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COMPARISON OF HERPESVIRUS ISOLATES FROM FALCONS, PIGEONS AND PSITTACINES BY RESTRICTION ENDONUCLEASE ANALYSIS

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ABSTRACT: Field isolates of herpesviruses recovered from falcon, pigeon, and psittacine birds were compared by restriction endonuclease (RE) analysis using four separate enzymes. Pigeon and falcon herpesviruses had strikingly similar DNA cleavage patterns, while DNA cleavage pattern of virus isolates from a double-yellow headed Amazon and an African grey parrot had different genomic patterns to both the pigeon and falcon herpesviruses. These findings support the field observations that pigeon herpesvirus causes a fatal herpesviral infection in the livers of pigeon-eating falcons.

Key words: Herpesvirus, restriction endonuclease analysis, prairie falcon, pigeon, psittacines, *Falco mexicanus*, *Columba livia*, *Falco peregrinus*.

INTRODUCTION

Herpesviral infections have been reported in domestic poultry and in a wide variety of pet and wild birds (Kaleta, 1989). Thirteen different avian herpesviral types are known (Gerlach, 1986). Herpesviruses have been isolated from affected falcon species and pigeons (Kaleta, 1990). Hepatic disease in falcons caused by a suspected virus is usually acute and invariably fatal (Graham and Halliwell, 1986). Pigeons are a common prey of many falcons. The pigeon appears to be the natural host of pigeon herpesvirus 1 (PHV1), but overt clinical disease is primarily seen in squabs lacking maternal antibody or in adult carriers in which exacerbation of the hepatic disease is due to other debilitating factors (Vindevogel and Pastoret, 1980). The herpesviruses of pigeons and falcon are serologically related (Gerlach, 1986). Graham and Halliwell (1986) suggested that pigeons may be the reservoir of the herpesvirus causing the fatal falcon hepatic disease.

Use of DNA restriction endonuclease (RE) analysis is common in molecular studies to differentiate herpesviral strains in both humans (Skare et al., 1975; Summers, 1980; Gerdes et al., 1981) and nonhumans (Paul et al., 1982; Osorio et al., 1985; Shih et al., 1989; Andreasen et al., 1990). Analysis of field isolates of falcon and pigeon herpesviruses by RE has not been reported, and serological studies can not differentiate between these two herpesviral isolates (Gerlach, 1986). Our objective was to determine the similarities and differences between falcon and pigeon herpesviruses, and psittacine herpesviral isolates, using an RE analysis.

MATERIALS AND METHODS

The origins and designations of avian herpesviruses used in this study are listed in Table 1. The sources of the pigeon and falcon viruses all were from cases submitted to the California Veterinary Diagnostic Laboratory System at Davis, California (USA), through the efforts of Dr. W. T. Ferrier of Woodland, California.

Virus sources

A herpesviral isolate (D8904689) was obtained from a captive prairie falcon (*Falco mexicanus*) from California which died from an acute infection 7 days after being fed pigeons. The bird had a clinical course and histologic lesions in the liver (intracellular inclusions) compatible with a herpesviral infection. The virus

TABLE 1. The origins and designations of herpesviruses used for restriction endonuclease analysis.

Virus source and designation	Origin[a] year	Tissues submitted for virus isolation	Titer[b] $\log_{10}$	IFA test[c]
Prairie falcon (D8904689)	1989	Liver	$10^{5.8}$	—
Prairie falcon (D8904641)[d]	1989	Liver	$10^{5.9}$	—
Pigeon (D9006983)	1990	Liver, spleen	$10^{5.7}$	—
Pigeon (D8900906)	1989	Liver	$10^{7.8}$	—
African grey parrot (D9100448)	1991	Liver, spleen	$10^{6.5}$	+++
Double-yellow headed Amazon (D9008842)	1990	Liver, spleen	$10^{7.5}$	+++

[a] All viral isolates originated from birds in California.
[b] Titration for each virus was at the third serial passage of stock virus. None of the field isolates underwent more than eight serial passages from the original tissue submitted.
[c] A direct immunofluorescence assay (IFA) with a conjugate specific to Pacheco's herpesvirus was done on virus infected cell cultures.
[d] A free living bird.

was isolated from a 10% (w/v) liver homogenate in secondary chick embryo fibroblast (CEF) cells prepared from specific-pathogen-free (SPF) 12-day-old chicken embryos (Hyvac, Gowrie, Iowa, USA) (Dotson and Castro, 1988). The virus was passed in CEF cells and the fourth passage virus was used in the RE analysis.

