MAb = monoclonal antibody; MHIS = mouse anti-rinderpest hyperimmune serum; MOI = multiplicity of infection; PEG = polyethylene glycol; PMSF = phenyl methyl sulphonyl fluoride; RHIS = rabbit anti-rinderpest hyperimmune serum; RIPA = radioimmunoprecipitation assay; RPV = rinderpest virus; SDS-PAGE polyacrylamide gel electrophoresis in the presence of SDS; TR-RPV = thermoresistant RPV; TR-Vero cells = thermoresistant Vero cells

Introduction

Rinderpest is one of the most dreaded, devastating and economically important diseases affecting livestock. The causative agent, RPV, is the species *Rinderpest virus,* a member of *Morbillivirus* genus and the family *Paramyxoviridae*. All members of the *Morbillivirus* genus share similar morphology, genomic structure, polypeptide profile and antigenic characteristics (Barrett *et al.,* 1991). A vaccine against rinderpest is currently produced from the Kabete 'O' strain of RPV grown in bovine kidney cells (Plowright, 1962). The Plowright's tissue culture rinderpest (TCRP) vaccine is an attenuated RPV vaccine, which after a single

dose application, provides lifelong protective immunity, produces no clinical signs and is not transmitted by natural means. TCRP vaccine has been extensively used in rinderpest control programs worldwide and has resulted in eradication of the disease in developed countries. However, a few countries in Africa, Near East and South Asia have experienced the disease till the recent past (Sahal, 1992; Moustafa, 1993). Although TCRP vaccine is very effective, it has a major limitation due to thermolability, posing logistical problems in delivery to the field since cold chain maintenance is required throughout from vaccine production till delivery. Thus, the most effective rinderpest vaccine must have characteristics like heat stability and easy production, storage, transportation as well as administration in the field. The thermolabile nature of TCRP vaccine virus is a major obstacle in eradication of rinderpest by vaccination, particularly in tropical countries. To overcome this difficulty, different approaches, e.g. use of various chemical stabilizers, diluents and improvisation of lyophilization procedures etc. have been tried in past, but with limited success (Languet *et al.*, 1985; Mariner *et al.*, 1990; Nayak *et al.*, 1995). Another approach to overcome the thermolability and other practical problems of TCRP vaccine, has been the development of vaccinia virus recombinant rinderpest vaccines (Yilma *et al.*, 1988, Barrett *et al.*, 1989, Belsham *et al.*, 1989, Tsukiyama *et al.*, 1989, Asano *et al.*, 1991, Giavedoni *et al.*, 1991) and a capripoxvirus recombinant rinderpest vaccine (Romero *et al.*, 1993), but because of the biosafety concerning these vaccines they could not be applied to field use. These reasons prompted us to develop a thermoresistant rinderpest vaccine virus (hereafter referred to as TR-RPV), derived from the Plowright's TCRP vaccine virus ("the vaccine virus").

Thus, in this paper, we report the development of a thermoresistant rinderpest vaccine virus by serial passaging the vaccine virus gradually from 37°C to 40°C. The antigenic characteristics of this TR-RPV were also studied and are being discussed.

Materials and Methods

Cells. Vero (African green monkey kidney) cell line grown in Glasgow's Modified Eagle's Medium (GMEM), supplemented with 10% (v/v) tryptose phosphate broth, 1mmol/l L-glutamine, 10 mmol/l HEPES, and 5% (v/v) of fetal bovine serum, was originally maintained at 37°C.

Virus. TCRP vaccine virus, RBOK strain, at passage level 92 in bovine kidney cells, was adapted to growth in Vero cells for 8 successive passages and used as seed virus for the development of TR-RPV.

Adaptation of Vero cells to higher temperature (40°C). Vero cells, originally grown at 37°C, were adapted to growth at 40°C by gradually increasing the cultivation temperature from 37°C to 40°C with an increment of 0.5°C per passage. Later, the cells were continuously grown at 40°C for 10 passages before using them for virus cultivation. The Vero cells adapted to growth at 40°C are referred to as thermoresistant Vero cells (TR-Vero cells).

