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Virologic and Serologic Studies of Zoo Birds for Marek's Disease Virus Infection[1]

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One hundred and eleven zoo birds representing 49 species in 14 orders were examined for Marek's disease (MD) herpesvirus (MDHV) infection. MDHV was isolated from 10 birds, all belonging to genus *Gallus*. The precipitating antibodies against MDHV were demonstrated only in the *Gallus* birds, when 51 selected birds including 34 *Galliformes* and 17 other birds representing 12 species from nine orders were examined. The 10 MDHV isolates all induced morphologically similar plaques in cell cultures closely resembling those of HN strain, a low pathogenic isolate of MDHV. Six of the 10 isolates, when inoculated into an experimental line of chickens highly susceptible to MD, caused only a minimal degree of histologic lesions without causing clinical MD, gross MD lesions, or deaths from MD. Natural hosts of MD are probably *Galliformes*, primarily affecting *Gallus* and less often other genera of *Galliformes*.

Except for chickens, Marek's disease (MD) herpesvirus (MDHV) has been demonstrated only in quails (12) and in turkeys (17). Although lesions resembling those of MD have been noted in pheasants (8), ducks (6), owls (7), swans (2), and partridges (11), attempts to demonstrate the presence of MDHV or MDHV antibodies in birds other than chickens, quails, and turkeys have been unsuccessful (1, 10, 12, 14). A recent report described only one guinea fowl positive for precipitins when 125 serum samples from 303 zoo birds were tested against MD feather follicle antigens (13).

This paper reports isolation and characterization of MDHV as well as demonstration of MDHV antibodies from zoo birds.

MATERIALS AND METHODS

Zoo birds for MDHV isolation. One hundred and eleven birds representing 49 species from 14 orders were examined for MDHV infection (Table 1). These were all zoo birds kept at a zoo maintaining 1,068 avian species.

The bird house area consisted of a series of display pens located on the periphery of a square with a group of holding pens arranged linearly inside the area. Display pens were constructed of a stucco-concrete material, with the fronts, part of the partitions between adjacent pens, and most of the roof being covered with a heavy wire mesh. Holding pens consisted of areas completely enclosed by wire and attached to a house constructed of wood and partitioned completely from adjacent units. Several series of other display pens were arranged linearly, 50 to 100 feet from the bird house area. The red jungle fowl had free access to the zoo grounds outside the display pens, hatching most of their eggs in nests hidden within the abundant bushes and trees. Caretakers were not restricted to any group of pens and often attended many types of birds. Free-flying wild birds, pigeons, and doves were commonly noted in the zoo grounds. Migratory waterfowl frequented the ponds on the zoo grounds. The Zoo Veterinary Hospital area where various *Aves* were held for treatment or observation, consisted of a series of wire-enclosed pens or wire cages arranged along the walls of separate rooms. An individual caretaker was responsible for the care of a number of hospital cases during the day.

Cell cultures. Chicken embryo fibroblast (CEF) and chicken kidney (CK) cell cultures were prepared as described previously (3, 4). Fertile eggs obtained from a commercial, specific, pathogen-free flock (H&N Inc., Redmond, Wash.) served as a source of chicken embryos for CEF cell cultures. Chicks (4 to 6 weeks old) hatched from the same eggs and reared in Horsfall-Bauer type isolators were employed for CK cell cultures.

Experimental chickens. The pathogenicity of the MDHV isolates was tested in the WSU-VS chicken, an experimental line of White Leghorns highly susceptible to MD. The chickens, along with the procedures of hatching and rearing, have been previously described (5).

MDHV isolation and detection of antibodies. Virus isolations were attempted from heparinized blood samples which were collected aseptically by venipuncture from various species of *Aves* held in the bird house display area or zoo veterinary hospital. Blood specimens from the San Diego Zoo were ob-

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TABLE 1. *Orders and species examined for MDHV and antibodies from zoo birds*

