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Influenza virus variants with reduced susceptibility to inhibition by a polyphenol extract from *Geranium sanguineum* L.

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A polyphenol extract obtained from the Bulgarian medicinal plant *Geranium sanguineum* L. (PC) inhibited influenza virus reproduction *in vitro*, *in ovo* and *in vivo*. The inhibitory activity of the plant preparation was proved to be selective and specific. Two variants of the virus A/chicken/Germany/34, strain Rostock (H7N1) (A/Rostock) with reduced drug sensitivity were selected by following passages in chicken embryo fibroblast (CEF) cells in the presence of inhibitory concentrations of the substance and by subsequent purification of resistant plaques (A/Rostock-R). PC affected to a lesser extent the biological activity and the infectivity of the resistant variants compared to the sensitive parent. The lower susceptibility of the variants was consistently observed in contact assay, ELISA, cytopathogenic effect reduction, plaque reduction and infectious virus yield reduction assays. Virus-specific protein synthesis in CEF cells infected with the resistant variants, determined by ^{35}S-labelling and following SDS-PAGE, was also less sensitive to inhibition with PC.

1. Introduction

In the search of new viral inhibitors naturally occurring preparations of plant origin might be an alternative source of biologically active compounds of diverse structure. A large number of extracts and pure substances have been tested and a selective antiviral effect has been proved for some of them (for review see [1, 2]).

Geranium sanguineum L. is wide spread in Bulgaria. Aqueous and alcoholic extracts from its root are used in traditional medicine to treat gastrointestinal disorders and various infections and inflammatory conditions [3]. A methanol extract from the aerial roots of the plant, characterised as a polyphenolic complex (PC), was shown to inhibit the reproduction of influenza A and B viruses [4]. PC administered intranasally or by aerosol, significantly reduced the mortality of white mice resulting from experimental infection with influenza A/Aichi/2/68 (H3N2) [5]. Phytochemical investigation revealed the presence of flavonoids, catechins, gallotannins and phenolic acids in PC preparations [6, 7]. To investigate the active components, PC was fractionated and a n-butanol fraction was shown to contain the majority of the *in vitro* antiviral activity [7].

The current study was undertaken to provide further evidence for the selectivity of the antiviral effect of the plant preparation by the selection of influenza virus variants with reduced susceptibility to PC-inhibition. To my knowledge this is the first report on the selection of resistance to an inhibitor of plant origin.

2. Investigations and results

2.1. Cellular toxicity

Confluent CEF cell monolayers treated for 72 h with PC at concentrations up to 50 μg/ml did not show any visible changes in cell morphology or cell density. 50% toxic concentration (CC_{50}) of PC was estimated to 100 μg/ml. The effect of PC on proliferating CEF cells was determined by the tetrazolium-based colorimetric MTT assay [8] and the 50% cell inhibitory concentration (IC_{50}) of PC was evaluated at 72 μg/ml [9].

2.2. Selection of PC resistant mutants

Methods which readily select rimantadine-resistant mutants were used [10]. After 11 passages of A/Rostock in CEF in the presence of increasing inhibitory concentrations of PC (10–50 μg/ml) the virus (R-11), harvested from the 11th passage, was less susceptible to inhibition by PC. Direct selection of resistant plaques from the sensitive virus stock by three successive plaque titrations in the presence of 50–100 μg/ml of PC yielded a more resistant variant (A/Rostock-R). Similar results were obtained for R-11 and A/Rostock-R with regard to their growth characteristics (production of HA, expression of HA, cytopathogenicity and plaque formation, total protein synthesis) and susceptibility to the inhibitory effects of PC. Further experiments were performed with A/Rostock-R. After three passages of A/Rostock-R in drug-free growth medium their growth characteristics, infectivity and reduced susceptibility to inhibition by PC remained unaltered.

The lower susceptibility of A/Rostock-R to inhibition by PC compared to parent virus was consistently observed in various assays.

