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ULTRASTRUCTURAL, PROTEIN COMPOSITION, AND ANTIGENIC COMPARISON OF PSITTACINE BEAK AND FEATHER DISEASE VIRUS PURIFIED FROM FOUR GENERA OF PSITTACINE BIRDS

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ABSTRACT: Psittacine beak and feather disease (PBFD) virus, was purified from diseased tissues of a lesser sulphur-crested cockatoo (*Cacatua sulphurea*), a black palm cockatoo (*Probosiger aterrimus*), a red-lored Amazon parrot (*Amazona autumnalis*), and a peach-faced lovebird (*Agapornis roseicollis*). The histopathology of diseased feathers and follicular epithelium from the different species was compared; basophilic intranuclear inclusion bodies were identified in the follicular epithelium and intracytoplasmic globular inclusions were observed within macrophages located in the feather pulp from the four species. Psittacine beak and feather disease virus antigen was specifically detected by colloidal gold immunoelectron microscopy. The different preparations of purified virions displayed an icosahedral symmetry, were non-enveloped, and had a mean diameter that varied from 12 to 15 nm when negatively stained. Two major viral-associated proteins with approximate molecular weights of 26 and 23 kilodaltons (kd) were consistently demonstrated from the four viral preparations. Purified virions from the four genera were antigenically related. These findings suggest that the PBFD virus purified from numerous genera of diseased birds is similar based on ultrastructural characteristics, protein composition and antigenic reactivity.

Key words: Psittacine beak and feather disease virus, Diminuviridae, ultrastructure, proteins, antigenic similarities, virus characterization, experimental study.

INTRODUCTION

In the last several decades, psittacine beak and feather disease (PBFD) has been diagnosed increasingly in a variety of psittacine birds based on clinical and histologic lesions. The syndrome generally is characterized by relatively symmetric feather dystrophy and loss, development of beak deformities and eventual death. A 14 to 16 nm icosahedral, non-enveloped virion with a single stranded circular DNA genome of approximately 1.7 to 2.0 kilobases can be consistently recovered from birds with PBFD (Ritchie et al., 1989a). Gross and histologic lesions consistent with PBFD have been reproduced experimentally by exposing neonatal psittacine birds to feather follicle homogenates containing suspected PBFD viral particles (Wylie and Pass, 1987), as well as by inoculation with purified concentrated PBFD virions (Ritchie et al., 1989a, b). We have suggested the name Diminuviridae for the family to which the PBFD virus and a similar nonpathogenic virus, porcine circovirus, may belong (Tischer et al., 1982; Ritchie et al., 1989b).

While the host range of the PBFD virus remains largely unknown, histologic or clinically suggestive lesions of the disease have been described in the following species: sulphur-crested cockatoo (*Cacatua galerita*), Major Mitchell's cockatoo (*C. leadbeateri*), galah (*C. roseicapilla*), little corella (*C. sanguinea*), long-billed corella (*C. tenuirostris*), budgerigar (*Melopsittacus undulatus*), cockatiel (*Nymphicus hollandicus*), rainbow lorikeet (*Trichoglossus haematodus*), eastern rosella (*Platycercus eximius*), western rosella (*P. icterotis*), hooded parrot (*P. dissimilis*), mallee ring-neck parrot (*Barnardius bar-*

nardi), red-rump grass parrot (*Psephotus haematonotus*), Port Lincoln parrot (*B. zonarius*), Bourke's parrot (*Neophema bourkii*), Eclectus parrot (*Eclectus roratus*), princess parrot (*Polytelis alexandrae*), peach-faced lovebird (*Agapornis roseicollis*), nyassa lovebird (*A. lilianae*), Fisher's lovebird (*A. fischeri*), masked lovebird (*A. personata*) and king parrot (*Alisterus scapularis*) (Pass and Perry, 1984a, b; Perry and Pass, 1985; Harrison, 1986). Other reports have shown that numerous additional species may develop PBFD including: Moluccan cockatoo (*C. moluccensis*), umbrella cockatoo (*C. alba*), lesser sulphur-crested cockatoo (*C. sulphurea*), Philippine red-vented cockatoo (*C. haematuropygia*), triton cockatoo (*C. galerita triton*), citron cockatoo (*C. cinrinocristata*), goffin cockatoo (*C. goffini*), (Harrison, 1984; Lowenstein, 1984; Jacobson et al., 1986); and vasa parrot (*Coracopsis vasa*) (Cooper et al., 1987). The disease has been documented in several wild populations of psittacine birds in Australia including sulphur crested cockatoos, galahs, little corellas, Major Mitchell's cockatoo and rainbow lorikeets (McOrist et al., 1984; Perry and Pass, 1985). There is undocumented discussion of endemic PBFD in populations of Moluccan cockatoos, Philippine red-vented cockatoos, umbrella cockatoos and budgerigars.