Order	Birds examined (species)	MDHV isolation	MDHV antibody[a]
Anseriformes	Australian shelduck (*Tadorna tadornoides*)	0/1[b]	NT
	Black cygnet swan (*Cygnus atratus*)	0/1	NT
	Cereopsis goose (*Cereopsis novaehollandiae*)	0/1	NT
	Fulvous tree duck (*Dendrocygna bicolor*)	0/1	NT
	Laysan teal (*Anas platyrhynchos laysanensis*)	0/3	0/3
Casuariformes	Emu (*Dromiceius novaehollandiae*)	0/3	0/1
Charadriiformes	Common sandpiper (*Actitis hypoleucos*)	0/1	0/1
	Murre (*Uria aalge*)	0/1	0/1
Ciconiiformes	Night heron (*Nycticorax nycticorax*)	0/2	0/2
Columbiformes	Feral pigeon (*Columbia livea*)	0/1	0/1
Coraciiformes	Abyssinian ground hornbill (*Bucorvus abyssinicus*)	0/4	NT
	Black-casqued hornbill (*Ceratogymna atrata*)	0/1	NT
Cuculiformes	Eastern giant plantain eater (*Corythaeola cristata yalensis*)	0/1	NT
	Roadrunner (*Geococcyx californianus*)	0/1	NT
Falconiformes	Coopers hawk (*Accipiter cooperi*)	0/1	0/1
	Gabar goshawk (*Melierax gabar*)	0/1	0/1
	Kestrel hawk (*Falco tinnunculus*)	0/1	NT
	Northern white-tailed kite (*Elanus leucurus majusculus*)	0/3	NT
	Red-tailed hawk (*Buteo jamaicensis*)	0/2	NT
Galliformes	Ceylon jungle fowl (*Gallus sonnerati*)	1/6 (GS-1)[c]	1/4
	Golden pheasant (*Chrysolophus pictus*)	0/2	NT
	Green peafowl (*Pavo muticus imperator*)	0/1	NT
	Indian peafowl (*Pavo cristatus*)	0/6	0/2
	Indian chukar (*Alectoris chukar*)	0/1	NT
	Japanese silky (black and red face) (*Gallus gallus*)	5/7 (JS-1, JS-2, JS-3, JS-4, JS-5)	1/5
	Japanese Yokohama (*Gallus gallus*)	0/1	NT
	Japanese Yokohama × silky (*Gallus gallus*)	0/8	2/8
	Red jungle fowl (*Gallus gallus murghi*)	4/17 (GM-1, GM-2, GM-3, GM-4)	4/10
	Razor-billed curassow (*Mitu mitu*)	0/1	NT
	Swinhoe's pheasant (*Lophura swinhoeii*)	0/5	0/3
	Swinhoe (female) × fireback (male) (*L. swinhoeii* × *L. diardi*)	0/2	0/1
	Wild turkey (*Meleagris gallopavo*)	0/1	0/1
Gruiformes	Clapper rail (*Rallus longirostris*)	0/1	0/1
Passeriformes	Bali mynah (*Leucopsar rothschildi*)	0/1	NT
	Central Europe magpie (*Pica pica pica*)	0/3	0/3
	Common crow (*Corvus brachyrhynchos*)	0/1	NT
	Raven (*Corvus corax*)	0/2	0/1
	Steller's jay (*Cyanocitta stelleri*)	0/1	NT
Pelecaniformes	Brown pelican (*Pelecanus occidentalis*)	0/1	NT
Psittaciformes	Cockatiel (*Nymphicus hollandicus*)	0/3	NT
	Double yellow-head amazon (*Amazona ochrocephla oratrix*)	0/1	NT
	Ornate lorikeet (*Trichoglossus ornatus*)	0/1	NT
	Red lory (*Eos bornea bornea*)	0/1	NT
	Red-vented amazon (*Pionus menstruus*)	0/1	NT
Strigiformes	Burrowing owl (*Speotyto cunicularia*)	0/1	NT
	Great horned owl (*Bubo virginianus pacificus*)	0/2	0/1
	Mottled owl (*Ciccaba virgata*)	0/1	NT
	Spectacled owl (*Pulsatrix perspicillata*)	0/1	NT
	Western barn owl (*Tyto alba practincola*)	0/1	NT

[a] MDHV precipitins. NT, Not tested.
[b] Number of positive/number of birds examined.
[c] MDHV isolate designation.

tained by Kenzy. The samples, which were shipped by air with a frozen jelly ice pack and arrived at the laboratory in 13 to 29 h after collection, were immediately processed for virus isolation.