A herpesvirus also was isolated from the liver of a free-living prairie falcon (D8904641) at the fourth serial passage in CEF culture. The falcon's liver had histopathologic lesions typical of a herpesviral infection. The prairie falcon was found in Yolo County, California (38°44'N, 121°48'W) and had died of unknown causes; however, the practitioner submitting the specimen suspected that the bird had been fed pigeons from a flock with carriers of a herpesvirus. Livers and spleens from 16 domestic pigeons (*Columba livia*) from that flock were homogenated into a 10% suspension (w/v) in Dulbecco's minimal essential medium (DMEM) containing 200 μg/ml gentamicin (Gibco BRL, Grand Island, New York, USA) using a polyethylene lab bag in stomacher blender unit (Fisher Scientific, Pittsburgh, Pennsylvania, USA). A 0.2-ml volume of the homogenates of each tissue was inoculated onto CEF cell cultures, adsorbed for 30 min and refed with DMEM supplemented with 10% fetal bovine serum (Hyclone Lab, Logan, Utah, USA) and 200 μg/ml gentamicin.

A herpesvirus (D9006983) was isolated from a California pigeon (*Columba livia*) which died of a suspected exposure to dioxin. On necropsy, numerous 1- to 2-mm white foci were seen in the liver. A 10% suspension (w/v) of liver and spleen was passed in CEF cells prior to virus purification.

A liver from a dead female domestic pigeon, Lahore breed (D8900906), was submitted. This bird and others in the flock had vomiting, green droppings, and recumbency followed by death. A viral cytopathic effect was observed at the second serial passage of the liver homogenate in CEF cultures. At cell passage two, the prepared supernatant was examined by negative electron microscopy (EM) (Castro et al., 1989); an enveloped herpesvirus was found. The bird was negative for chlamydia by isolation and immunofluorescence on specimen-inoculated McCoy (mouse) cells (Woods et al., 1989).

Herpesviral isolates also were made from clinical submission of two "pet" psittacine birds, an African grey parrot (*Psittacus erithacus erithacus*) and a double-yellow headed Amazon parrot (*Amazona ochrocephala oratrix*). Both birds appeared in good health and showed no clinical signs prior to sudden death. On necropsy, the liver was pale and the spleen was congested in the African grey parrot and the lesions showed evidence of an acute pneumonia, thoracic airsacculitis; the liver was slightly enlarged, soft, and friable in the double-yellow headed Amazon.

Reference strain herpesviruses for psittacine and pigeon were obtained from American Type Culture Collection (ATCC), Rockville, Maryland (USA). The parrot herpesvirus (ATCC VR-915) had been isolated from liver of psittacine birds (*Rosella*), and the pigeon herpesvirus (ATCC VR-705) had been isolated from feces of a pigeon (*Columba livia*).

Experimental procedures

Each herpesviral isolate was propagated in CEF cells prior to DNA isolation. The CEF cells were prepared from 9- to 11-day-old SPF chick-

en embryos. Cells were grown in DMEM supplemented with 10% fetal bovine serum and 200 μg/ml of gentamicin. Cell cultures with a complete viral cytopathic effect (CPE) were harvested by three cycles of freezing at −70 C and thawing at 22 to 25 C; spun at 800 × g for 10 min; the supernate was stored at −70 C as stock virus (Burleson et al., 1992).

The presence of herpesvirus harvested from infected CEF cells was confirmed by negative staining (Castro et al., 1989) using a Zeiss 10C electron microscope (Zeiss, New York, New York). Morphologic identification of herpesvirus was based on its size, icosahedral symmetry and the presence of an envelope (Morgan and Rose, 1968; Castro and Daley, 1982). Direct immunofluorescence testing with a conjugate provided by National Veterinary Service Laboratory at Ames, Iowa to Pacheco's herpesvirus was done on virus-infected cell cultures (Marks, 1971; Liu and Llanes-Rodas, 1972; Gardner and McQuillin, 1980). The virus-infected cells were viewed using an Olympus fluorescent microscope (Olympus Corporation, Lake Success, New York, New York).

Titration of each herpesvirus at the third serial passage in cell culture was done by using the 50% tissue culture infectious dose ($TCID_{50}$) method (Kuchler, 1977; Burleson et al., 1992). Microtiter platers with 96 wells were monolayered with CEF cells followed by inoculation of 50 μl of each dilution (10^{-1} to 10^{-8}) of the test virus. The first row of eight wells was used as uninoculated cell controls. The plates were then incubated at 37 C and reading of positive or negative cytopathic effect (CPE) were recorded at 24-hr intervals beginning at 48-hr post-infection, for 7 days. The $TCID_{50}$ titration was calculated by the Reed and Muench (1938) method.