Development of thermoresistant RPV (TR-RPV). Vero cell adapted RPV RBOK strain was inoculated to TR-Vero cells at a multiplicity of infection (MOI) of 0.5. The virus adsorption was done at 37°C for 1 hr and then the infected cells were incubated at 37°C (passage 1). These infected cells were passaged at 38°C (passage 2). In subsequent passages the incubation temperature was increased by an increment of 0.5°C per passage until it reached 40°C at passage 6. Ten-fold serial dilutions of the virus from passage 6 were inoculated to TR-Vero cells and single plaques were isolated for plaque purification. Additional 2 passages (passages 7–8) were performed at 40°C. The simultaneous culture method of Singh *et al.* (1995) was used in all 8 passages. According to this method, uninfected and infected cells were trypsinized separately, mixed 1:1 and incubated at given temperature.

After passage 10, the virus was used for determining its thermoresistance. Finally, TR-RPV was prepared in bulk and used for further studies. In our experiments described here, after passage 2 onwards, the CPE was strong.

Assessment of thermoresistance and virus infectivity titration. Aliquots of TR-RPV-infected cell culture fluids were incubated at 37°C for 0, 0.25, 0.5, 1, 2, 4, 7, and 12 hrs and stored at -40°C until the virus infectivity titration. Infectivity titrations were carried out on Vero cells as follows. Ten-fold dilutions of each virus sample were mixed with Vero cells (3×10^4 cells/well) in six replicates and incubated at 37°C in 5% CO_2 for 7–10 days. The plates were observed for RPV-specific CPE and $TCID_{50}$ titers were calculated in a standard manner. The vaccine virus treated in similar manner was used as control. Standard regression curves (Snedecor, 1956) were constructed to compare the thermoresistance of the vaccine virus and TR-RPV. The half-life was calculated as the time required for a decrease of the $TCID_{50}$ titer by 0.3 log based on the degradation rate constant K by adopting the formula

$$t_{1/2} = 0.3/b$$

where b is the slope value of regression curve.

Serological tests. Samples of TR-RPV grown at the 6th, 8th and 10th passage levels were precipitated with 8% (v/v) polyethylene glycol (PEG) 8000 and used as antigen in agar gel precipitation (AGP) (Josi *et al.*, 1972) and counter-immunoelectrophoresis (CIEP) tests (Bansal *et al.*, 1981). TR-RPV-infected cells on coverslips in Leighton tubes or 4-well plastic tissue culture plates were used for immunoperoxidase (IP) test. The cells were fixed during early and late stages of infection, treated with a specific antiserum and the antigen-antibody reactions were detected by a conjugate and chromogen substrate by the method of Wohlseen *et al.* (1993).

Reactivity of antigens in capture ELISA. Capture ELISA was performed by a standard procedure to characterize TR-RPV using MAbs 12AD10.1.1; 12BD7.1.1; 12DG7.1.1, and 12BF8.1.1 directed to RPV N protein and a mouse anti-rinderpest hyperimmune serum (MHIS). Each well was coated with 50 µl of rabbit anti-rinderpest hyperimmune serum (RHIS) (1:5000 dilution) and blocked with 5% skim milk in PBS. A 50 µl aliquot of antigen (PEG-8000-precipitated vaccine virus or TR-RPV) was added to each well and incubated at 37°C for 1 hr. After washing, either a MAb

or MHIS was added, followed by rabbit anti-mouse IgG-horse radish peroxidase conjugate (ICN Biologicals, USA) and orthophenylenediamine substrate.

In vivo labeling of proteins with ^{35}S-methionine and preparation of cell lysates was done by a standard procedure. Vero cell monolayers in tissue culture microplates were infected separately with the vaccine virus and TR-RPV and incubated at 37°C and 40°C, respectively. After 3–4 days, the cells were starved for methionine for 3 hrs by incubating in a methionine-free medium. Thereafter, the medium was changed for a medium containing ^{35}S-methionine (50 µCi/ml). Later, this medium was removed and the cells were lysed in the RIPA buffer (10 mmol/l NaH_2PO4/Na_2HPO_4, pH 7.4), 1% Triton X-100, 1% sodium deoxycholate, 15 mmol/l NaCl, 3 mmol/l PMSF, 5 µg/ml aprotenin) (100 µl per well) for 3 mins on ice. The lysates were collected, centrifuged at 12,000 x g for 20 mins at 4°C and the supernatants were stored at -40°C until use.

Radioimmunoprecipitation assay (RIPA). The supernatants (see above) containing virus-specific proteins were subjected to RIPA using both RHIS and BHIS according to Grubman *et al.* (1988).