MDHV isolation in cell cultures from heparinized blood was done by the method previously described (5) with the following minor modifications. Blood samples of 0.5 ml or more were centrifuged at 1,000 $\times$ *g* for 10 min to separate the buffy coat. The buffy coat cells, with as few erythrocytes as possible, were then suspended in 1 ml of maintenance medium and inoculated (0.25 ml) into each of two dishes of secondary CEF (24-h culture) and primary CK monolayers, respectively. Of those samples with less than 0.5 ml of blood, whole blood (0.1 ml) was inoculated into CK or CK and CEF monolayers. Uninoculated cell cultures served as controls. The inoculated cultures were examined daily for cytopathic effects. After 7 to 8 days, those cultures without any cytopathic effects were passaged and observed for 8 to 10 days; CEF cell cultures were subcultured from one dish to two dishes, and CK cultures were passaged by inoculating one-half of the cells of one dish onto each of two normal CK monolayers.

The plasma samples, collected from heparinized blood of 51 selected birds, including 34 *Galliformes* and 17 other birds representing 12 species from nine orders, were tested for MDHV precipitins by the micro agar gel precipitin test as described elsewhere (5).

Identification of the MDHV isolates. Any isolates inducing plaques suggestive of herpesvirus were cloned by three successive isolations of a single plaque, and then identified as MDHV by examining its cytopathology in cell cultures, nucleic acid type, cell association of infectivity, and antigenic relationship to MDHV and turkey herpesvirus (HVT; FC 126 strain) by an indirect fluorescent antibody staining. The procedures of cloning and identification have been detailed previously (5).

Pathogenicity of the MDHV isolates to chickens. Six of the 10 isolates were examined similarly for their pathogenicity to the WSU-VS chicken in three separate trials, two isolates per trial.

A group of 15 WSU-VS chicks (3 days old) was employed for each isolate, 10 of them being inoculated with the isolate and five uninoculated serving as contacts. In each trial, a group of 15 hatchmates similarly treated with a known virulent strain of MDHV (Id-1 strain) (5) and a control group consisting of five untreated hatchmates were included. Each of the inoculated and control groups was housed in a separate Horsfall-Bauer type isolator. The virus was inoculated subcutaneously in the dorsal cervical area with a dose ranging from 159 to 401 plaque-forming units for the zoo isolates and 132 to 213 plaque-forming units for the Id-1 strain (see Table 3). The inoculated birds were observed for development of clinical MD for 6 weeks when surviving birds were examined for MDHV viremia and MD lesions. Ten birds, five each of the inoculated and contacts from each of the group inoculated with the zoo isolate, all surviving birds of the group inoculated with the Id-1 strain, and all control birds were examined for MDHV viremia by the procedures previously described (5). All birds dying during the observation period and surviving birds were necropsied to examine for gross and histologic MD lesions. Tissues (right and left brachial and sciatic plexuses, and gonads) for histologic examination were taken from the same birds examined for viremia. The lesions were interpreted as described by Payne and Biggs (15).

RESULTS

Isolation and characterization of MDHV. Plaque-forming agents were isolated in cell cultures from 10 of the 111 zoo birds examined, five isolates from seven Japanese silkies, four isolates from 17 red jungle fowl, and one isolate from six Ceylon jungle fowl. Two Ceylon jungle fowl chicks from the same holding pen died when 5 to 6 weeks old with gross and histologic lesions of acute MD. The plaques, closely resembling those of MDHV, started to develop both in CEF and CK cell cultures on initial isolation 5 to 6 days after inoculation of heparinized blood or buffy coat cells (Table 1). The cell cultures inoculated with buffy coat cells from a Swinhoe's pheasant developed cytopathic effects that were different from those of MDHV and characterized by syncytium formation in CEF cultures. Those inoculated similarly with heparinized blood or buffy coat cells from the rest of the birds, as well as uninoculated control cultures, did not show any cytopathic effects through the second passage.

