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Characterization of A New Coronavirus-like Agent Isolated from Parrots

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SUMMARY

Characterization of a new virus from parrots showed it to be sensitive to lipolytic solvent treatment, and labile to heat and low pH. It passed through a 220-nm but not a 100-nm filter. The particles, in ultrathin sections of cells, resembled coronaviruses in morphology and ranged from 90 to 120 nm. No cytopathic effect was noted in cell cultures. The virus did not agglutinate chicken or human O erythrocytes. The virus was tentatively classified as a coronavirus. No antigenic relationship to infectious bronchitis virus (IBV), transmissible gastroenteritis virus (TGEV), or canine coronavirus (CCV) could be demonstrated. The virus was pathogenic for young chickens and budgerigars but not for Coturnix quail. The lesions were characterized by hemorrhagic necrosis in the liver and spleen. The severity of the lesions varied according to the route of inoculation and the age of the host.

INTRODUCTION

Members of the coronavirus group are etiologic agents of a variety of respiratory-tract, enteric, hepatic, and neurologic diseases of poultry, mice, swine, cattle, dogs, cats, and man (1,3,8,13, 15). No published information is available on coronavirus infections of parrots.

During the virological investigation of diseased psittacine birds, the authors isolated apparently similar unidentified viruses, designated PRT-1, -2, and -3, from three parrots. Virus growth characteristics and the lesions of infected chicken embryos were described (12) but the agents were not characterized. This report

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describes the physicochemical and morphological properties of isolates PRT-1 and PRT-2, which suggest that they represent a new coronavirus of parrots. Also reported are preliminary data from experimental infections in chickens, budgerigars and quail.

MATERIALS AND METHODS

Virus. The two isolates studied were from liver and spleen suspensions of two diseased parrots from different sources. They were respectively designated as the PRT-1 and -2 isolates. Both isolates produced similar lesions in the liver and spleen of infected chicken embryos (12). Materials for this study, prepared from the 3rd passage of the viruses in chicken embryos, consisted of 10% (w/v) suspensions of liver and spleen in phosphate-buffered saline (PBS). The suspensions were prepared by homogenizing typically affected livers and spleens harvested on the 5th day after inoculation. After homogenization, the materials were clarified by successive low- and high-speed centrifugation. The supernatants were collected as virus stocks and stored frozen at -80°C until use.

Chicken embryos and virus titrations. Ten-day-old chicken embryos were from the Departmental PDRC strain specific-pathogen-free (SPF) flock (4). Virus titrations were carried out by inoculating 0.2 ml of serial 10-fold dilutions of the isolates into the allantoic cavity. All embryos were incubated at 37°C and examined daily to determine embryo deaths. Endpoints were determined after the 9th day postinoculation on the basis of embryo death and/or the appearance of characteristic lesions in surviving embryos. Most mortality occurred between the 3rd and 9th days postinoculation. Embryos that died four or more days postinoculation had lesions consisting of mottled necrosis and hemorrhages of the liver, and enlargement and paleness of the spleen, with necrotic foci. The embryo-infective-dose-50% (EID₅₀) was calculated by the method of Reed and Muench (17).

Physicochemical characterization. Chloroform sensitivity tests were conducted by adding one part of chloroform to nine parts of virus suspension. The mixture was thoroughly shaken, chilled to 4°C , and held for 15 min, after which the chloroform was removed by centrifugation and the residual virus was titrated in embryos. Ether sensitivity was tested as described previously (9). Acid sensitivity was assayed by holding virus suspensions in medium adjusted to pH 3.0 at 25°C for 30 min, while control virus suspensions were similarly held at pH 7.4. The virus suspensions were then titrated in embryos. The isolates were passed through mem-

brane filters (Millipore Filter Corp., Bedford, Massachusetts) with average pore diameters of 220 and 100 nm, and the filtrates were titrated in embryos.

