

Biophysical Studies of Respiratory Syncytial Virus

I. Density of Respiratory Syncytial Virus and Associated Complement-Fixing Antigens in a Cesium Chloride Density Gradient

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ABSTRACT

COATES, HELEN V. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), BEN R. FORSYTH, AND R. M. CHANOCK. Biophysical studies of respiratory syncytial virus. I. Density of respiratory syncytial virus and associated complement-fixing antigens in a cesium chloride density gradient. *J. Bacteriol.* 91:1263-1269. 1966.—Concentrated fluids from respiratory syncytial (RS) virus-infected tissue cultures (HEp-2 and BEK) were subjected to equilibrium sedimentation in cesium chloride. When two antigenically distinct strains of RS virus (Long and 18537) were tested, approximately 90% of the infectious virus was recovered in a sharp, symmetrical peak with a density of 1.22 to 1.24. In a similar study, unconcentrated virus had a density of 1.25 to 1.27. Two immunologically distinguishable complement-fixing antigens (antigens A and B) were detected at densities of 1.28 to 1.32 and 1.23 to 1.37. In addition, the existence of a third antigen (density of 1.22 to 1.30) was suggested. The possible origin of these antigens is discussed relative to the known properties of RS virus and the other myxoviruses.

Despite the recognition of respiratory syncytial (RS) virus as one of the major causes of severe respiratory-tract disease in infants and young children, no vaccine is yet available to prevent this infection. In fact, this virus has proved difficult to study because of the relatively low yield of virus and viral antigens from infected cell cultures (4, 7, 15). The infectious particle can be sedimented by high-speed centrifugation; however, centrifugation has proved of limited value as a means of concentrating the virus, since 80 to 90% of the infectivity is lost during sedimentation (7). In infected tissue culture fluids, a major proportion of the total antigenic material reactive in the complement-fixation (CF) test is present in a form which is not easily sedimented in the ultracentrifuge (7, 8). The relationship between this "soluble" material which fixes complement and the antigens of the virion is not known.

Isopycnic banding in a density gradient has been widely applied to the separation and biophysical characterization of a variety of viruses and viral antigens. The successful separation of multiple antigenic components of three myxoviruses [influenza (3), measles (17, 24), and Newcastle disease (22) viruses] in a cesium chloride density gradient suggested that similar results

might be obtained with RS virus. In the studies which follow, fluids from RS virus-infected cell cultures were subjected to equilibrium sedimentation in cesium chloride. Data which suggest the presence of four biologically different components in such fluids were obtained, and form the subject of this report.

MATERIALS AND METHODS

Virus. The Long strain of RS virus was isolated from a throat swab specimen of a child with bronchopneumonia in 1956 (7). It was passaged 11 to 13 times in heteroploid human cell lines (KB, Chang liver, HEp-2) before being used in these studies.

The 18537 strain of RS virus was isolated from the throat swab specimen of a child with upper respiratory disease in 1962 (8). It was recovered in a human diploid cell strain (Wistar 26 cells; 12), and was subsequently passaged eight times in human heteroploid cell lines before being used in these studies.

Quantities of 0.5 to 2 liters of virus were grown in HEp-2 cells or primary bovine embryonic kidney cells. Infected tissue culture cells and fluids were harvested when 75 to 100% of the cell sheet exhibited cytopathic effects (CPE). Harvests were concentrated 10- to 20-fold by vacuum dialysis by use of an LKB ultrafilter. The concentrates were then clarified by centrifugation for 20 min at 2,000 rev/min in an International PR2 refrigerated centrifuge.

Tissue cultures. HEP-2 cells were grown in 32-oz (ca. 1-liter) prescription bottles or in 5-liter Povitsky bottles with a medium consisting of Eagle's minimum essential medium (MEM) containing Hanks' balanced salt solution (BSS) and supplemented with 10% heat-inactivated (56 C, 30 min) calf serum and 0.1 mM glutamine. Virus infectivity was assayed in HEP-2 cell roller-tube cultures, purchased from Flow Laboratories, Inc., Rockville, Md., or with HEP-2 cell monolayers which were prepared in tissue culture dishes (60 by 15 mm; Falcon Plastic, Los Angeles, Calif.).