SDS-PAGE and fluorography. The labeled proteins in the lysates of virus-infected cells and the virus-specific proteins after RIPA were analyzed by SDS-PAGE (10% gels) by the method of Grubman *et al.* (1988). A mixture of ^{14}C-methylated proteins (Amersham, UK) ranging from 13 K to 200 K was used as size marker. The gels were fluorographed by a standard procedure (Boenner and Lasky, 1974).

Results

Development of TR-RPV

Development of TR-RPV was carried out by cultivation of the vaccine virus in TR-Vero cells at gradually increasing temperatures from 37°C up to 40°C in 6 passages (see Materials and Methods). The virus from the passage 6 was plaque purified. Using the plaque purified virus 2 additional passages were performed at 40°C.

Cultivation of the vaccine virus in TR-Vero cells resulted in poor CPE in the passage 1, but the extent of CPE increased with subsequent passages, when the infected cells were cultured with uninfected cells. Passage 2 resulted in a moderate CPE 24 hrs post infection (p.i.); such a CPE was typical for the vaccine virus in normal Vero cells. After the passage 10, the log $TCID_{50}$/ml titer was 5.80. RPV antigen was demonstrable in infected TR-Vero cells by an immunoperoxidase (IP) test using RHIS and MAbs to RPV-N protein. RPV antigen could also be demonstrated in PEG-8000-precipitated TR-RPV by an agar gel precipitation (AGP) test and a counterimmunoelectrophoresis (CIEP) using RHIS.

Assessment of thermoresistance of TR-RPV

The infectivity titers of the vaccine virus and TR-RPV at different times of incubation at 37°C are depicted in Fig. 1. Initially, the infectivity titers (log $TCID_{50}$/ml) of both the vaccine virus and TR-RPV were 6.07 and 5.80, respectively. With increasing incubation time, titers of both viruses began falling. After 4 hrs, the titer of the vaccine virus was reduced by 1.77 log units while that of TR-RPV by only 1.0 log unit. At the end of the experiment, i.e. after 12 hrs, the titer of the vaccine virus fell by 3.77 log units, while that of TR-RPV by 2.0 log units. Statistical analysis (regression lines) of these results is shown in Table 1. The half-lives ($t_{1/2}$) for the viruses, as calculated from slope values of regression curves, were 1.11 hr and 1.81 hr for the vaccine virus and TR-RPV, respectively. TR-RPV exhibited a higher thermoresistance (with a half-life of 1.81 hr and degradation rate constant K of 0.1656) as compared to the vaccine virus (with a half-life of 1.11 hr and degradation rate constant K of 0.2686).

Serological tests

The PEG-8000-precipitated TR-RPV gave precipitin lines with RHIS in AGP test and CIEP. In IP test, the staining was very strong during early CPE but weak during advanced CPE.

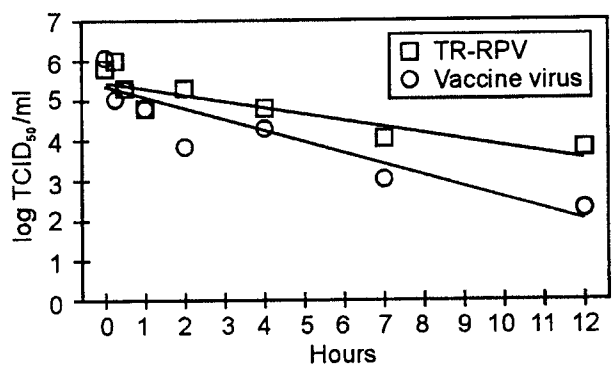

Fig. 1
Thermal degradation of vaccine virus and TR-RPV at 37°C

Table 1. Statistical assessment of thermostability of wt RPV and TR-RPV

Virus	Regression equation	Regression coefficient (b)	Half life ($t_{1/2}$) (hrs)	Degradation rate constant (K)
wt RPV	$y = 5.2356–0.2686x_1$	b = –0.2686	1.11	0.2686
TR-RPV	$y = 5.5394–0.1656x_2$	b = –0.1656	1.81	0.1656