While many species of white and pink cockatoos are included in the reported list of susceptible birds, there has been no previous documentation of PBFD in any of the black cockatoo genera. Prior to a recent report which described histologic lesions consistent with PBFD in a blue-fronted Amazon parrot (*Amazona aestiva*), a new world Psittaciforme, the disease was thought to be restricted to Old World and South Pacific psittacine species (Pass and Perry, 1984b; Huff et al., 1988). Psittacine beak and feather disease has only been reported in birds within the order Psittaciformes.

Diagnosis of PBFD previously has required the identification of basophilic intracytoplasmic or intranuclear inclusion bodies in feathers or follicular epithelium from birds with clinical signs of necrotic, dystrophic, nonviable feathers that stop growing shortly after emerging from the follicle (McOrist, 1984; Pass and Perry, 1984a; Jacobson et al., 1986; Huff et al., 1988).

In this study, purified concentrated virus was recovered from a sulphur-crested cockatoo, a black palm cockatoo (*Probosiger aterrimus*), a red-lored Amazon parrot (*Amazona autumnalis*), and a peach-faced lovebird. Viral preparations were compared based on ultrastructural characteristics, protein composition, and immunological homology with antibodies generated against PBFD virus in rabbits. The pathology associated with diseased feather follicles from these birds was compared both at the light and at the electron microscopic level for reactivity of intracytoplasmic inclusion bodies with viral specific antibody. This study was designed to firmly establish the association of clinical and histologic lesions with virion recovery from various psittacine species, to refine the characteristics of the PBFD virus based on the commonality of ultrastructural features and viral protein composition, to establish the presence of common antigenic determinants among viruses purified from different diseased birds and to document the expanding host range of the PBFD virus.

MATERIALS AND METHODS

Experimental samples were collected from birds presented to the teaching hospital at the University of Georgia College of Veterinary Medicine (Athens, Georgia 30602, USA). Skin biopsies containing affected feathers were obtained from a sulphur-crested cockatoo, a black palm cockatoo, a red-lored Amazon parrot, and a peach-faced lovebird and preserved in 10% neutral buffered formalin solution. Formalin-fixed tissues were processed routinely, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. The induced pathologic changes were compared microscopically.

Skin biopsies for immunoelectron microscopy were fixed, processed routinely, and embedded

in L. R. White resin (Polysciences, Inc., Warrington, Pennsylvania, 18976, USA). Thin sections were placed on high-transmission nickel grids and a 1:10 dilution of normal goat serum (Vectastain ABC Kit, Vector Laboratories, Burlingame, California 94010, USA) was applied to inhibit nonspecific background staining. Sections were incubated overnight in a 1:10 dilution of purified rabbit IgG prepared against the 23 and 25 kilodalton proteins of the PBFD virus. Primary antibody binding subsequently was labelled with a 1:20 dilution of goat-anti-rabbit IgG conjugated with 10 nm colloidal gold particles (Janssen Life Sciences Products West Chester, Pennsylvania, 19380, USA). The sections were counterstained with uranyl acetate and lead citrate and examined by transmission electron microscopy (Jeol JEM-100S Peabody, Massachusetts 01960, USA) at 80 kV.