Electron microscopy (EM). Livers and spleens with lesions were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, embedded in Epon 812 resin, sectioned on a Porter ultramicrotome, and stained with lead citrate and uranyl acetate before examination (11). Uninoculated control tissues were tested in the same way. For partial purification of the isolate the centrifuged concentrate of the virus-containing chorioallantoic fluid was banded 2 times in a discontinuous 20 and 50% (w/w) sucrose gradient by ultracentrifugation (Spinco SF 25 I rotor) at 30,000 rpm for 3 hr at 4 C. The virus, banded at the interface, was collected, and dialyzed in the cold against 0.01M phosphate buffer to remove sucrose as described previously (10). All EM examinations were made on a Hitachi 11A electron microscope.

Antisera. Antiserum against the PRT-1 isolate was prepared by injecting chickens intravenously with $10^{6.0}$ EID₅₀ of the isolate at weekly intervals. Seven days after the fourth injection, the chickens were bled. The serum was heat-inactivated at 56 C for 30 min and then stored at -20 C. Antisera against various IBV strains were obtained from B. S. Cowen in this laboratory. The antisera against TGEV and CCV were obtained from L. E. Carmichael of Cornell University.

Neutralization tests. The sera were diluted in PBS in twofold steps. Aliquots of virus, diluted to contain about 100 EID₅₀ per 0.2 ml, were mixed with equal volumes of each serum dilution and incubated at 37 C for 1 hr. The virus-serum mixture was then inoculated in 0.2-ml amounts into each of 5 embryos. Neutralizing-antibody titers were expressed as the reciprocal of the highest serum dilution that neutralized the virus.

Experimental birds and inoculation. One-day-old and 2-week-old White Leghorn chicks were from the Departmental PDRC-strain SPF flock. One-week-old quail (*Coturnix coturnix japonica*) were kindly supplied by the Department of Poultry Science at Cornell University. Mature budgerigars were from a Departmental colony. Except for the budgerigars, all the birds were bled for serum before virus inoculation and were shown to be free of neutralizing antibodies to the PRT-1 isolate. The dose of virus inoculated ranged from 10^5 to 10^6 EID₅₀ per bird. Inoculation was made by intramuscular (IM) or intraperitoneal (IP) injection or by ocular instillation (OI). An uninoculated group of room-contact (RC)