2.3. Virucidal activity

To determine the direct inactivating effect of PC on extracellular virus ten fold diluted allantoic fluid of A/Rostock

Table: Inactivating effect of PC on influenza viruses A/Rostock and A/Rostock-R

Virus	PC (μg/ml)	HA titre	Infectious titre	
			$TCID_{50}$/0.2 ml	PFU/0.5 ml
A/Rostock	0–50	32	10^6	10^7
	100	16	10^4	10^5
	150	8	$10^{3.5}$	10^4
	200	2	n.d.	n.d.
A/Rostock-R	0–50	32	10^6	10^7
	100	32	10^6	10^7
	150	16	10^6	10^7
	200	4	10^4	10^4

n.d. not done

and A/Rostock-R were treated directly with equal volumes of PC (12.5–200 μg/ml) with subsequent determination of HA-titres and virus infectivity (Table). Up to 50 μg/ml no inactivating effect was observed. 100 μg/ml reduced HA-titre (2 log_2) and infectious titre (2 log_{10}) of the parent virus, but did not affect the biological activities of A/Rostock-R. 150 μg/ml affected slightly A/Rostock-R and reduced HA-titre (1 log_2) but not infectious titres. 200 μg/ml of PC inactivated significantly A/Rostock-R and abolished completely the biological activity of the parent virus. Minimal inhibitory concentrations (MICs) were 200 and 100 μg/ml PC respectively.

2.4. Effect of PC on A/Rostock-R replication

In all antiviral experiments non drug treated, mock infected cells were used as cell control and non drug treated, virus infected cells as virus control. Rimantadine hydrochloride (0.1–1 μg/ml) was used as a positive control.

2.4.1. Reduction of protein expression on infected cells surface

In some experiments low multiplicities of infection were used (1–3 log_{10} $TCID_{50}$/0.2 ml) and the expression of viral glycoproteins and viral yields was determined after multiple cycles of reproduction. In one cycle growth experiments high multiplicities of infection were used (7 log_{10} $TCID_{50}$/0.2 ml).

The inhibitory effect of PC on both parent virus and resistant variant was dose-related and highly depended on the virus inoculum. The comparative expression of hemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) on the infected cells surface was determined by ELISA with the corresponding monoclonal antibodies (Mab). The results are presented in Fig. 1–a, b, c. HA was most susceptible to the effect of the substance. 50% effective concentrations (EC_{50}-s) for A/Rostock-R they were about 10 times higher than the respective EC_{50}-s for the parent A/Rostock (20 and 2.1 μg/ml of PC respectively). In one cycle growth experiments 50 μg/ml, respectively 25 μg/ml inhibited the expression of all three proteins.

2.4.2. Reduction of virus induced cytopathogenic effect

PC inhibited significantly A/Rostock-induced CPE at concentrations >3.6 μg/ml. At least 25 μg/ml of the extract were needed to reduce A/Rostock-R-induced cytopathogenic effect (CPE). EC_{50}-s were 3.6 and 25 μg/ml of PC respectively.