Feather follicle tracts from a sulphur-crested cockatoo, a black palm cockatoo, a red-lored Amazon parrot, and a peach-faced lovebird diagnosed clinically with PBFD were excised, minced with scissors, and stored at −70 C. Virus purification was performed as described previously (Ritchie et al., 1989a). Virus suspension (10 μl) from each bird was mixed with 1 μl of tobacco mosaic virus (1 mg/ml Cedric Kuhn, Department of Plant Pathology, University of Georgia, Athens, Georgia 30602, USA). The virus suspension was adsorbed onto 0.25% formvar-coated grids for 5 min, negatively stained with 1% phosphotungstic acid, and examined at 80 kV (Jeol JEM-100S).

Samples of purified virus were suspended in sample buffer composed of 62.5 mM Tris-HCL, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.5% 2-mercaptoethanol, 0.008% bromphenol blue and 0.001% phenol red. Samples were then heated (35 C) for 5 min, and loaded onto 14% SDS-polyacrylamide gels (40:1 acrylamide-acrylaide cross-linker (FMC Bioproducts Rockland, Maine 04841, USA)), in a Laemmli buffer system (Laemmli, 1970). Electrophoresis was conducted at 20 ma for 3 hr. Proteins were visualized by silver staining (Bio-Rad Laboratories Richmond, California 94804, USA). Molecular weights were determined from densitometer tracings of photographic negatives with a Beckman DU-64 spectrophotometer (Beckman Instruments, Inc., Fullerton, California 92631, USA). The molecular weights of the viral-associated polypeptides were compared.

Virus recovered from the skin of a Moluccan cockatoo was purified by isopycnic and rate zonal centrifugation as previously described (Ritchie et al., 1989a), and used to generate anti-PBFD virus-specific antibodies. An adult New Zealand white rabbit (Myrtels Rabbitry, Thompson Station, Tennessee 37179, USA) was inoculated intramuscularly with 200 μl (402 ng total protein) of purified virus mixed with 200 μl of Freund's complete adjuvant (Sigma Chemical Company, St. Louis, Missouri 63178, USA). Booster inoculations of purified virus and adjuvant were given 2 and 4 wk later. Blood was collected 1 wk after the last booster injection and rabbit IgG was purified from serum by ammonium sulfate precipitation (Hudson and Hay, 1980). The purified IgG was stored at −70 C.

The 26 and 23 kd viral associated proteins were separated using a 14% SDS-polyacrylamide gel as described above. The area of the gel containing these proteins was sliced out of the gel, diced, mixed with 1.5 ml of sterile water for injection and administered intramuscularly to a New Zealand White Rabbit (Myrtels Rabbitry). Booster inoculations were performed at 2 and 4 wk. IgG was purified as described above.

Immunologic detection of viral antigen was performed by spotting purified virus suspensions onto nitrocellulose filters (0.45 μm pore size, Bio-Rad Laboratories). Filters were blocked overnight at 4 C using 2% bovine serum albumin (BSA, Sigma Chemical Company) and then blocked for 30 min at room temperature with biotin (20ug/ml, Sigma Chemical Company) and normal goat serum (1% Vector Laboratories, Burlingame, California 94010, USA). Filters were washed repeatedly with tween-20 tris buffered saline (TTBS) and incubated in a 1:400 dilution of rabbit anti-PBFD antibody (in 0.1% Tween 20, 1% BSA, TTBS) for 30 min. Filters were washed repeatedly with TTBS and incubated in biotinylated goat anti-rabbit IgG (Zymed Laboratories, Inc., McGaw Park, Illinois 60085, USA). Filters were washed repeatedly with tris buffered saline (TBS) and incubated in streptavidinalkaline phosphatase (Zymed Laboratories, Inc.) for 20 min. Filters were washed with TBS then veronal buffer (0.15 M, pH 9). Filters were developed with 5 bromo-4 chloroindoxyl phosphate and nitro blue tetrazolium substrates according to established procedures (Blake et al., 1984). Normal rabbit serum (Vector Laboratories) was used in place of rabbit anti-PBFD virus antibody as a negative control.