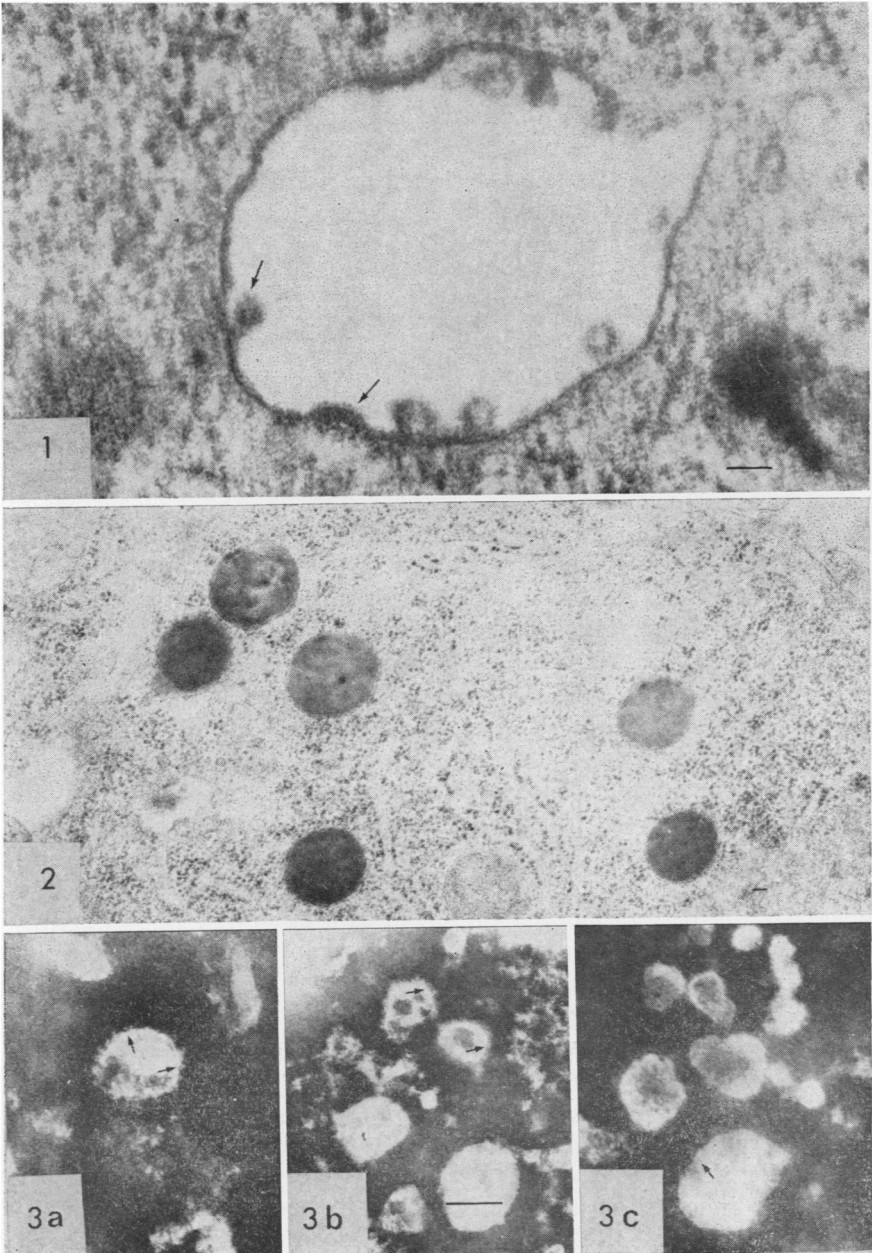


Fig. 1. Various stages of budding into a large cytoplasmic vesicle (arrows). $\times 80,000$. The bar represents 100 nm.

Fig. 2. Electron-dense areas surrounded by a double membrane are frequently seen in the cytoplasm. $\times 22,500$. The bar represents 100 nm.

Fig. 3. Negatively stained viral particles of the PRT-1 isolate. Club-shaped projections are evident (arrows). $\times 100,000$. The bar represents 100 nm.

Table 1. Physicochemical properties of the PRT-1 and PRT-2 isolates.

Treatment	Infectivity (\log_{10} EID ₅₀ /0.2 ml)	
	PRT-1	PRT-2
None	7.2	6.4
Chloroform (10%, 4 C, 15 min)	0	0
Ether (20%, 4 C, 18 hr)	0	0
Acid (pH 3.0, 30 min)	3.2	2.8
56 C, 5 min	3.4	3.2
10 min	0	0
Filtration 220 nm	1.4	2.0
100 nm	0	0

birds was held in the same room with the inoculated group. All experimental birds were held in isolation units for 4 weeks. Dead birds and survivors at 4 weeks were subjected to laboratory investigations.

Cell cultures and hemagglutination tests. Primary monolayer cultures of chicken embryo fibroblasts (CEF) and chicken kidney (CK) were used. Methods of preparation, cell-culture media, and incubation have been described (5). The isolates were inoculated on CEF and CK cell cultures maintained free of serum. After 5 days of incubation at 37 C, the cultures were frozen and thawed, and supernatant fluids harvested. This procedure was repeated through ten cell-culture passages. Hemagglutination tests using chicken and human O erythrocytes were attempted with the original virus suspensions and after passage of those materials in CEF and CK cell cultures.

RESULTS

Chemical and physical properties. As shown in Table 1, chloroform and ether treatment of the PRT-1 and -2 isolates resulted in complete inactivation of infectivity of the viruses. The virus was considered to be sensitive to acid treatment since the titers obtained with the acid-treated virus were respectively $10^{4.0}$ and $10^{3.6}$ logs lower than those obtained from untreated virus. The infectivity of both viruses was reduced by incubation at 56 C for 5 min; after exposure for 10 min, no viable virus was detectable. The viruses passed through 220-nm but not through 100-nm filters.

Electron microscopy. Characteristic viral particles were seen in the liver and spleen of the infected embryos. These particles were observed in the cytoplasm but not in the nucleus of the infected

Table 2. Neutralization tests with various coronavirus antisera against PRT-1.