The 10 isolates were all identified as MDHV on the basis of cell culture cytopathology characteristic of herpesvirus, cell association of infectivity, inhibition of plaque formation by a deoxyribonucleic acid inhibitor, and closer antigenic relationship to a known MDHV than to HVT (Table 2). The plaques induced by each of the 10 isolates were essentially the same in morphological characteristics. The plaques were relatively large, ranging from 1 to 2 mm in diameter at 6 to 7 days after inoculation, and were composed of round refractile cells of variable sizes with a few dark-appearing cells which were found to be polykaryocytes when stained with May-Grünwald-Giemsa (Fig. 1A). Those round cells of plaques stained dark by May-Grünwald-Giemsa without discernible cell structure and polykaryocytes were quite common within and around the plaques, but intranuclear inclusions (type A) were seen only in a few cells. In all isolates, the infectivity was cell associated as indicated by the absence of infectivity in the culture fluids of an infected CEF culture which had a titer of 2×10^4 to 3.3×10^5 plaque-forming units when cells were assayed. The plaque-forming activity was completely inhibited in the presence of 5-iododeoxyuridine (100 μg/ml of medium), whereas control cul-

TABLE 2. *In vitro characteristics of the 10 isolates of MDHV from zoo birds*

Isolate	Source birds	Cytopathology[a]			Cell association of infectivity[b]		Inhibition of plaque formation by an inhibitor[c]	
		PF	PK	NI	Culture fluids	Cells	Treated	Nontreated
JS-1	Japanese black-face silky (1.5-year-old female)	+	+	+	0	3.3×10^5[d]	0	330[d]
JS-2	Japanese red-face silky (1.5-year-old female)	+	+	+	0	3×10^5	0	300
JS-3	Japanese black-face silky	+	+	+	0	2.8×10^4	0	285
JS-4	Japanese black-face silky (male)	+	+	±	0	3.7×10^4	0	756
JS-5	Japanese red-face silky (female)	+	+	+	0	5.1×10^4	0	514
GM-1	Red jungle fowl (male)	+	+	+	0	2.9×10^4	0	299
GM-2	Red jungle fowl (6-month-old female)	+	+	+	0	6×10^4	0	1214
GM-3	Red jungle fowl (6-month-old male)	+	+	+	0	3.4×10^4	0	679
GM-4	Red jungle fowl (6-month-old female)	+	+	+	0	2×10^4	0	413
GS-1	Ceylon jungle fowl (2-year-old male)	+	+	+	0	6.5×10^4	0	438

[a] PF, Plaque formation; PK, polykaryocytes; NI, intranuclear inclusions (type A).
[b] The culture fluids and cells of an infected CEF cell culture were respectively assayed.
[c] 5-Iododeoxyuridine (100 μg/ml of medium). Newcastle disease virus (B_1 strain) used as a ribonucleic acid virus control was not affected.
[d] In plaque-forming units.

tures yielded 299 to 1,214 plaques. The CEF cell cultures grown on cover slips and infected with each isolate, when stained by indirect fluorescent antibody using chicken anti-MDHV (Id-1 strain) serum and fluorescein-conjugated horse anti-chicken globulin, exhibited specific fluorescence in both the nucleus and cytoplasm virtually limited to those cells of plaques (Fig. 1B). The stained antigens of the nucleus and cytoplasm generally appeared finely granular but nuclear antigens were occasionally particulated. Uninfected control cultures similarly stained showed no fluorescence and infected replica cultures of each isolate exhibited only a weak fluorescence with chicken anti-HVT serum which gave a strong fluorescence to HVT-infected cells.

When plasma samples from 51 selected birds, including 34 *Galliformes* and 17 other birds representing 12 species from nine orders, were tested for MDHV precipitins, the antibodies were demonstrated only in *Gallus* birds, such as red jungle fowl, Ceylon jungle fowl, Japanese silkies, and Japanese silky and Yokohama cross (Table 1).

Pathogenicity of the MDHV isolates. Six isolates (JS-1, JS-2, JS-3, GM-1, GM-3, and GS-1) tested for their pathogenicity in the WSU-VS chicken, highly susceptible to MD, all failed to cause clinical MD, gross MD lesions, or MD deaths, whereas the hatchmates inoculated with a virulent strain of MDHV (Id-1) developed clinical and pathologic MD in almost all inoculated, as well as in contact, cagemates in 6 weeks after inoculation (Table 3). Although none of the isolates caused gross MD lesions, some of the inoculated and contacts developed a minimal degree of histologic MD lesions in nerves. The lesions were mostly milder than C-type MD lesions (15) and characterized by few areas with 10 or less small lymphoid cells occasionally together with mild edema. The six isolates tested were considered to be apathogenic or mild strains of MDHV since they caused only minimal histologic lesions even in a highly susceptible line of chickens.