RESULTS

Feather lesions from a sulphur-crested cockatoo, a black palm cockatoo, a red-lored Amazon parrot and a peach-faced lovebird diagnosed clinically with PBFD were identical (Fig. 1). Feathers occasionally appeared clubbed, constricted, misshapen or otherwise deformed. Feather follicles and or sheath epithelium were

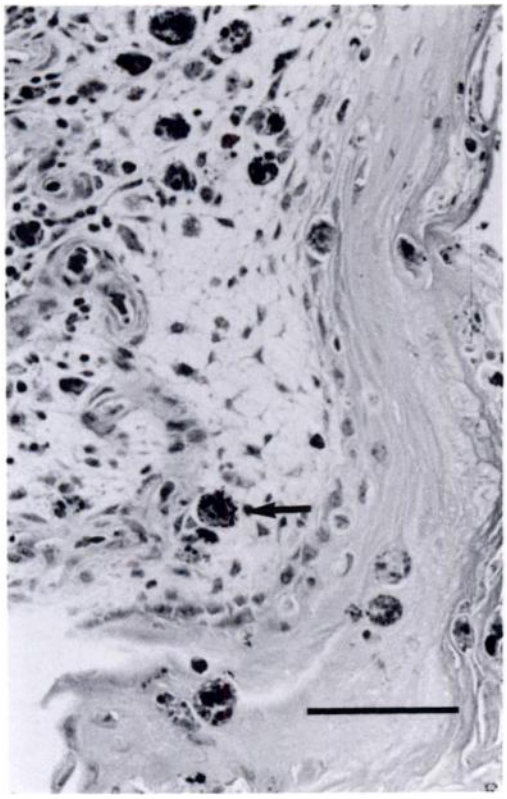

FIGURE 1. Photomicrograph of skin biopsy from a red-lored Amazon parrot with psittacine beak and feather disease. Globular intracytoplasmic inclusion bodies are present within macrophages in the feather pulp (arrow). Bar = 10 μm.

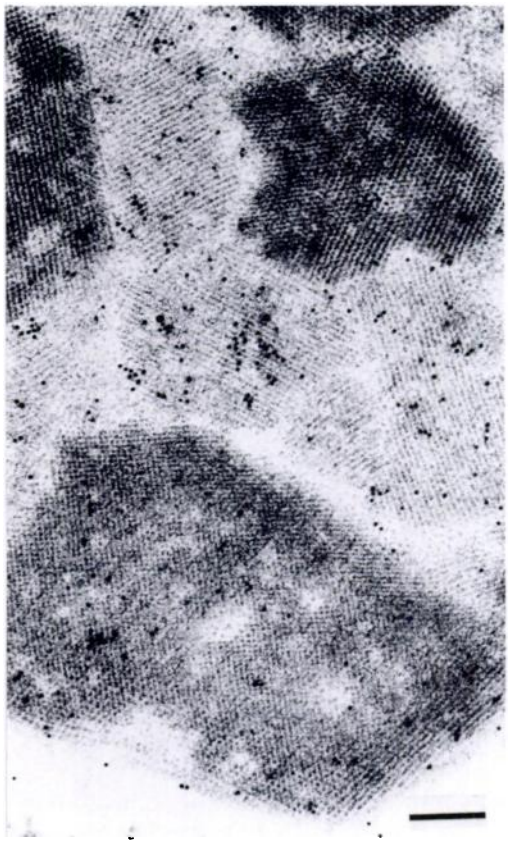

FIGURE 2. Electron micrograph of macrophage inclusion from a peach-faced lovebird with PBFD. Viral particles are arranged in paracrystalline arrays. Colloidal gold labelling (dark dots) indicate specific antigenic staining of 23 and 26 kilodalton PBFD viral proteins. Bar = 100 nm.

characterized by necrosis of individual epithelial cells, intra-and intercellular edema and occasional intranuclear basophilic inclusion bodies within epithelial cells. In rare instances, vesicle formation was noted within the follicular epithelium. Also present were macrophages with multiple, basophilic, intracytoplasmic, globular inclusion bodies (Fig. 1). These cells were observed often in the feather pulp and less frequently within the epithelium. The feather pulp occasionally was necrotic with infiltration of mononuclear and heterophilic cells of variable severity.

Ultrastructurally, inclusion bodies in epithelial cells and macrophages appeared as viral particles arranged in paracrystalline arrays. Colloidal gold binding indicated the presence of specific PBFD viral antigen within the inclusions (Fig. 2).