Primary bovine embryonic kidney (BEK) cell cultures in 32-oz bottles were purchased from Flow Laboratories. They were grown in Eagle's basal medium with 5% calf serum.

Prior to inoculation with virus, the cell sheets were washed with Hank's BSS and refed with maintenance medium composed of Eagle's MEM, 5% heat-inactivated agamma calf or chicken serum, 0.1 mM glutamine, 100 μ g of streptomycin, and 100 units of penicillin per ml. HEP-2 cells were maintained with Eagle's MEM containing chicken serum, and the BEK cells were maintained with medium containing calf serum.

Density gradient centrifugation. The cesium chloride stock solution consisted of a saturated solution of commercial CsCl in 0.01 M phosphate buffer (pH 7.2). The impurities in the commercial CsCl were either adsorbed with activated charcoal or neutralized by addition of 1% bovine albumin to the stock solution prior to use. Concentrated RS virus was mixed with sufficient CsCl stock solution to yield a starting density of approximately 1.25 to 1.35 g/cm³. A 4.5- or 5-ml amount of the CsCl-virus mixture was then placed in a Lusteroid centrifuge tube and overlaid with sufficient mineral oil to completely fill the tube. The sucrose gradient was performed by hand. A stock solution of 60% reagent-grade sucrose in distilled water was diluted in 5% steps to 40% in phosphate-buffered saline (pH 7.2). Five layers, of 0.75 ml each, were added to the centrifuge tube, and 1.25 ml of virus was layered on top of the gradient. Centrifugation was carried out at 4 C in the SW 39 rotor of a Spinco model L ultracentrifuge at approximately 115,000 \times g for 36 hr or longer, as specified in Results. After centrifugation, the tubes were examined for the presence of visible bands, and the contents were collected in uniform fractions of 5 to 10 drops from the bottom of the tube. The bouyant density of each fraction was determined by weighing a 100- μ liter sample of the fraction on a Sartorius analytical balance. All densities were expressed in grams per cubic centimeter.

Virus assay. Plaque assays were performed as previously described (Coates, Alling, and Chanock, *Am. J. Epidemiol.*, *in press*). HEP-2 cell monolayers were used at the end of a 24-hr growth period before the cell sheet was confluent. As a rule, serial 100-fold dilutions of the suspensions to be tested for infectivity were prepared and inoculated onto two or three plaque plates per dilution (0.2 ml per plate). After a 2-hr adsorption period, the cell sheet was overlaid with a fluid medium consisting of Eagle's MEM, 1% methylcellulose (4,000 cps), 1% heat-inactivated chick

serum plus glutamine, and antibiotics as above. Cultures were incubated in an atmosphere of 5% CO₂, 85% relative humidity, for 2 to 3 days until plaques developed. At that time, the overlay was replaced with 10% formalin. After fixation, the cells were stained with Giemsa stain, and plaques were counted with the aid of a Bausch & Lomb Stereozoom dissecting microscope. The titer of virus in the suspension was calculated from the geometric mean number of plaques per plate and expressed as plaque-forming units (PFU) per milliliter. The standard error of each titration was estimated by dividing the range of counts at a specified dilution by the number of replicate plates counted. In no case did the estimated standard error exceed 10^{0.3}.

Assays were also performed in roller-tube cultures of HEP-2 cells. Serial 10-fold dilutions of each suspension were prepared and inoculated into two cultures per dilution (0.2 ml per culture). Inoculated cultures were observed over a 2-week period for typical syncytial cytopathic effects. The titer of the virus suspension was calculated as that dilution at which 50% of the inoculated cultures were infected (TCD₅₀), according to the method of Reed and Muench (18).

Immune sera. Serum was obtained from an adult male volunteer after experimental infection with the 11657 strain of RS virus (14). The 11657 strain of RS virus has been shown to be closely related to the Long strain in reciprocal plaque-reduction neutralization tests (Coates, Chanock, Alling, *in press*). This serum had a CF antibody titer of 1:64 when tested with the standard RS virus CF antigen. In addition it had a CF antibody titer of 1:32 when tested with RS virus antigen A, the first antigen to be eluted when RS virus concentrated by vacuum dialysis was chromatographed on a Sephadex G200 column. A CF antibody titer of 1:32 was obtained when this serum was tested with antigen B, the second antigen to be eluted from the Sephadex column. Details of the separation and purification of antigens A and B are reported in a separate communication (10).

