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CHARACTERIZATION OF POXVIRUSES FROM FOREST BIRDS IN HAWAII

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ABSTRACT: Two strains of avian pox viruses were isolated from cutaneous lesions in Hawaiian crows (*Corvus hawaiiensis*) examined in 1994 and a third from a biopsy obtained in 1992 from an infected bird of the Apapane species (*Himatione sanguinea*) by inoculation of the chorioallantoic membranes (CAM) of developing chicken embryos. The resulting proliferative CAM lesions contained eosinophilic cytoplasmic inclusion bodies characteristic of pox virus infection. The pathogenicity of these three viruses in domestic chickens was mild as evidenced by the development of relatively minor lesions of short duration at the sites of inoculation. Their virulence in this host was similar to that of a fowlpox virus (FPV) vaccine strain and contrasted greatly with the ability of two field strains of FPV to produce extensive proliferative lesions. One of the Hawaiian crow pox virus isolates as well as the one originating from the Apapane species could be propagated in two secondary avian cell lines, QT-35 and LMH. A comparison of the restriction fragment length polymorphisms (RFLP) of the genomes of the two cell line-adapted viruses, generated by *Eco*RI digestion, revealed a limited degree of similarity. Moreover, neither profile was comparable to those of the two field isolates of FPV, which were almost indistinguishable from each other. Thus, based on the genetic distinctness of the two Hawaiian bird viruses, they appear to represent different strains of avipoxvirus.

Key words: Avian pox viruses, *Corvus hawaiiensis*, fowlpox virus, genetic differences between pox viruses, *Himatione sanguinea*, mild pathogenicity in chickens.

INTRODUCTION

Natural pox virus infections have been reported in nearly 60 species of wild birds comprising 20 families (Kirmse, 1967; Karstad, 1971). The pox viruses which infect birds belong to the genus *Avipoxvirus* of the Poxviridae family. Although a few members (fowlpox, turkeypox, pigeonpox, canarypox, and quailpox virus) of the genus have been characterized, most of the information about this genus is derived from studies on fowlpox virus, a common pathogen of chickens and turkeys. Despite the variety of hosts, pox virus infection is generally manifested either cutaneously or diphtheritically, although both forms of the disease may occur in the same bird. In the cutaneous form, the proliferative lesions are primarily confined to unfeathered areas of skin, e.g., legs, head, and eyelids, while in the more lethal diphtheritic form, the lesions may be found in the mouth, esophagus and trachea. Because of the economic importance of this disease in commercial poultry, vaccines of fowlpox virus (FPV) or pigeonpox virus (PPV) origin have been used for more than 50 yr.

While avian malaria (van Riper III, 1991) and pox virus infections (Warner, 1968) have been considered to be important factors in limiting the population of Hawaiian forest birds, little detailed knowledge is available about the direct effects of these pathogens on the survival of Hawaiian species or why these birds are more susceptible than their mainland counterparts (Atkinson et al., 1995). Although evidence of pox virus infection in Hawaiian crows has been reported previously (Jenkins et. al., 1989), no attempt was made to characterize the responsible virus. Therefore, the initial objective of

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this study was to characterize pox viruses from native Hawaiian avian species in order to determine their relationships to each other and to avian pox viruses that affect domestic poultry, especially FPV. If they represent novel strains, then, in view of their ability to infect endangered species, the findings of this study can form the basis for the generation of a vaccine(s) for amelioration of the manifested disease.

MATERIAL AND METHODS

Suspected pox lesions from naturally infected birds

Six samples suspected of harboring pox virus were used as inocula for virus isolation. Three, identified as ʻAlala/Mahoa, ʻAlala/Hulali, and ʻAlala/Lanakila were skin lesions from separate Hawaiian crows (*Corvus hawaiiensis*). The first was collected in 1994 at the Peregrine Fund's hacking facility in South Kona (19°22′N, 155°49′W) on the island of Hawaii, while the other two were obtained in 1994 from the Olinda Endangered Species Propagation facility (20°51′N, 156°18′W) on Maui. The ʻAlala/Mahoa and ʻAlala/Lanakila samples were excisional surgical biopsies, while the ʻAlala/Hulali one consisted of superficial scrapings. Three other specimens, designated as 4263-23, 4263-219 and 4263-277, were collected from Apapane (*Himatione sanguinea*) birds found dead in Hawaii Volcanoes National Park (19°25′N, 155°14′W) during an epizootic outbreak of pox and malaria in 1992. All lesions were excised during necropsy and stored frozen at −20 C.