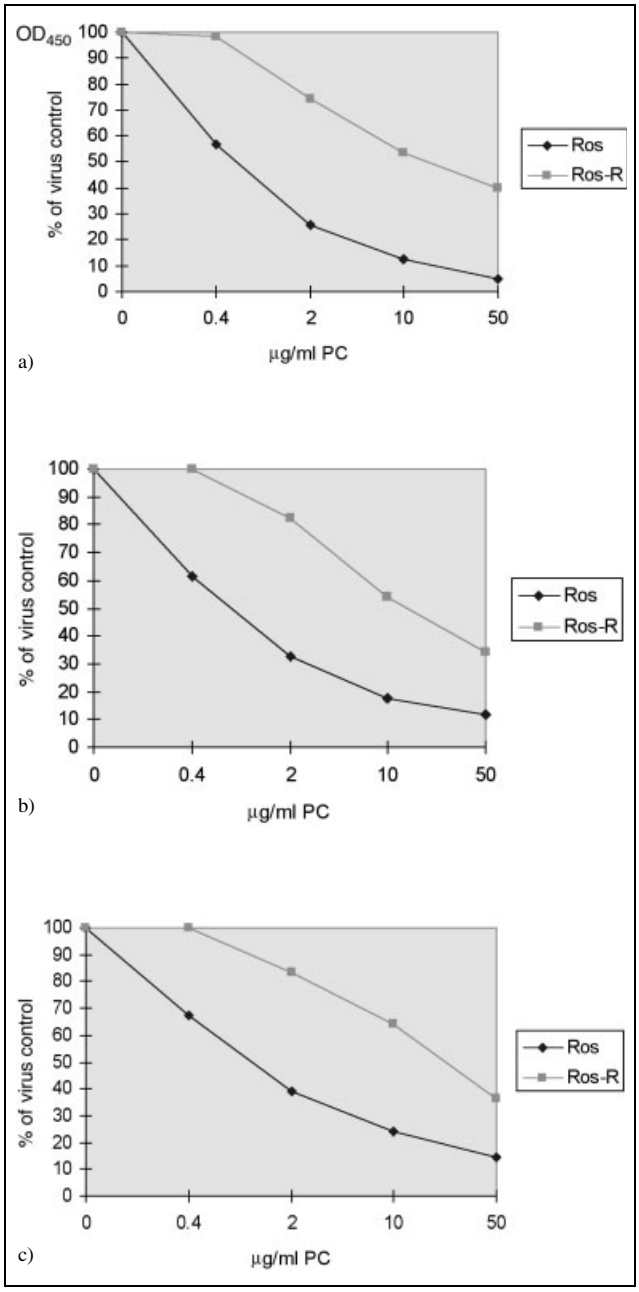

Fig. 1: Effect of PC on the expression of viral proteins on the surface of CEF, infected with influenza viruses A/Rostock and A/Rostock-R – hemagglutinin (a), neuraminidase (b), mucleoprotein (c)

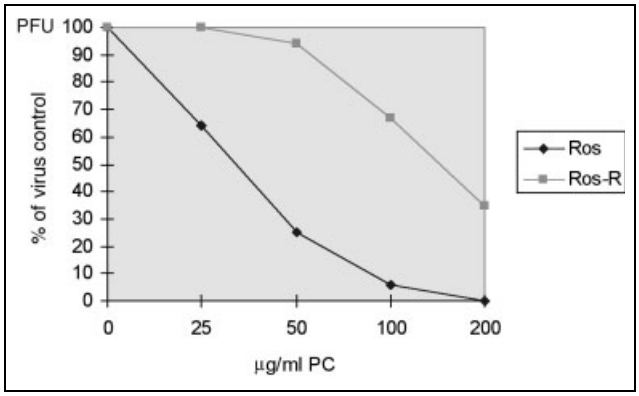

Fig. 2: Dose-dependence of the inhibition of virus-induced plaque formation in CEF, infected with influenza viruses A/Rostock and A/Rostock-R by PC.

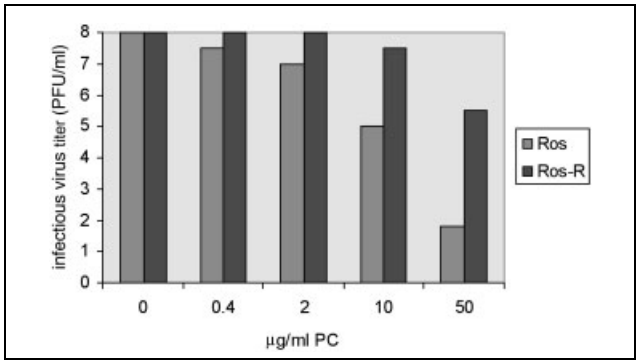

Fig. 3: Dose-dependence of the inhibition of infectious virus yield production in CEF, infected with A/Rostock and A/Rostock-R by PC.

2.4.3. Reduction of infectious plaque formation

The reduced susceptibility of A/Rostock-R to PC-inhibition was demonstrated also in the infectious plaque reduction assay. 50 µg/ml of PC did not affect the plaque formation activity of A/Rostock-R while 25 µg/ml already reduced significantly the size and number of A/Rostock plaques (Fig. 2). EC_{50}-s were respectively 32 and >100 µg/ml of PC.

2.4.4. Reduction of infectious virus yields

In infectious virus yield reduction assay 2 µg/ml of PC inhibited the reproduction of A/Rostock and partially reduced HA yields and plaque formation by 1 $\log_{10}$ PFU/0.5 ml, 10 µg/ml decreased infectivity with up to 3 $\log_{10}$ PFU/0.5 ml (Fig. 3). A/Rostock-R was much more resistant to inhibition with PC – 2 µg/ml were not effective, while 10 µg/ml insignificantly reduced infectivity. In single cycle growth experiments the HA-yields and infectious virus plaque formation were inhibited by 10 µg/ml and 50 µg/ml respectively. 90% effective concentrations (EC_{90}-s) for A/Rostock and A/Rostock-R in this assay were 3.6 and 25 µg/ml of PC respectively.