Antisera to antigens A and B were prepared by the hyperimmunization of guinea pigs with antigens prepared from chromatographic fractions of the soluble CF antigen of the Long strain of RS virus grown in HEP-2 cells. These sera reacted specifically, in the CF test, with the homologous antigen prepared from BEK-grown RS virus. BEK-grown antigens were used in the CF test to avoid reactions with antibodies against HEP-2 cell constituents and chicken serum. The anti-A serum had a homologous CF antibody titer of 1:80, and the anti-B serum had a homologous CF antibody titer of 1:40. Heterologous antibody was not detected in either serum at a dilution of 1:10, nor was CF antibody to BEK cells or to calf serum detected.

CF test. The CF test was carried out by a modified Bengston procedure as previously described (6), with the use of 1.6 to 1.8 units of complement, 4 units of antiserum, and overnight fixation at 4 C. One unit of CF antigen was taken as the highest dilution of the antigen which produced 75 to 100% fixation of complement. The titer of the CF antigen was ex-

pressed in CF units per 0.025 ml (CFU). The standard RS virus CF antigen was prepared from infected tissue culture cells and fluid as previously described (5).

RESULTS

Density of the virion and CF antigen of the Long strain of RS virus. In the first experiment, the Long strain of RS virus (concentration, 10 times) was centrifuged for 36 hr at 35,000 rev/min. As shown in Fig. 1A, two white bands were observed in the gradient prior to collection of the fractions. The upper band was 4 mm in depth and floccular in appearance. The lower band had a distinct, opaque upper zone 1 mm in depth and a lower opalescent zone 5 mm in depth.

Eleven fractions of approximately 0.4 ml each were collected, and the pellet was resuspended in 0.5 ml of tissue culture medium. As shown in Fig. 1A, 90% of the infectious virus was recovered in a single fraction (fraction 8), which had an infectivity titer of $10^{6.5}$ TCD₅₀ per 0.2 ml and a density of 1.23. In contrast, 80% of the CF antigen, as

determined by reaction with human serum, was distributed over six fractions with a range in density of 1.23 to 1.32. Fraction 5, with a density of 1.28, contained 160 CFU which represented 25% of the recovered CF antigen. The CF antigen detected by human RS immune serum was skewed toward the top of the gradient, overlapping the infectious virus band.

Within the limits of the assay systems used, no loss of infectivity or CF antigen was detected. The starting material had a total infectivity of $10^{6.9}$ TCD₅₀ and contained 80 CFU. When the biological activity of all of the fractions, including the resuspended pellet, was totaled, 99% of the virus input ($10^{6.8}$ TCD₅₀) and 54% of the CF antigen was recovered. The resuspended pellet contained $10^{5.4}$ TCD₅₀ of virus, but less than 10 CFU.

This experiment was repeated with a 20-fold concentrate of the Long strain of RS virus. After centrifugation for 48 hr at 39,000 rev/min, the gradient contained two visible bands, as shown diagrammatically in Fig. 1B. In contrast to the preceding experiment, the lower band appeared homogeneous. Eighty per cent of the infectious virus was recovered in a single fraction (no. 9, Fig. 1B) with a density of 1.22. This fraction had an infectivity titer of $10^{7.2}$ PFU per 0.2 ml. Seventy-six per cent of the recovered CF antigen, as determined by reaction with human serum, was collected in four fractions which ranged in density from 1.23 to 1.30. Each of these fractions contained 160 CFU.

Density of the virion and CF antigen of the 18537 strain of RS virus. It has been reported that strains of herpes simplex virus which differ antigenically as well as in plaque morphology have a different buoyant density in CsCl (19). For this reason, density gradient centrifugation was repeated with the 18537 strain of RS virus, since this strain differs from the Long strain in antigenic properties and in plaquing behavior (8; Coates, Alling, and Chanock, *in press*). In this experiment, centrifugation was carried out at 39,000 rev/min for 72 hr. Two bands, similar to those which had been seen in the preceding experiments, were observed in the gradient after centrifugation. As can be seen in Fig. 2, 97% of the recovered infectious virus was present in two fractions (no. 9, density 1.23; and no. 10, density 1.22). These fractions contained $10^{4.3}$ and $10^{4.0}$ PFU/0.2 ml, respectively. Sixty per cent of the CF activity was recovered in fractions 6 through 8, which ranged in density from 1.25 to 1.28. Each of these fractions contained 80 CFU reactive with human RS antiserum. In a second experiment, the 18537 strain was mixed with the Long strain to give a final mixture with approximately equal quantities of each virus. The prepa-