The six isolates spread readily from the inoculated to contact cagemates as indicated by viremia with the respective isolate in all or most of the contact cagemates (Table 3).

DISCUSSION

The 10 isolates from zoo birds were all identified as MDHV on the basis of in vitro characteristics of herpesvirus, cytopathology in cell cultures, closer antigenic relationship to MDHV than to HVT, and development of histologic MD lesions, although minimal, in the inoculated and contact cagemates. The fluorescent antibody staining of both the nuclear and cytoplasmic antigens of infected cells also indicated these isolates were MDHV. Purchase et al. (16) reported that only the nuclear antigen stained in HVT-infected cells when stained with anti-MDHV serum.

It was interesting to note that the plaque morphology of these 10 isolates closely resembled those of HN strain, a low pathogenic strain of MDHV (5), and six isolates tested were found to be apathogenic or mild strains. It is not known whether the 10 MDHV isolates represent one strain or different strains of MDHV.

In view of housing and management practices at the zoo, there was likelihood of direct or indirect contact among those zoo birds exam-

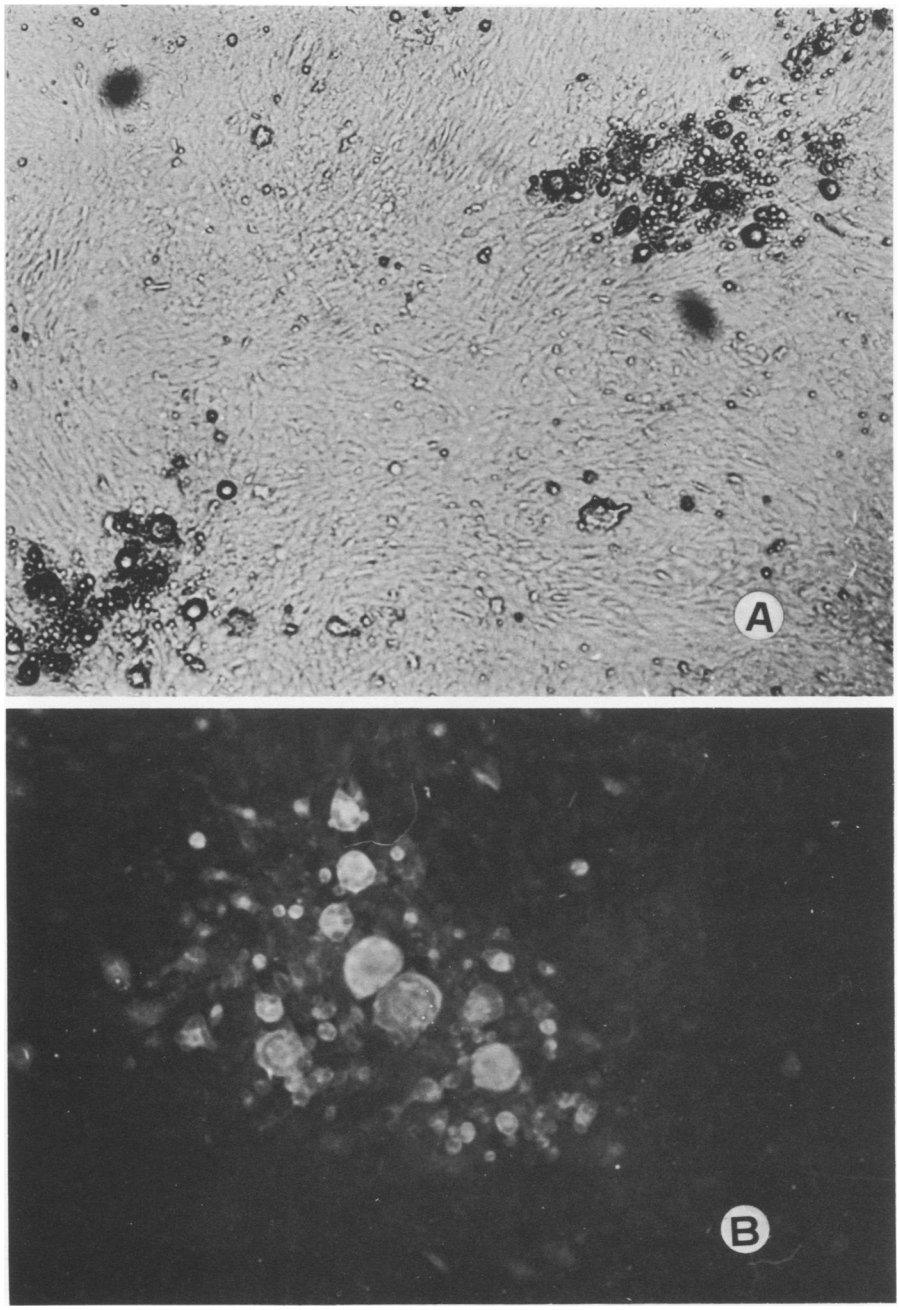

FIG. 1. *(A) Plaques formed by the MDHV isolate from a red jungle fowl (GM-1 isolate) in CEF cell cultures 7 days after inoculation. All 10 isolates induced morphologically similar plaques. ×50. (B) Indirect fluorescent antibody staining of a plaque with anti-MDHV serum. ×200.*

ined. Nevertheless, the demonstration of MDHV and/or antibodies only in *Gallus* birds in this study, together with other earlier reports of natural MDHV infection only in quails (12) and turkeys (17) besides chickens, may suggest that natural hosts of MD are *Galliformes* birds, primarily affecting *Gallus* birds and less often other genera of *Galliformes*. Natural MDHV infection among *Gallus* birds at the zoo was also indicated by the diagnosis of MD, through gross and histologic examinations, in two Ceylon jungle fowl several weeks before virus isolation