Hawaiian bird pox virus isolation attempts

All lesions were ground separately with sterile alundum (60 mesh Norton Alundum "RR"; Fisher Scientific Company, Pittsburgh, Pennsylvania, USA) in Hank's balanced salt solution containing 1,000 units penicillin, 1 mg streptomycin and 2.5 μg amphotericin B/ml (Life Technologies, Gaithersburg, Maryland, USA). Following incubation at 37 C for 1 hr and clarification by low speed centrifugation at 850 × *g* for 5 min, approximately 0.1 ml of the supernatant from each sample was separately inoculated onto the chorioallantoic membrane (CAM) of specific-pathogen-free 10-day-old developing chicken embryos (Hy-Vac, Adel, Iowa, USA) as described by Tripathy and Reed (1998). Following inoculation, the embryos were incubated at 37 C and checked daily for mortality. At 7 days after infection, CAMs were examined and portions of those showing lesions were frozen at −20 C while the remainder was fixed in 10% buffered formalin and embedded in paraffin. Thin sections of the fixed lesions were stained with hematoxylin and eosin and by the Fuelgen reaction (Tripathy et al., 1973). Stained sections were examined microscopically for the presence of cytoplasmic inclusion bodies. Frozen lesions were ground as described above to be used as inocula for passaging either on the CAM or in permanent avian cell lines.

Fowlpox viruses

Two virulent strains of FPV, isolated from chicken flocks located in Minnesota and Nebraska, were initially propagated on CAMS as described above for the Hawaiian bird pox viruses. In addition, a vaccine strain (POXVAC-TC®) provided by Schering-Plough Animal Health Corp. (Elkhorn, Nebraska, USA) was included in the pathogenicity trials.

Propagation of viruses in avian cell lines

"Blind passaging" of the Hawaiian bird pox viruses was performed in the Japanese quail fibroblastic cell line, QT-35 (Schnitzlein et al., 1988), as well as the chicken liver cell line, LMH (Schnitzlein et al., 1994). The initial inocula were ground suspensions of CAM lesions and subsequent ones consisted of cells that had been lysed by two cycles of freezing and thawing at 4–14 days post infection. Likewise, both field strains of FPV were also grown in QT-35 monolayers.

Viral pathogenicity in chickens

Pairs of adult susceptible chickens obtained from a specific-pathogen-free flock (Hy-Vac) were inoculated cutaneously (comb, leg and wing web) with either one of the three Hawaiian bird viruses which had been confirmed as pox by histopathology of the infected CAM's, one of the two virulent field strains of FPV or the mild FPV vaccine. Inocula (approximately 100 μl per bird) consisted of ground CAMS which had contained confluent pocks arising from virus infection. The birds were housed in separate cages in different isolation units under negative pressure in the laboratory animal facility of the College of Veterinary Medicine, University of Illinois under the care of American Association of Laboratory Animal Science (AALAS) certified animal care staff. Each bird was examined daily for the development and regression of lesions. The birds were euthanized at 5 wk after inoculation.

Genetic analysis of pox viruses

Viral DNA was isolated from infected QT-35 and LMH cells as previously described (Schnit-

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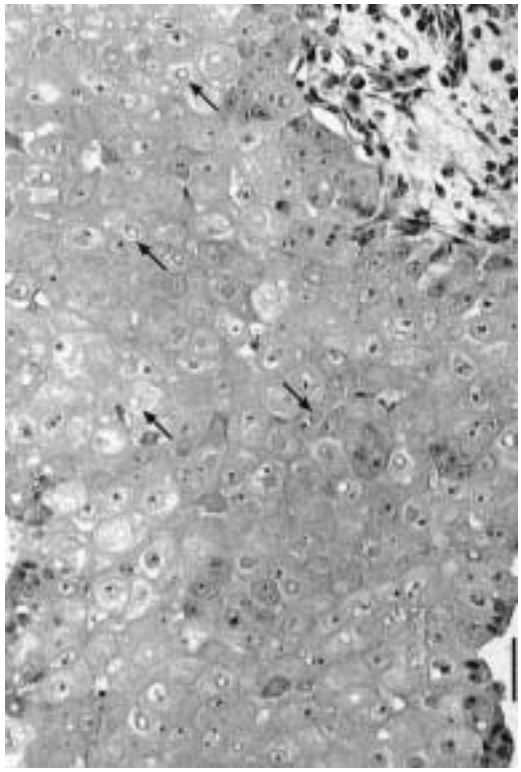