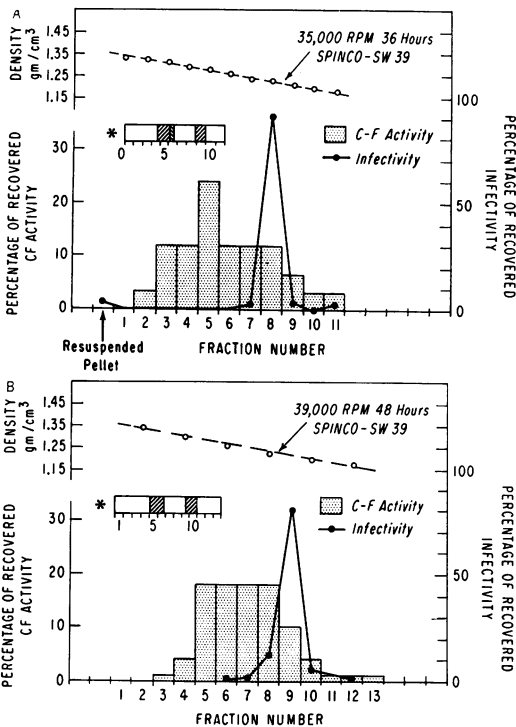


FIG. 1. Distribution of infectious virus and complement-fixing antigen of respiratory syncytial virus (Long strain) in a CsCl density gradient. (A) Column length equals 40 mm. (B) Column length equals 42 mm. Diagram of gradient tube (indicated by asterisks) showing approximate relationship of each fraction to visible bands.

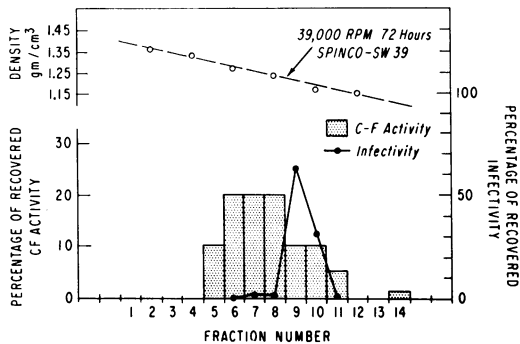


FIG. 2. Distribution of infectious virus and complement-fixing antigen of respiratory syncytial virus (CH 18537 strain) in a CsCl density gradient.

ration was centrifuged at 39,000 rev/min for 44 hr. In contrast to the previous experiments, the virus-containing fluids used in this experiment were not concentrated, but consisted of unconcentrated fluids from infected tissue cultures which had been frozen and stored at -70°C prior to use. After centrifugation, the gradient, which contained two visible bands, was collected in 22 fractions. Approximately 90% of the infectious virus input ($10^{6.8}$ PFU) was recovered from the gradient. Ninety-three per cent of the recovered virus was present as a single peak with a density of 1.25 to 1.27. Furthermore, large and small plaque variants coincided in distribution within this peak. Although a difference in density could not be detected between the two strains in either experiment, the density of the infectious virus mixture was higher than that observed when concentrated preparations of either strain were studied. The reason for this difference was not determined.

Density of the CF antigens of RS virus. The soluble CF antigenic material present in infected tissue culture fluid contains two immunologically distinct antigens which can be separated by chromatography on a G200 Sephadex column (10). It was of interest to determine the position of these antigens in a density gradient relative to (i) infectious virus and (ii) the CF activity detected in the preceding experiments with antibodies in human serum. Replicate tubes, containing a concentrate of the Long strain of RS virus, which had been grown in BEK cells, were centrifuged for 45 hr at 38,000 rev/min. After centrifugation, two visible bands were observed in the gradient similar to those seen previously. The contents of one of the tubes were collected in 24 fractions for CF assay, while the contents of a second tube were collected in 12 fractions for assay of infectivity. As can be seen in Fig. 3, 90%