2.5. Inhibition of viral protein synthesis

The effect of PC on virus specific protein synthesis was studied following a single cycle of virus replication by labelling with ^{35}S -methionine followed by SDS-PAGE of the cell lysates (Fig. 4). The inhibitory effect was dose-dependent and was more pronounced when PC was applied after viral infection (results not shown). The inhibition of virus-specific protein synthesis was selective; MIC (10 µg/ml) was tenfold lower than that which inhibited cell protein synthesis. PC caused visible inhibition of A/Rostock-R-specific protein synthesis at concentration 50 µg/ml.

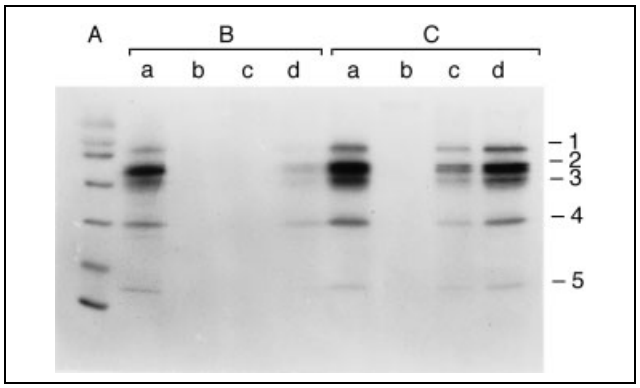

Fig. 4: Inhibitory effect of PC on synthesis of ^{35}S-methionine labelled proteins in CEF infected with influenza viruses A/Rostock and A/Rostock-R. 1 – P – proteins of the polymerase complex, 2 – HA – hemagglutinin glycoprotein, 3 – NP – nucleoprotein, 4 – M – matrix protein, 5 – NS – nonstructural protein; A – molecular weight markers, B – A/ Rostock, C – A/Rostock-R; lanes: a – PC absent, b – 100, c – 50, d – 10 µg/ml PC

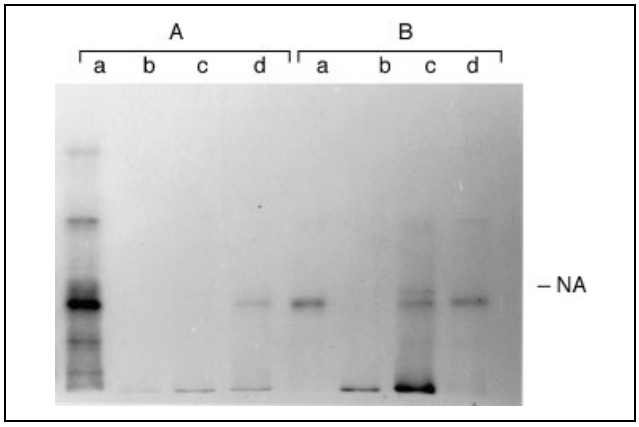

Fig. 5: Inhibitory effect of PC on viral NA synthesis in CEF, infected with influenza viruses A/Rostock and A/Rostock-R. NA – neuraminidase; A – A/Rostock, B – A/Rostock-R, lanes: a – PC absent, b – 100, c – 50, d – 10 µg/ml PC.

The effect of PC on the synthesis of the individual virus proteins HA, NA and NP was determined by immunoprecipitation with MAbs followed by SDS-PAGE of the immune complexes. The results obtained for NA synthesis are presented on Fig. 5. To abolish NA synthesis in CEF, infected with the parent virus A/Rostock 10 µg/ml of PC were enough, while NA synthesis of A/Rostock-R was inhibited only at doses >50 µg/ml. (Fig. 5). Similar results were obtained for HA and NP synthesis (results not shown).

3. Discussion

The principal objective of the study was to further characterise the antiinfluenza virus activity of the plant polyphenolic complex isolated from the Bulgarian medicinal plant *Geranium sanguineum* L. with respect to its selectivity by selection of variants with reduced drug sensitivity. In previous studies it was shown that the inhibitory effect was strain-dependent, consistent with its apparent selectivity [4, 9].