Identical purification schemes were employed for all the virus isolates using a procedure modified from that of Pritchett (1980). Virus-infected cells were clarified by centrifugation at 4 C for 15 min at 3,000 × g in a GSA Sorvall rotor (Du Pont Company, Wilmington, Delaware, USA). Virus suspensions then were centrifuged at 35,000 × g for 90 min in a SS-34 Sorvall rotor. Virus pellets were resuspended in 1 mM potassium phosphate buffer (pH 7.5) and layered onto 10 to 60% sucrose linear gradient, centrifuged at 4 C for 1 hr at 79,000 × g using a Beckman SW41 swinging bucket rotor (Beckman Instruments, Inc., Fullerton, California, USA). One or two light scattering bands were visible which were pooled and pelleted at 79,000 × g for 90 min.

The purified virus pellets were resuspended in Tris-saline buffer. Viral DNA was isolated from purified virions by treatment with 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulphate (SDS), 1% Sarkosyl, 0.5% sodium deoxycholate and 0.1% proteinase-K and followed by one butanol and two phenol/chloroform extractions as described by Pritchett (1980). The preparations were then dialyzed overnight at 4 C against two changes of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Viral DNA was precipitated with ethanol and resuspended in TE buffer.

DNA restriction endonuclease (RE) analysis using EcoRI, HindIII, PstI, and XbaI enzymes (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA) was done according to the manufacturer's recommendations. Extracted viral DNA was digested with each restriction enzyme and the fragments generated were electrophoretically separated in 0.7% agarose gels for 18 hr at a constant voltage of 50 volts (Maniatis et al., 1982). A HindIII digest of bacteriophage lambda DNA was used as the standard size marker. The DNA fragments then were stained with a 0.5-μg/ml ethidium bromide solution (Maniatis et al., 1982) and photographed using an ultraviolet light source.

RESULTS

Each herpesviral isolate produced CPE within 2 to 3 days postinoculation in CEF monolayers. The herpesviral CPE was characterized by rounded, highly refractile cells and syncytial formation. On examination by EM of the supernatant obtained from infected cells with CPE, enveloped herpesviruses were seen in all the isolates. The range of infectious titers for the six isolates was between $10^{5.7}$ to $10^{7.8}$ $TCID_{50}$/ml after three serial passages in CEF cells (Table 1).

Based on an immunofluorescence assay, monolayers of CEF cells infected with either the pigeon or falcon herpesviruses did not have nuclear or cytoplasmic fluorescence when stained with a fluorescent conjugate specific for psittacine (Pacheco's) herpesvirus (Table 1).

The herpesviral DNA cleavage patterns of EcoRI digest are shown in Figure 1. Striking similarities were observed among two pigeon and two falcon isolates (lanes 6 to 9) with the exception that one fragment located at 8.5 kilobases (kb) in the pigeon isolates (lanes 6 and 7) was not detected in falcon isolates, and one fragment

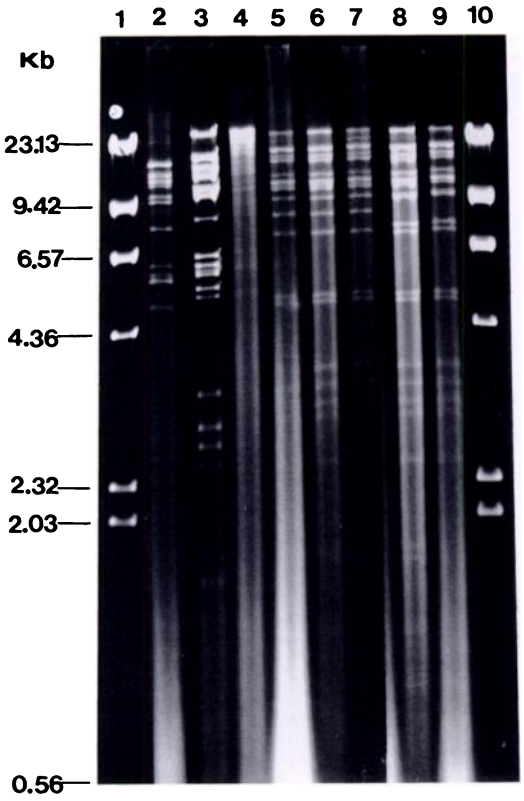

FIGURE 1. EcoRI cleavage patterns of herpesvirus DNA given in kilobases (kb). Lane 1: a lambda DNA HindIII digest as a size marker; land 2: reference strain of parrot herpesvirus (ATCC VR-915); lane 3: double-yellow headed Amazon parrot virus (D9008842); lane 4: African grey parrot virus (D9100448); lane 5: reference strain of pigeon herpesvirus (ATCC VR-705); lane 6: pigeon virus isolate (D9006983); lane 7: pigeon virus isolate (D8900906); lane 8: falcon virus isolate (D8904689); lane 9: falcon virus isolate (D8904641); and lane 10: lambda DNA HindIII digest as size marker.