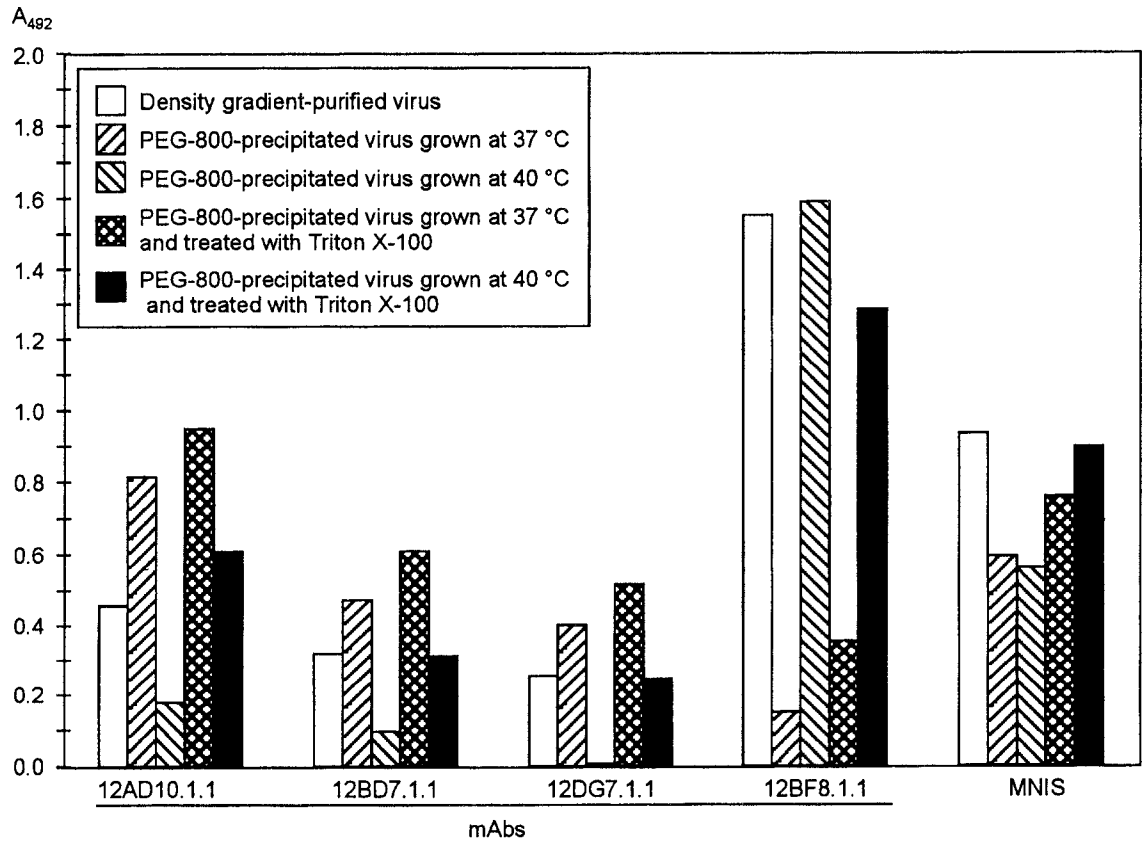

Fig. 2
Reactivity of vaccine virus and TR-RPV in capture ELISA

Reactivity in capture ELISA using MAbs and MHIS

Comparison of the reactivities of TR-RPV and the vaccine virus antigens with MAbs 12AD10.1.1, 12BD7.1.1, 12DG7.1.1, and 12BF8.1.1 (directed to RPV N polypeptide) and MHIS by capture ELISA (Fig. 2) revealed that wt RPV grown at 37°C showed at least 4–5 times higher A_{492} with MAbs 12AD10.1.1, 12BD7.1.1, and 12DG7.1.1 than those obtained with TR-RPV grown at 40°C. Whereas MAb 12BF8.1.1 showed at least 10 times lower A_{492} with the vaccine virus than with TR-RPV. Serum antibodies did not differ much when reacted with the vaccine virus or TR-RPV. The vaccine virus treated with Triton X-100 did not show any differences when reacted with the three MAbs (12BD7.1.1, 12DG7.1.1, and 12AD10.1.1) and MHIS. However, MAb 12BF8.1.1 showed an increase in reactivity with the vaccine virus. On the other hand, the treatment of TR-RPV with Triton X-100 gave 2–4 times higher A_{492} with MAbs 12AD10.1.1, 12BD7.1.1, and 12DG7.1.1, while MAb 12BF8.1.1 showed a slightly decreased A_{492}. MHIS gave slightly higher A_{492} with the treated than with the untreated TR-RPV. In comparison to the Triton X-100-treated vaccine virus (corresponding to 1.5 μg of the density gradient-purified vaccine virus), TR-RPV-treated with Triton X-100 showed dramatic increase in reactivity (over 200%) with MAbs 12AD10.1.1, 12BD7.1.1, and 12DG7.1.1. Such an increase in reactivity was not observed with MAb 12BF8.1.1 and MHIS. In the case of the vaccine virus grown at 37°C, only mAb 12BF8.1.1 showed an 130% increase in reactivity after treatment with Triton X-100.