PC-resistant variants were selected by multiple passages in the presence of the drug and purification of resistant plaques. It should be noted that their growth characteristics, infectivity and reduced susceptibility to inhibition by PC remained unaltered after three passages in drug-free growth medium. PC affected to a lesser extent the expression of HA, NA and NP on infected cell surface analysed by ELISA (Fig. 1), the virus-induced CPE, the production of infectious virus in plaque reduction assay (Fig. 2) and viral protein synthesis in CEF cells infected with the resistant variants compared to the sensitive parent (Figs. 4, 5).

Although the lower sensitivity of the variants was consistently observed the difference in drug sensitivity was insufficiently discrete to provide a convincing basis for full measure genetic studies. Preliminary results from the sequencing analysis of HA and M genes did not reveal any changes. It might therefore be that the reduced sensitivity of the variants was due to conformational changes of the viral glycoproteins (HA and NA) which could have occurred during the multiple passages in the presence of PC.

Our studies concerning the specificity of the antiviral effect of PC in respect to viral infection showed that its selectivity was limited [9]. The present studies were important and necessary in view of the pronounced therapeutic effect of PC found in experimental influenza infection in mice [5]. PC administered intranasally (1–3 mg/kg) applied 6 h before infection reduced mortality rate (index of protection = 33–67%) and prolonged survival time (1–3 days). When PC was administered by aerosol in a dose of 20 mg/ml, applied 2, 24, 48 and 72 h after virus infection the protection index was 64% Administration of PC in combination with rimantadine hydrochloride (1–10 mg/kg) produced a synergistic therapeutic effect and the protection index reached 77.8% [11].

It has been found that the plant preparation possessed some other biological activities. PC exhibited a stimulating effect on cell type immune response and induced low production of interferon after intraperitoneal application [12]. In a model system PC demonstrated an O_2^- radical scavenging activity [13].

The determination of biologically active substances showed that the quantity of tannins was 34.02%, of flavonoids – 0.141%, of catechins and proanthocyanidines – 2.177 mg/kg [6]. To investigate the active components the mixture was fractionated and an n-butanol fraction was shown to contain the majority of the *in vitro* antiviral activity [7]. Phytochemical investigation revealed the presence of flavonoids (quercetin, morin, myricetin, kaempferol, apigenin, rhamnasin, retusin), (+) and (−) catechins, gallotannins and phenolic acids (caffeic, ellagic, quinic, chlorogenic) [6, 7]. A schedule of standardised phytochemical and biological parameters was developed to control the biological properties of the preparation [14]. The therapeutic effect of PC on the course of experimental influenza infection *in vivo* might be attributed to the combination of the expression of more than one biological activities of the plant product – selective antiviral action, immunostimulating effect, some important non specific biological and pharmacological interactions, typical for plant polyphenols such as protein binding [15] antioxidant effect [16] and radical scavenging activity [17].

4. Experimental

4.1. Compounds

The medicinal plant *Geranium sanguineum* L. (Geraniaceae) has been introduced into the experimental field of the Institute of Botany, Bulg. Acad. Sci., Sofia and a voucher specimen was deposited in the Herbarium of the same institute (N SOM 5/86). The preparation of the polyphenolic complex has been described previously [5]. In short – ground air-dried aerial roots, collected during the flowering period, were defatted with petroleum ether and treated with methanol to fully extract the polyphenolic components. The extract was lyophilised (yield 16%). The obtained preparation (PC) was a dark red powder, odourless, soluble in water. The polyphenol content of PC was controlled by thin layer chromatography and by quantitative determination of tannins, flavonoids and catechins [6]. PC was prepared and kindly provided by Dr. Stefka Ivancheva from the Institute of Botany, Bulg. Acad. Sci. A 1% stock solution was prepared in sterile distilled water. For the *in vitro* experiments further dilutions were made in cell culture medium *ex tempore*.

Rimantadine hydrochloride was obtained from Hoffman – La Roche Inc., Nutley, NJ.

4.2. Cells and viruses

Primary CEF cell cultures were prepared according to Portfield [18] and maintained in tris-buffered growth medium, containing 45% MEM (minimal Eagle's medium), 45% Hank's solution, 5% LAH (lactoalbumin hydrolysate) and supplemented with 5% calf serum and antibiotics (100 IU/ml benzylpenicillin and 100 μg/ml streptomycin).