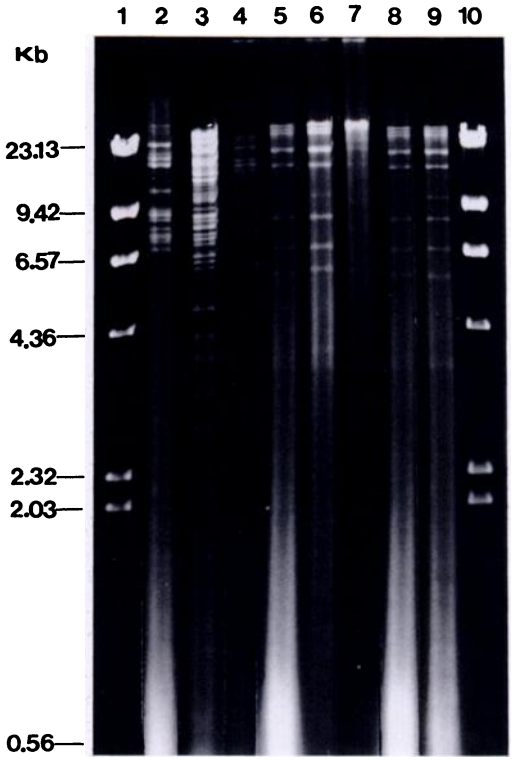

FIGURE 2. HindIII cleavage patterns of herpesvirus DNA given in kilobases (kb). The samples (lanes 1 to 10) are in the same order as Figure 1.

located at the 8.0-kb position in the falcon isolates (lanes 8 and 9) was not observed in the pigeon isolates. These patterns coincided well with that of the reference pigeon herpesvirus (lane 5) but were different from a reference psittacine herpesvirus (lane 2). Furthermore, the DNA patterns of pigeon and falcon isolates were distinctly different from three other psittacine herpesviral isolates which included a reference psittacine strain (lanes 2 to 4). Of interest, there was a marked difference in DNA cleavage pattern among three psittacine isolates (lanes 2 to 4).

The DNA cleavage patterns using different restriction enzymes HindIII (Fig. 2), PstI (Fig. 3) and XbaI (Fig. 4) further supported observations with the EcoRI digestion. In particular, the results with PstI digests (Fig. 3) most clearly demonstrated the similarities and the difference of the DNA cleavage patterns among the herpesviruses examined.

DISCUSSION

From the restriction endonuclease analysis of four herpesviral isolates from pigeons and falcon species using four distinct restriction enzymes, we believe that the DNA migration profiles were strikingly similar to each other, but visibly different from those of three separate psittacine herpesviruses. Furthermore, the cleavage patterns of the DNA within the three separate

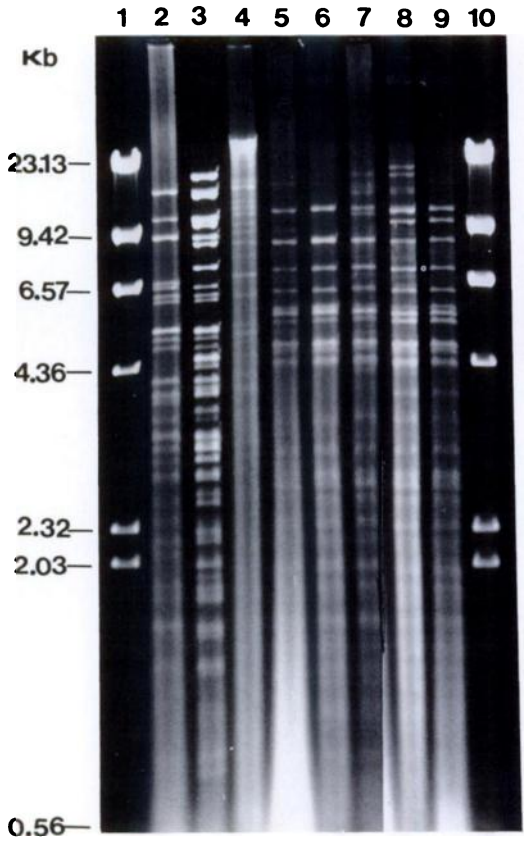

FIGURE 3. PstI cleavage patterns of herpesvirus DNA given in kilobases (kb). The samples (lanes 1 to 10) are in the same order as Figure 1.