TABLE 3. *Pathogenicity of the MDHV isolates from zoo birds*

Isolate	Virus dose per bird[a]	Incidence of MD in inoculated and contact-exposed chickens[b]						MDHV viremia	
		Clinical MD		MD lesions (gross and histologic)		MD deaths			
		Inoculated	Contacts	Inoculated	Contacts	Inoculated	Contacts	Inoculated	Contacts
JS-1	279	0/10[c]	0/5	0/10(2/5)	0/5(1/4)	0/10	0/5	5/5	5/5
JS-2	227	0/10	0/5	0/10(4/5)	0/5(1/4)	0/10	0/5	5/5	5/5
Id-1[d]	213	9/10	1/5	10/10(5/5)	5/5(5/5)	8/10	1/5	2/2	4/4
None[e]	0								
JS-3	159	0/10	0/5	0/10(5/5)	0/5(3/5)	0/10	0/5	5/5	4/5
GM-1	388	0/10	0/5	0/10(3/5)	0/5(1/5)	0/10	0/5	5/5	4/5
Id-1	132	10/10	2/5	10/10	5/5(5/5)	10/10	0/5		5/5
None[e]	0								
GM-3	401	0/10	0/5	0/10(2/5)	0/5(4/5)	0/10	0/5	5/5	5/5
GS-1	133	0/10	0/5	0/10(3/5)	0/5(5/5)	0/10	0/5	5/5	5/5
Id-1	167	9/9	4/5	9/9(9/9)	5/5(5/5)	8/9	0/5	1/1	5/5
None[e]	0								

[a] Virus dose in plaque-forming units.
[b] An experimental line of White Leghorn chickens highly susceptible to MDHV.
[c] Number of birds positive/number of birds examined.
[d] A highly virulent strain of MDHV.
[e] There were no birds positive for five birds examined in all conditions.

attempts. A serological survey of the 303 zoo birds reported recently described only one guinea fowl positive for precipitins when serum samples were tested against chicken feather follicle antigens (13). It is questionable whether the precipitins were specific for MDHV in view of the recent observation on the coexistence of herpesvirus and type C virus in chicken feather follicle epithelium (9).

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