Avian influenza virus A/chicken/Rostock/34 (H7N1) (A/Rostock) and resistant variant (A/Rostock-R) were grown in 11-days old fertile hen's eggs and allantoic fluids were used as virus inoculum. The virus stocks were stored at −70 °C. For plaque assay, CEF were overlaid with 1% agarose in 199 medium supplemented with 10% bovine serum albumin (BSA) and 1% dextran sulphate. The HA assay was performed as described [5].

4.3. Cellular toxicity

The toxicity of the preparation for CEF cells was evaluated by two assays – cytopathogenic effect (CPE) and neutral red dye uptake – as described [9]. 50% cell toxicity concentration (CC_{50}), the concentration causing visible changes in 50% of intact cells, was evaluated from graphic plots.

4.4. Selection of resistant variants

A/Rostock was serially passaged in the presence of inhibitory concentrations of PC (10–50 μg/ml) and resistant plaques were isolated in the presence of drug (50–100 μg/ml) [10].

4.5. Virucidal activity

Virucidal activity was assayed as described [9]. The HA titres and virus infectivity in CPE and plaque assays were determined (see below). The minimum concentrations (MIC) that reduced the HA titre by 2 $\log_2$ and infectious titre by 1 $\log_{10}$ PFU/0.5 ml or 1 $\log_{10}$ $TCID_{50}$/0.2 ml were determined.

4.6. Antiviral assays

The inhibitory effect of PC on the replication of A/Rostock and A/Rostock-R in CEF was studied by a number of antiviral assays. PC was diluted two fold in serum-free medium.

4.6.1. Enzyme linked immunosorbent assay (ELISA) of HA, NA and NP expression

The ELISA method was applied as described [19]. After 16–20 h incubation with 2× drug-containing medium at 37 °C cell monolayers were fixed with 0.05% glutaraldehyde in PBS and assayed for HA, NA and NP on the cell surface. ELISA was performed with MAbs to HA, NA and NP and protein A-horseradish peroxidase conjugate (Bio Rad Lab., Ca). The optical densities (OD_{450}) were measured and expressed as% of non drug-treated virus infected cells (virus control). MAbs were kindly provided by Mr. Alan Douglas of the WHO Influenza Collaborative Centre, Mill Hill, London. The concentration causing 50% reduction in optical density values (EC_{50}) was evaluated from graphic plots.

4.6.2. Cytopathogenic effect (CPE) reduction assay

CPE was performed and the virus-induced CPE was scored as described [9]. The concentration reducing CPE by 50% (EC_{50}) with respect to virus control was estimated from plots of the data.

4.6.3. Infectious plaque reduction assay

The method was described earlier [9]. The concentration reducing plaque number by 50% (EC_{50}) was evaluated.

4.6.4. Infectious virus yield (IVY) reduction assay

The method was described earlier [9]. Virus titres were determined by plaque assay or CPE assay by endpoint titration [20]. The significance of differences in infectious virus titres was estimated using the Student's t-test. The concentrations that reduced virus infectivity by 90% (1 $\log_{10}$ PFU/0.5 ml, 1 $\log_{10}$ $TCID_{50}$/0.1 ml; EC_{90}) and reduced HA titres with >2 $\log_2$ were determined.

4.7 Viral protein synthesis

A modification of the method described in [21] was used. The cell monolayers in duplicate wells of 24-well plates were pre-treated for 1 h at room temperature with PC, washed twice with PBS and challenged with undiluted infectious allantoic fluid (MOI = 10–50 PFU/cell). After adsorption for 1 h at room temperature in the presence of PC, the cells were washed twice with PBS and drug-containing medium added. After incubation for 5 h at 37 °C the monolayers were labelled for 1 h with 10 μCi of ^{35}S-methionine/well, lysed and proteins were analysed by electrophoresis on a 12% polyacrylamide gel.

4.8. Immuneprecipitation

Cell lysates were immuneprecipated with anti-HA, anti-NA and anti-NP MAbs and immune complexes analysed by SDS gel electrophoresis [22].

4.9. Nucleotide sequencing analyses

Sequences of the HA and M (matrix protein) RNA genes were determined by the deoxynucleotide chain-terminating method, using ^{32}P-labelled primers and AMV reverse transcriptase [10].

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