Antiserum	Antibody titer ^A against:		
	Homologous virus	PRT-1	PRT-2
PRT-1		20	10
IBV Mass 41	80	-- ^B	ND ^C
Conn 46	40	--	ND
Iowa 609	20	--	ND
Holte	400	--	ND
Gray	100	--	ND
TGEV	32	--	ND
CCV	64	--	ND

^ATiters expressed as the reciprocal of the highest serum dilution that neutralized 100 EID₅₀ of virus.

^B-- = negative at 1:2 serum dilution.

^CND = not done.

cells. Infected cells often contained numerous particles that were spherical, had an average diameter of 90 to 120 nm, and contained a doughnut-shaped nucleoid with a central lucent core. The outer envelope consisted of a dilaminarlike unit membrane with numerous spikelike projections. The particles were often found in dilated cisternal structures in the cytoplasm (Fig. 1). The cisternae were constructed of what appeared to be smooth endoplasmic reticulum. The process of development of individual particles appeared to be that of budding from intracytoplasmic membranes. The earliest event appeared to be the development of a crescent. An altered segment of cisternal or vesicular membrane bulged out and pulled away from cytoplasm, extending into the vesicle. Electron-dense areas with well-defined but incomplete margins were frequent in the cytoplasm (Fig. 2).

In negatively stained preparations, particles were approximately circular in outline and had club-shaped projections. They ranged from 90 to 180 nm in diameter (Fig. 3a-c).

Neutralization. Considering the characteristics described above, the isolates were tentatively classified as coronaviruses. To test the immunological relationship of the isolates with each other and with other coronaviruses, virus-neutralization tests were conducted (Table 2). Since the infectivity of the PRT-2 isolate was neutralized by anti-PRT-1 serum, both isolates were considered to be

the same virus. The PRT-1 isolate, however, was not neutralized by antisera prepared against the other coronaviruses tested.

Experimental infection. Table 3 summarizes data on pathogenicity tests with the PRT-1 isolate. Clinical signs in inoculated one-day-old chickens and mature budgerigars usually appeared within 7 days. They were greenish diarrhea, ruffled feathers, anorexia, depression, and prostration. During the period of diarrhea, the virus was recovered from the feces of the chickens and budgerigars. Seven one-day-old chickens, which were inoculated intramuscularly with the isolate, died between the 10th and 13th days postinoculation, after a brief period of anorexia and depression. Postmortem examination revealed characteristic lesions including hepatic and splenic necrosis. In severe cases, the liver had a large number of greenish foci, surrounded by hemorrhagic rings, on the external surfaces (Fig. 4). In moderate and mild cases, the lesions varied from case to case. Generally, greenish foci and some hemorrhages were found in the livers. No lesions were found in any organs other than liver and spleen. All chickens which survived the 4-week experimental period were found to have characteristic lesions as described above. The virus was reisolated from the liver and spleen of all chickens; virus titers ranged from $10^{2.8}$



Fig. 4. Livers showing characteristic lesions produced by the PRT-1 isolate. Affected livers had large numbers of greenish foci, surrounded by hemorrhagic rings.

Table 3. Experimental infection of the PR1-1 isolate.

Experimental birds	No. of birds	Route of inoculation ^A	Clinical signs	Reisolation of the virus from feces	Occurrence of liver and spleen lesions ^B		Incidence ^C	Neutralizing-antibody titer ^D
					++	+		
Day-old chicks	20	IM	+	+ ^E	14 ^F	6	20/20	10
	10	IP	+	ND ^G	4	6	10/10	10
	10	OI	+	ND	3	7	10/10	8
	10	RC	+	ND	2	8	10/10	4
Mature budgerigars	8	IM	+	+	8	8	8/8	8
	4	IP	+	ND	4	4	4/4	4
	4	OI	+	ND	3	1	4/4	4
	4	RC	+	ND	4	4	4/4	2
2-week-old chicks	5	IM	-	+	1	4	1/5	10
1-week-old Coturnix quail	10	IM	-	-	10	10	0/10	< 2

^ABirds were inoculated with 10^5 to 10^6 EID₅₀ of the PR1-1 isolate. Inoculation was made by intramuscular (IM), intraperitoneal (IP), and ocular instillation (OI). For room contact (RC), uninoculated birds were held in the same room as the inoculated group.