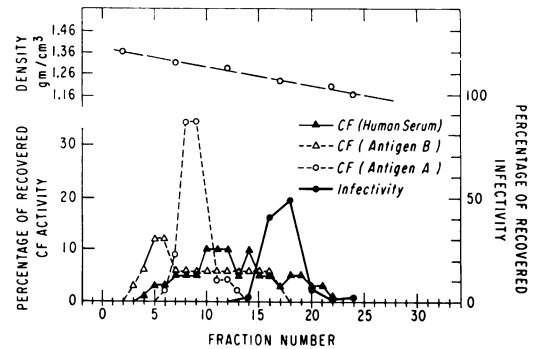


FIG. 3. Distribution of infectious virus and complement-fixing antigens of respiratory syncytial virus (Long strain) in a CsCl density gradient (38,000 rev/min, 45 hr, Spinco SW39).

of the recovered infectivity appeared in two fractions which had densities of 1.22 and 1.23. These fractions contained 10^3 and $10^{3.1}$ PFU per 0.2 ml, respectively. Forty-five per cent of the recovered CF activity reactive with antibody in human serum appeared in five fractions with a range in density of 1.26 to 1.30, whereas 65% of the recovered activity reactive with specific guinea pig antiserum for antigen A appeared in two fractions with a range in density of 1.30 to 1.32. Twenty-four per cent of recovered CF antigen reactive with specific guinea pig antiserum for antigen B appeared in two fractions having densities of 1.33 and 1.34, and 60% of the recovered CF antigen was detected in 10 additional fractions ranging in density from 1.23 to 1.32.

Similar results were obtained in a second experiment (Table 1). With human serum, 90% of the CF activity was detected in five fractions which ranged in density from 1.22 to 1.30. Eighty-nine per cent of the recovered antigen A was found in three fractions ranging in density from 1.28 to 1.32. Fifty-four per cent of the recovered antigen B was present in two fractions with a range in density of 1.32 to 1.37. Forty-two per cent of the recovered antigen B CF activity was detected in three additional fractions ranging in a density from 1.30 to 1.26.

In a preformed sucrose gradient, on the other hand, antigens A and B and antigen reactive with antibody in human serum appeared to have similar densities (Table 1).

DISCUSSION

When concentrated fluids from RS virus-infected tissue cultures were examined by equilibrium sedimentation in a CsCl density gradient, four zones of biological activity were detected.

TABLE 1. *Distribution of the complement-fixing antigens of respiratory syncytial virus (Long strain) in a density gradient (SW 39 rotor; 37,000 rev/min, 68 hr)*

Fraction no.	Sucrose gradient				Cesium chloride gradient			
	Density (g/cm ³)	Reciprocal CF titer with indicated serum			Density (g/cm ³)	Reciprocal CF titer with indicated serum		
		Guinea pig antigen A	Guinea pig antigen B	RS human convalescent		Guinea pig antigen A	Guinea pig antigen B	RS human Convalescent
1	1.284	<2	<2	2 or <	(1.39)*	10 or <	20	10 or <
2	1.266	64	4	4	1.373	10 or <	2,560	10 or <
3	1.250	512	128	16	(1.32)	1,280	2,560	20
4	1.240	512	512	32	1.298	5,120	1,280	80
5	1.218	1,024	512	32	(1.28)	2,560	1,280	80
6	1.191	1,024	512	64	1.255	640	1,280	40
7	1.174	1,024	1,024	64	(1.23)	160	320	80
8	1.132	64	512	32	1.216	160	80	80
9	1.102	32	8	<4	(1.20)	80	20	20
10	1.079	32	8	<4	1.168	40	10	<10
11	1.070	64	32	2 or <	(1.14)	80	10	<10

* Figures in parentheses estimated assuming a linear density gradient.

Infectivity was concentrated in a sharp peak with a density of 1.22 to 1.24, and CF activity, as detected by antibody in human serum, occurred as a broad zone ranging in density from 1.22 to 1.30. The distribution of this antigen was asymmetrical, suggesting that the antigen was heterogeneous with respect to density (16). However, the asymmetry might also be the result of complement-fixation by the virion, since the skew was toward the less-dense portion of the gradient, overlapping the virus band. A similar distribution of infectious virus and CF activity, with human serum, was seen with two antigenically distinct strains of RS virus (Long and 18537) and with one strain (Long) grown in two different tissue culture systems (HEp-2 and BEK).