Protein profile of vaccine virus and TR-RPV

RIPA of the vaccine virus- and TR-RPV-infected cell lysates with RHIS and BHIS revealed the presence of four antigenic proteins, namely H (79 K); N (60 K); F (42 K), and M (36 K). The bands corresponding to L and P protein could not be observed in any of the reactions. Two other proteins of 55 K and 50 K were, however, observed in all reactions. The reaction with an uninfected Vero cell lysate

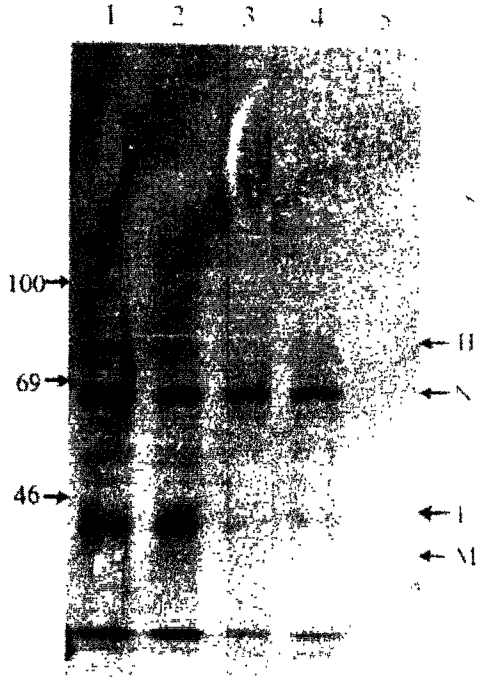

Fig. 3
Fluorogram of RIPA of vaccine virus and TR-RPV using RHIS and BHIS
Vaccine virus-infected Vero cell lysate using RHIS (lane 1), TR-RPV-infected Vero cell lysate using RHIS (lane 2), vaccine virus-infected Vero cell lysate using BHIS (lane 3), TR-RPV-infected Vero cell lysate using BHIS (lane 4), and uninfected Vero cell lysate (lane 5).

revealed no protein band (Fig. 3). From these results it follows that the TR-RPV proteins are of the same or very similar size as the vaccine virus proteins.

Discussion

The extensively used TCRP vaccine (RBOK strain) against rinderpest was developed in 1959 (Plowright, 1962). Since then, the work on improvement of TCRP vaccine, especially its thermostability has been in progress. Substitution of water by 1 mol/l $MgSO_4$ (Provost, 1982) or saline (Plowright *et al.*, 1971; Languet *et al.*, 1985) as diluent of the vaccine has been reported. Likewise, use of various stabilizers and a modified lyophilization procedure has increased the thermostability of the vaccine (Mariner *et al.*, 1990; Nayak *et al.*, 1995). However, there was no report on improvement of thermoresistance of the virus itself.

Hence, we attempted a systematic approach to developing a clone (mutant) of thermoresistant virus by passaging the vaccine virus in Vero cells at gradually elevated temperatures. The virus could withstand the final 40°C by increasing the temperature slowly by an increment of 0.5°C per passage. The virus growth under these conditions resulted in selection of thermoresistant clone (mutant) that may prove to be basis for a more thermostabile vaccine in field conditions than the parent vaccine virus, RPV Rebok strain. The data on TR-RPV presented in this paper confirm our success in the development of a more thermoresistant clone (mutant).

In another approach, different from that described above, native vaccine virus was inoculated at similar MOI (0.5) to TR-Vero cells, left to adsorb at 37°C and then incubated straight at 40°C. On day 5 p.i., the CPE was RPV-specific but only partial. Therefore, we abandoned this approach and used the simultaneous culture method (Singh *et al.*, 1995) that allowed a cell-to-cell virus transfer and hence infection of more cells and, thereby, enhanced CPE. As reported by Singh *et al.* (1995), the simultaneous culture method also reduced the time of adaptation of virus to grow at 40°C.