FIGURE 1. Section of avian pox virus lesion on the CAM. Infected cells are enlarged and show cytoplasmic inclusion bodies (arrowheads). H&E, Bar = 50 μm.

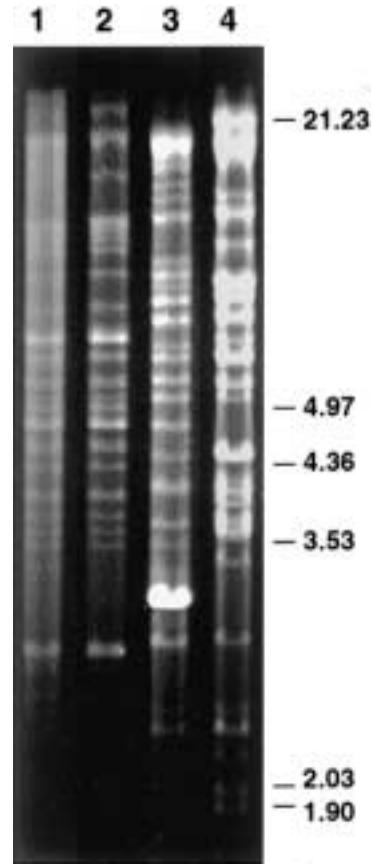

FIGURE 2. Agarose gel electrophoresis of *Eco*RI-generated fragments of the genomes of two FPV field strains (Minnesota, lane 1; Nebraska, lane 2), *Apapane* isolate 4263-219 (lane 3) and ʻAlala Lanakila isolate (lane 4). FPV were grown in QT-35 cells, while the Hawaiian bird pox viruses were propagated in LMH cells. The relative mobilities of the *Eco*RI/*Hin*dIII-generated fragments of lambda DNA used as molecular-weight markers (X1,000) are indicated on the right-hand side.

zlein et al., 1988) and digested with *Eco*RI (Life Technologies) according to manufacturer's instructions. The resulting fragments were separated by electrophoresis in a 0.8% agarose gel and then stained with ethidium bromide. The stained gel was photographed using a red filter on an IBI UVT 750-M transilluminator (International Biotechnologies, Inc., New Haven, Connecticut, USA).

RESULTS

In an attempt to isolate the agent(s) responsible for the lesions in the Hawaiian birds, CAM's were inoculated with portions of the original samples. By 7 days post infection only three, ʻAlala/Mahoa, ʻAlala/Lanakila and 4263-219, of the six inocula had produced morphological changes consisting of individual pocks in the CAM and/or marked thickening of the membrane. In all three cases, histopathological examination of the altered CAM areas revealed marked cellular hyperplasia and eosinophilic cytoplasmic inclusion bodies within enlarged cells (Fig. 1) after staining with hematoxylin and eosin. These inclusions which appeared as solid or ring-like structures stained pink with Fuelgen reaction and thus were comprised of DNA-containing virus (not shown). Many affected cells contained only viral inclusions and seemed to have lost their nuclei.

Although the three pox virus isolates originated in Hawaiian birds, it is not clear whether they could pose a threat to com-

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TABLE 1. Comparative pathogenicity of pox virus isolates in susceptible chickens.