^BBirds were killed for examination at 4 weeks postinoculation. Degree of lesions: ++, marked gross lesions; +, mild gross lesions; -, no lesions.

^CNo. of birds with lesions/no. of birds inoculated.

^DGeometric mean of all birds tested.

^EReisolation of the virus was carried out 4th, 6th, and 8th days postinoculation.

^FSeven birds died between 10th and 13th days postinoculation.

^GNot done.

to $10^{4.2}$ EID₅₀ per 0.2 ml. Neutralizing antibodies against the virus were found in sera from the chickens.

All of the budgerigars survived for 4 weeks postinoculation. On postmortem examination they were found to have gross lesions in the spleen consisting of marked enlargement (4 to 5 times) with surface striation and hemorrhages. Livers had mild lesions consisting of discoloration and some enlargement. Virus was recovered from the spleen and liver of all birds. The budgerigars were found to possess virus-neutralizing antibodies; titers ranged from 2 to 8.

While only one of five 2-week-old chickens which were inoculated intramuscularly with the virus had lesions (mild liver necrosis), the virus could be reisolated from livers in all chickens. Neutralizing antibody titers of the chickens ranged from 4 to 16.

No signs of disease were observed in the exposed quail, and both pre- and postinoculation sera taken from the quail were shown to be free of the virus-neutralizing antibodies.

Horizontal spread of infection, evidenced by clinical signs, gross lesions, and positive serology in room contacts, was observed with both chickens and budgerigars (Table 3).

Cell-culture and hemagglutination tests. No cytopathic effect was seen in CK and CEF cell cultures inoculated with either isolate. Hemagglutination activity also was not detected when the liver and spleen homogenates or cell-culture fluids were tested with chicken and human O erythrocytes.

DISCUSSION

This is the first report of a coronaviruslike agent from parrots. The evidence supporting such a classification is not wholly complete but is nevertheless substantial. First, the physical, chemical, and morphological properties reported in this communication are similar to published descriptions of coronaviruses (1,8,9,13,14, 15). Also, the appearance of virus particles in cytoplasmic vesicles and in the cisternae of the endoplasmic reticulum, and the absence of budding from the cytoplasmic membrane, are in agreement with published work on the morphogenesis of coronaviruses (2,3,11,13, 14,15,18,19,21). The presence of particles in the cytoplasmic vesicles and the bulging of the membrane over underlying densely staining material are consistent with intracellular bud formation as the principal means of virus maturation. The significance of the electron-dense areas in the cytoplasm is not known, but their fre-

quent appearance denoted cell pathology associated with the virus infection. Examination of allantoic fluids and liver homogenates of the infected embryos revealed a very small number of particles with morphology resembling that of coronaviruses, although the surface projections of the particles were not sufficiently well preserved to establish their identity.

From this evidence, it seems reasonable to assume that the PRT isolate virus represents a new coronavirus infecting parrots in nature. Final classification of the virus, however, must wait until its physicochemical and morphological properties become more detailed.

It is known that coronaviruses are pathogenic viruses in all of their natural hosts, which range from fowl to man, and that they produce lesions in many organs in susceptible animals. The type of disease is generally influenced markedly by route of inoculation, the age and genetic background of the animal, and the virus isolate. Preliminary pathogenicity studies presented here revealed that the PRT-1 isolate is pathogenic for both chickens and budgerigars. The lesions were characterized by the hepatosplenitis. One-day-old chickens were especially susceptible and were the only group with mortality. Only one of the 2-week-old chickens developed mild lesions, although all chickens developed neutralizing antibodies. Thus, the susceptibility to disease seems to be lowered with age, and it may be that only inapparent infections develop in older chickens. Lesions of moderate or severe degree were recognized in most of the budgerigars killed at 4 weeks postinoculation, but there was no mortality. Of particular interest was the fact that infection was easily established by a natural route of exposure (ocular instillation), and contact spread occurred with both chickens and budgerigars. More detailed studies of the pathogenesis of the disease, including histopathologic findings, would be an interesting subject for the future. The potential risk to commercial chickens should certainly be considered. At this time, it is not known if infections with this virus occur naturally in chickens, but the possibility of adventitious introduction of a new virus is always worrisome and additional information is desirable.

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