No reports are available on the method of assessment of thermoresistance (thermostability) of viruses, especially RPV, except titrating the virus incubated without a stabilizer. However, there are several reports on the study of stability of the lyophilized virus as determined after its reconstitution. Also available are reports showing the effect of different chemical stabilizers/vaccine diluents as well as lyophilization procedure itself (Languet *et al.*, 1985; Mariner *et al.*, 1990; Nayak *et al.*, 1995). Usually, employment of certain diluents or addition of some stabilizers has positive effect on the thermoresistance of the vaccine virus, but we evaluated TR-RPV in this regard without addition of any stabilizer to see if the TR-RPV itself had acquired the intended property. In absence of stabilizers in cell culture supernatants, the observed half-life of the vaccine virus was 1.11 hrs in contrast to 8.4 hrs (Plowright *et al.*, 1971) and 7.0 hrs (Provost, 1982), reported earlier for a reconstituted vaccine virus. In our experiments, the half-life of TR-RPV was longer (1.81 hr) than that of the vaccine virus (1.11 hrs) in infected cell culture supernatants. The increased thermostability of TR-RPV over the vaccine virus may be due to its acquired property, as all the experimental conditions during the assessment of the thermoresistance were kept identical.

RPV is known for its antigenic stability and this is the reason for using a single vaccine strain worldwide in rinderpest control programs. Since TR-RPV achieved resistance to higher temperatures (40–41°C), it was essential to study its protein profiles and immunogenic potential in order to reveal effects of temperature stress, if any, on antigenicity of the virus. The PEG-8000-precipitated TR-RPV gave precipitin lines in AGP test and CIEP with RHIS, indicating preservation of antigenicity of the virus. In IP test, the very strong staining reaction observed during early CPE might be due to localization of viral antigen within the

infected cells. Weak staining during late CPE might be due to shedding viral antigen from infected cells or due to the excessive degradation of N protein of mutant virus.

There was an increased binding of Triton X-100-treated TR-RPV with MAbs 12AD10.1.1, 12BD 7.1.1, and 12DG7.1.1. MHIS also exhibited similar patterns of reactivity. This might be due to the disaggregation of the protein in the presence of Triton X-100. An increased reactivity has been reported for Triton X-100-solublized protein in AGP test, CIEP, single radial haemolysis and solid phase agglutination of coated erythrocytes (Sarkar *et al.*, 1993). In the present study, the effect of Triton X-100 on TR-RPV was more pronounced than that on the vaccine virus grown at 37°C. This could be due to a reduced formation of viral aggregates at 37°C. It is known that certain stress/heat shock proteins are produced by the cells grown at higher temperatures. These proteins, being non-specific, bind to cellular and non-cellular proteins to protect them from heat and other abnormal conditions. Being non-specific, they may also bind to virus particles and mask epitopes. The virus particles may also tend to form large aggregates when subjected to higher temperatures during their propagation in cell cultures. We do not have any evidence on the latter possibility. The Triton X-100 treatment might have resulted in releasing the bound stress/heat shock proteins as well as in disaggregating the virus particles resulting in better reactivity of MAbs in capture ELISA.

In RIPA of the labeled proteins, using either RHIS or BHIS, four virus-specific proteins, namely H (79 K), N (60 K), F (42 K) and M (36 K) were observed in both the vaccine virus-and TR-RPV-infected cell lysates. Similar results have also been reported earlier (Sato *et al.*, 1981; Diallo *et al.*, 1987). However, immunoprecipitation reaction of RPV-infected cell lysates has shown five viral proteins, namely P, H, N, F, and M (Barrett *et al.*, 1987; Grubman *et al.*, 1988). The difficulty in demonstration of P and L proteins in all immunoprecipitation reactions in the present study might be due to their low concentration or high susceptibility to proteolysis as reported by Diallo *et al.* (1987). We observed two extra bands in all immunoprecipitation reactions despite the use of protease inhibitors, PMSF and Aprotenin. M_r of these proteins (50 K and 55 K) correspond to the proteolytic products of RPV N proteins as reported earlier (Rima, 1983; Singh, 1990). Summing up, TR-RPV proteins are of the same or very similar size as the vaccine virus proteins.

To conclude, the present study shows that though TR-RPV in contrast to the vaccine virus multiplies at higher temperature (40°C), the biochemical and immunogenic protein profiles of both the viruses are similar, indicating that there was no change as regards these characteristics. Thus, the acquired thermoresistance of TR-RPV might be due to minor amino acid changes that were observed by us by nucleotide sequences analysis (data not shown). It also implies that the method for developing TR-RPV may also be used for other viruses, e.g. the peste des petits ruminants (PPR) virus, antigenically very closely related to RPV, which might be a suitable thermoresistant PPR vaccine candidate.

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