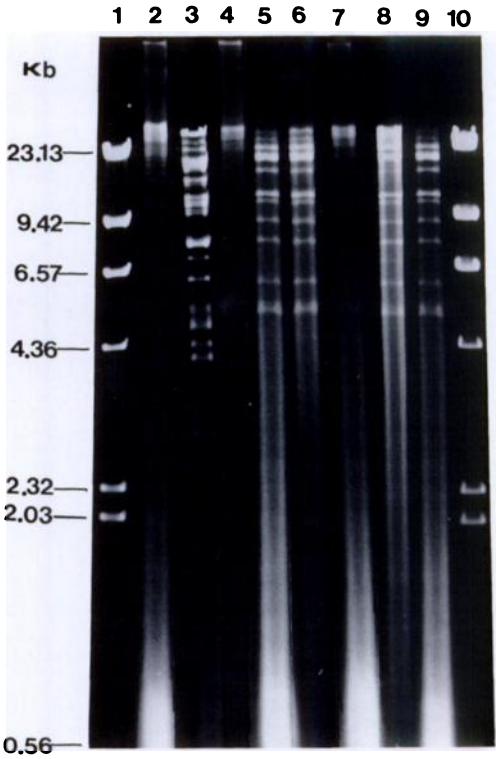

FIGURE 4. XbaI cleavage patterns of herpesvirus DNA given in kilobases (kb). The samples (lanes 1 to 10) are in the same order as Figure 1.

psittacine herpesviruses (one a reference strain) were different in their migration profiles.

The genomic profiles of two psittacine herpesviral isolates, from a double yellow-headed Amazon parrot and an African grey parrot, respectively, had different migration patterns from a reference psittacine (Pacheco's) herpesvirus. Psittaciformes are infected with three serologically distinct and unrelated herpesviruses, namely, Pacheco's virus, Amazon-tracheitis virus and Budgerigar herpesvirus (Kaleta, 1990). This serologic distinction offers one explanation for the differences observed in the DNA patterns in our study. We speculate that the existence of species-specific serotypes of such herpesviruses could account for different avian disease syndromes seen with these herpesviruses. This initial comparison of the RE analysis of these three psittacine herpesviruses to two isolates of pigeon and two isolates of falcon herpesviruses was done to ascertain that the viruses isolated from the latter two species were not psittacine-type herpesviruses or cross-contaminants produced by serial passage in virus-infected cells within the laboratory. Additionally, it was imperative to demonstrate the constancy of the RE migration profiles of the viral DNAs of the falcon and pigeon viruses after serial passing in cell cultures and their similarity to the DNA to a reference pigeon herpesviral strain. In contrast, the DNA's of the psittacine viruses, including the reference Pacheco virus, had much variation among themselves using RE analysis.

The antigenic distinction of pigeon and falcon viruses from psittacine herpesviruses was demonstrated by the absence of

specific fluorescence in cell cultures infected with pigeon or falcon viruses when a psittacine fluorescent conjugate to Pacheco's herpesvirus was used. The herpesviral isolates from falcon and pigeon in the present study were different in their RE profiles from that of infectious laryngotracheitis (ILT) virus (Andreasen et al., 1990). This finding eliminates the possibility that ILT viruses from poultry may have been transmitted to the affected pigeons.

The finding that the herpesviral DNA from a pigeon isolate and from fatal cases of herpesviral infection in a falcon were strikingly similar is significant. It is particularly relevant to the management practices used in rearing pigeon-eating falcon species, especially endangered ones. Our RE analysis is the first which implicates pigeon herpesvirus as the potential etiology of fatal herpesviral liver infections in falcons and further argues in favor of empirical field observations. Pigeons from unknown flocks should not be used as food sources for falcons reared in captivity and for hunting until a serologic method is available to determine the pigeons' carrier status. Since pigeons are an integral part in training birds used in falconry, vaccination (killed herpesviral vaccine) of falcon could become a viable approach for the control of this disease. The homing pigeon and squab industries also would benefit from such a vaccine.

Further studies are required using DNA sequencing of these viral DNAs from pigeon and falcon to ascertain if their genomes are identical: a DNA probe could be developed to examine isolates of such avian carriers. Studies with additional herpesviral isolates from falcons and pigeons should further reveal the homogeneity of such isolates from other falcon or pigeon species and may also reveal interspecies diversities.

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