Two zones of CF activity were detected when CsCl gradient fractions prepared from BEK-grown RS virus (Long strain) were tested with guinea pig antisera prepared against Sephadex-purified antigens A and B prepared from HEp-2-grown RS virus. Antigen A was concentrated in a sharp peak with a density of 1.28 to 1.32. In contrast, antigen B tended to concentrate at a density of 1.34 to 1.37, but trailed toward the lighter portion of the gradient with 42 to 60% of the recovered CF activity lying between a density of 1.23 and 1.32.

From the density distribution of these components, it appears that the virion and antigen A are biologically different and possibly are physically distinct, fairly homogeneous materials. These observations are of particular interest since animals immunized with antigen A developed neutralizing antibody whereas those immunized with antigen B did not (10). Since the banding

density of antigen A in CsCl is that of protein (1.28 to 1.30), it probably represents virion coat antigen relatively free from lipid and nucleic acid (9). Although the virion did not react with anti-A serum in this study (Fig. 3), it is probable that this was because the virion concentration was insufficient to fix complement since we have demonstrated that virion concentrated by centrifugation onto a sucrose cushion reacts primarily with anti-A serum (10).

It is of interest that differences in density between antigens A and B were not observed when centrifugation was carried out in a preformed sucrose gradient. It is possible that the differences in density observed in cesium chloride may not reflect an actual difference in density but rather a difference in the reaction of cesium ions with these antigens.

The need for an effective vaccine against RS virus infection suggests that the immunogenicity of antigen A for man should be explored. Because of its sharp banding and apparent homogeneity with respect to density, equilibrium sedimentation might be used to concentrate antigen A for more extensive studies of its antigenicity, including its immunogenicity for man. However, antigen A obtained by this means would not be entirely free from the other CF antigens.

Antigen B and CF antigen detected by antibody in human serum are apparently more heterogeneous with respect to density than is antigen A or the virion. While column chromatographic studies suggest that antigen B is physically distinct from the other RS virus antigens, such information is not available for the somewhat lighter component which reacts with antibody in

human serum. Although human serum reacts equally well with Sephadex-purified antigens A and B, the fact that the major distribution of activity measured with this serum was consistently found at a density lower than that of either antigen A or antigen B (Fig. 3 and Table 1) suggests that a third CF antigen, antigen "C," is present in fluids from RS virus infected cell cultures. The density distribution of the hypothetical antigen "C" suggests further that it may be a protein, possibly containing lipid, but with little or no nucleic acid. Although the origin of this antigen is not known, it may be similar in its origin to the "T" antigens which are currently under investigation in virus-induced tumors (13). Such "T" antigens are defined as virus-induced antigens which are not incorporated into the infectious virion. Antigen B, on the other hand, appears to be a component of the virion, since it can be released from the intact particle by Tween-ether treatment (10). However, it is unable to stimulate neutralizing antibody, and thus is probably not present on the surface of the virion. Possibly antigen B is analogous to the "soluble" antigen of influenza and parainfluenza viruses, which has been demonstrated to be nucleoprotein (1, 21).

When Tween-ether-disrupted measles virus was examined in a density gradient of CsCl, by Norrby (17), a hemagglutinating and CF antigen was observed which had a density of 1.30. Adsorption of this hemagglutinin with erythrocytes revealed a nonhemagglutinating CF antigen with a density of 1.32. In a similar study Waterson et al. (25) found the hemagglutinin to have a density of 1.34 and the C-F antigen a density of 1.35. It is possible that these antigens are similar to antigens A and B of RS virus.

The densities of three other myxoviruses have been determined by equilibrium sedimentation in CsCl, i.e., influenza A virus, 1.25 (3); measles virus, 1.25 to 1.28 (17, 24, 25); and Newcastle disease virus, 1.28 (22). These densities are somewhat higher than that found for RS virus (1.22 to 1.24), and may indicate that the latter contains more lipid than do the other myxoviruses. However, the molecular weight of the viral nucleic acid must also be taken into account. Although influenza A virus and Newcastle disease virus have approximately equal percentages of lipid (2, 11), the molecular weight of the ribonucleic acid of influenza A virus is estimated to be only 0.6 that of the ribonucleic acid of Newcastle disease virus (3×10^6) (20, 23).

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