Ultrastructural analysis of negatively stained viral preparations from each bird revealed a highly concentrated, homogenous population of 12 to 15 nm diameter, icosahedral, nonenveloped virus particles (Fig. 3). Virtually all of the virions observed in the preparations excluded the negative stain. Consequently, few viral particles were observed with electron dense cores.

SDS-polyacrylamide gel electrophoresis revealed that the protein compositions of all four viral preparations were virtually the same (Fig. 4). The molecular weights

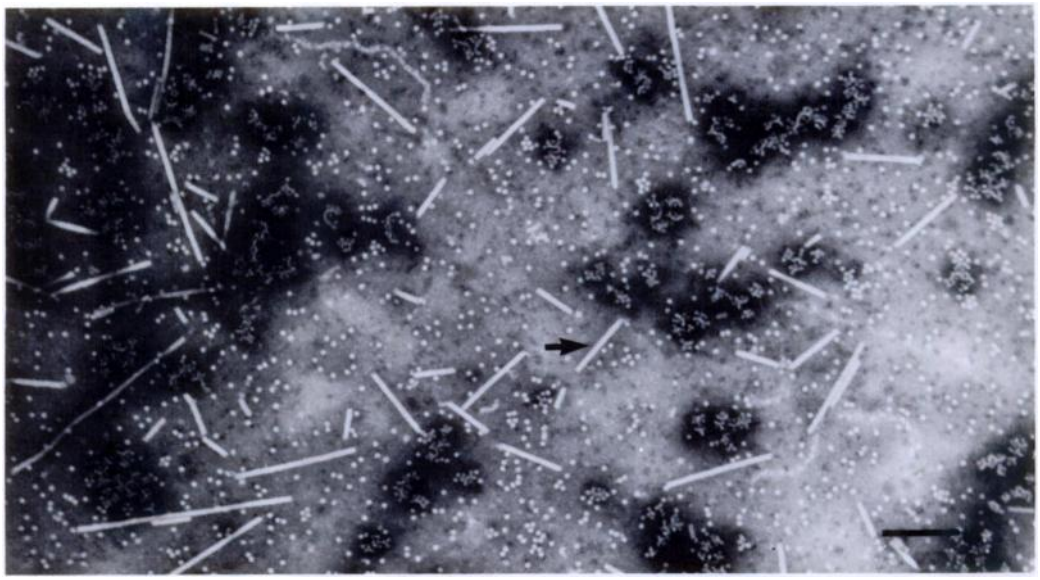

FIGURE 3. Electron micrograph of psittacine beak and feather disease virus particles isolated from a black palm cockatoo. Virus suspensions were negatively stained with 1% phosphotungstic acid and photographed at 80 kV. Tobacco mosaic virus (arrow: 15 nm mean diameter) is the internal standard. Bar = 275 nm.

and relative concentrations of the viral-associated proteins (VP) were determined for each isolate by analyzing densitometer tracings of gel electrophorograms (Fig. 5). Comparison of the apparent molecular weights of the major viral proteins (VP1, 26,000; VP2, 23,000; and VP3, 15,000 daltons) indicated only minor variation among the various viral preparations (Table 1). Minor proteins with molecular weights of 48,000 and 58,000 also were present in most of the purified virus preparations.

As an additional measure of the relatedness of these viral preparations, specific anti-PBFD virus antibody was made and used to detect viral antigens. The sensitivity of the system was demonstrated by probing decreasing amounts of purified PBFD virus obtained from an umbrella cockatoo (Fig 6). It was possible to detect <1 ng of viral protein while no signal was obtained in the absence of viral antigen. In addition, as much as 25 μg of soluble protein from nondiseased feather shaft extracts failed to yield detectable signal when probed with the rabbit anti-PBFD virus IgG (data not shown). Purified virus from all isolates examined in this study reacted positively when probed with the rabbit anti-PBFD virus antibody (Fig. 7). No signal was detected when normal rabbit serum was substituted for the anti-PBFD virus antibody.

DISCUSSION

The pathology associated with diseased feather follicles from these birds was compared both at the light microscopic level and for reactivity of intracytoplasmic inclusion bodies with viral specific antibody. The histologic lesions in these four species of birds were identical to those classically described in cases of PBFD (Pass and Perry, 1984a; Jacobson et al., 1986). Typical findings included deformed feathers, intranuclear and intracytoplasmic inclusion bodies, and inflammation of varying severity. Ultrastructural examination of the inclusion bodies, as observed in electron microscopic sections, indicated that they were composed of PBFD virus. Colloidal gold staining verified that antigenic determinates from the 23 or 26 kd viral proteins were present in the inclusion bodies identified in each bird.

Comparison of the ultrastructural char-

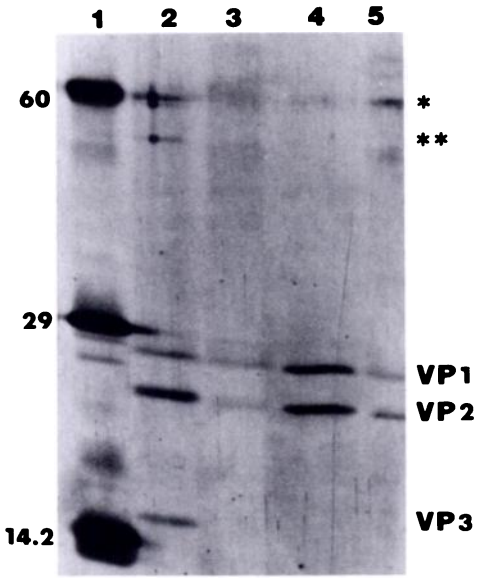

FIGURE 4. Silver stained SDS-polyacrylamide gel electrophoresis of purified PBFD viral associated proteins isolated from Lane 2 (sulphur-crested cockatoo), Lane 3 (red-lored Amazon parrot), Lane 4 (black palm cockatoo) and Lane 5 (peach-faced lovebird). Lane 1 bovine serum albumin (60,000 daltons), carbonic anhydrase (29,000 daltons) and @-lactalbumin (14,200 daltons) were used as molecular weight standards.

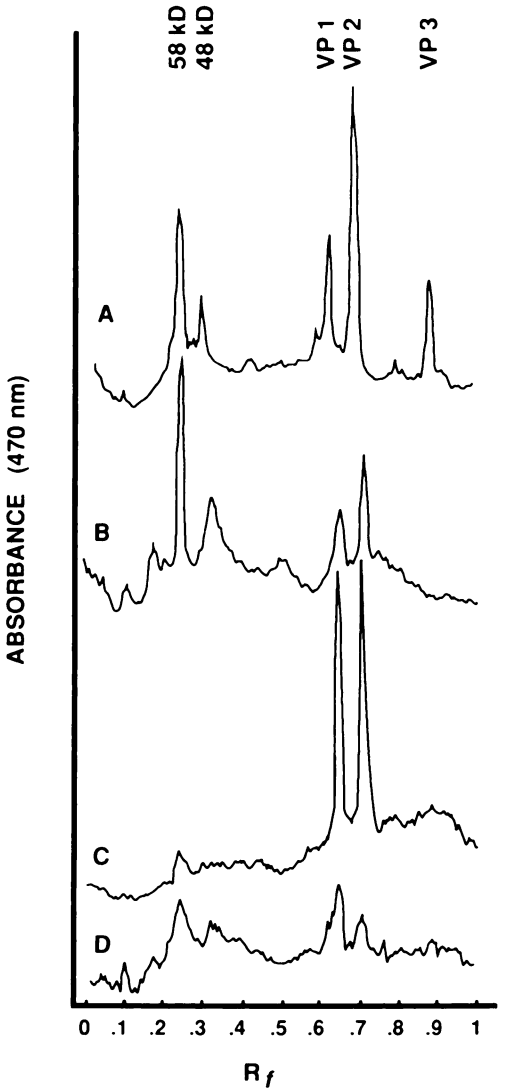

FIGURE 5. Identification of the major viral proteins. Photographic negatives of electrophoretically separated viral proteins were scanned densitometrically at 470 nm for determination of their relative concentrations and apparent molecular weights. Tracing A is of viral proteins from a sulphur-crested cockatoo. Tracing B is of viral proteins from a peach-faced lovebird. Tracing C is of viral proteins from a black palm cockatoo. Tracing D is of viral proteins from a red-lored Amazon parrot.

acteristics of virus purified and concentrated from diseased tissues of a sulphur-crested cockatoo, a black palm cockatoo, a red-lored Amazon parrot and a peach-faced lovebird revealed that the virus morphology was similar for each of the purified viral preparations. The viruses purified from birds reported in this study were also ultrastructurally similar to PBFD virus recovered from other cockatoo species (Ritchie et al., 1989a). While there is a slight difference in size between the virions in this study (12 to 15 nm) and the size previously reported for PBFD virus (14 to 16 nm), these differences are considered insignificant.

By comparing the protein composition (as determined by SDS-PAGE and dot immunoblot detection of viral antigens) of virus purified from a sulphur-crested cockatoo, a black palm cockatoo, a red-lored Amazon parrot and a peach-faced lovebird, it was determined that the major viral associated proteins from each of these species were similar. Minor protein bands in the 48,000 and 58,000 molecular weight range from some viral isolates are similar to those obtained from other sulphur-crested cockatoos, umbrella cockatoos and Mo-

TABLE 1. Molecular weights (in daltons) of PBFD viral proteins from various psittacine birds.

Protein	Sulfur-crested cockatoo	Black palm cockatoo	Red-lored Amazon parrot	Peach-faced lovebird
VP1	26,300	25,600	25,700	25,000
VP2	23,200	22,600	22,600	22,300
VP3	15,400	15,700	15,700	—
AP1*	58,700	58,600	58,600	58,000
AP2*	51,700	—	48,800	49,100

* AP1, AP2 represent minor viral-associated proteins at higher molecular weights.

luccan cockatoos (Ritchie et al., 1989a). When the molecular weights of the smaller proteins (VP1, VP2 and VP3) are summed, they approximate a total molecular weight of 60,000. One might speculate that these larger proteins could represent alternatively processed translation products, although it is possible that they are host cell proteins which become viral associated during virus maturation. Partial proteolytic sequencing of viral associated proteins and analysis of in vitro translation products will be required to clarify the relationship of the various viral associated proteins.

Using rabbit anti-PBFD virus antibody to compare the antigenic similarities between the four viral preparations, it was determined that the viruses recovered from each of these birds were antigenically related.

The ultrastructural characteristics, protein composition, and antigenic similarities of viruses isolated from a sulphur-crested cockatoo, a black palm cockatoo, a red-lored Amazon parrot and a peach-faced lovebird suggest that virus obtained from each of these genera belong to the same viral family. It is now clearly established that the novel virus previously isolated and characterized as PBFD virus can be consistently recovered from various psittacine species displaying PBFD lesions. Presently, no other viral agent has been consistently purified from follicles of infected birds utilizing the described techniques. This investigation also represents the first reported case of PBFD in a species of black cockatoo. Although histologic lesions of PBFD have been reported for numerous species of lovebirds and a blue-fronted Amazon parrot, this is the first report of PBFD virus recovery from these two genera.

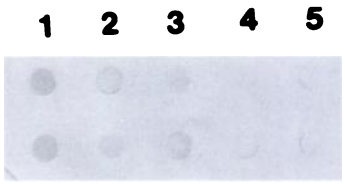

FIGURE 6. Dot immunoblot detection of purified PBFD virus from an umbrella cockatoo. Viral antigen was titrated against rabbit anti-PBFD virus IgG as described in material and methods: Lane 1 (6.7 ng total protein), Lane 2 (1.3 ng total protein), Lane 3 (0.67 ng total protein), Lane 4 (0.13 ng total protein), and Lane 5 (no antigen).

FIGURE 7. Demonstration of antigenic relationship between PBFD virus purified from different genera of psittacine birds. Lane 1 (umbrella cockatoo), Lane 2 (black palm cockatoo), Lane 3 (red-lored Amazon), and Lane 4 (peach-faced lovebird).

At the level of analysis described in these investigations, there is limited or no antigenic diversity detectable with PBFD virus recovered from numerous species of infected psittacine birds. With the many species of birds currently considered susceptible to the PBFD virus, the antigenic homology that can be demonstrated between purified virus from different genera could be significant in the development of an effective PBFD viral vaccine.

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