Virus isolate	Type of lesions[a]	Persistence of lesions at 3 wk[a]
ʻAlala/Lanakila	+	–
ʻAlala/Mahoa	+	–
Apapane 4263-219	+	–
FPV (Minnesota)	++	++, S
FPV (Nebraska)	++	++, S
FPV (POXVAC-TC®)	+	–

[a] – = absence of lesions at site of inoculation; + = mild localized lesion at site of inoculation; ++ = extensive, proliferative lesion at site of inoculation; S = secondary lesions at other sites.

mercial poultry. Therefore, their ability to infect domestic fowl was evaluated. Adult chickens inoculated with any of these viruses developed only relatively minor lesions which remained localized at the site of scarification and which disappeared within the following 3 wk. Likewise, limited pathogenicity was observed when a FPV vaccine strain was used (Table 1). Based upon the mild localized reaction in young chickens due to FPV immunization (Tripathy and Reed, 1997), it is unlikely that the age of the recipient would have influenced the virulence of the Hawaiian bird pox viruses. Thus, similar results would probably have been obtained if younger birds had been used.

In contrast to the moderate effects on the host due to infection by the Hawaiian bird pox viruses, severe lesions formed at the site of inoculation and persisted for >3 wk (Table 1) in those chickens which received either of the two virulent field isolates of FPV. Additionally, these afflicted birds also developed secondary lesions around the eyelids and legs which probably contributed to their apparent weight loss due to reduced feed consumption.

To examine further the host range of the Hawaiian bird pox viruses, they were "blind passaged" in two avian cell lines of chicken (LMH) or quail (QT-35) origin. Evidence of virus replication in both LMH (cell rounding, enlargement, and detachment) and QT-35 (plaque formation) cells was detected during the second and fourth consecutive passages of the Apapane isolate 4263-219 and the ʻAlala/Lanakila virus, respectively. The cytopathic effect (CPE) produced by both viruses in LMH cells occurred faster and was more extensive than that observed in QT cells. Interestingly, no CPE was detected by the fifth passage of ʻAlala/Mahoa in either cell line.

Since the two cell line-adapted viruses originated in different species of Hawaiian birds, a genetic analysis was performed to determine their relationship to each other and FPV. A comparison of the profiles generated by *Eco*RI digestion of their genomes revealed that the two Hawaiian bird pox viruses appeared to be more related to each other than to FPV. Of 24 fragments derived from the genomes of the Apapane isolate 4263-219 and the ʻAlala/Lanakila virus that ranged between approximately 1.9–21 kb, 12 or 50% of them had similar mobilities. In contrast, less than 25% of them were comparable in size to any of those generated from FPV DNA. Although the two field isolates of FPV originated from flocks in geographically distinct areas, the *Eco*RI-generated profiles of their DNAs were nearly indistinguishable.

DISCUSSION

This study was designed to establish criteria for the isolation and characterization of poxviruses associated with endangered Hawaiian forest avian species. Since such pathogens may play a role in limiting the population of these birds (Warner, 1968), this initial analysis will be an important step towards determining the epidemiology of these viruses and the consideration of measures designed to prevent their spread. Unfortunately, pox viruses were only successfully isolated from half of the submitted samples. It is doubtful that this failure can be attributed to an inability of the pathogen to grow on CAM's, since this host is commonly used for the initial isolation of avipoxviruses (Tripathy and Reed, 1997). Rather, the "negative" samples pre-

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sumably either lacked viable viral particles or arose due to an etiologic agent other than pox virus.

The apparent genomic differences (unique RFLP) between the two viruses isolated from an infected *Corvus* sp. and *Himitione* sp. and also FPV indicate that genetically distinct pox viruses do exist in Hawaiian forest birds, as postulated by Jenkins et al. (1989). That such genetic diversity with regard to the natural host exists is not surprising. Indeed, such variability has previously been observed between FPV and quailpox virus (Ghildyal et al., 1989). Whether the observed limited virulence these new viruses have in susceptible chickens extends to the Hawaiian birds is not known at this time. In this regard, the degree of viral pathogenicity may be a reflection of the species being afflicted and whether it has already acquired an immune resistance to the invader (Warner, 1968).

Since avian malaria and pox may be transmitted at the same time during some outbreaks in Hawaiian birds, interactions between the two pathogens may be important as was the case in infected young turkeys (Wright, 1986). Thus, minor cutaneous lesions, which appear on the birds' toes or feet and are caused by naturally attenuated strains of low pathogenicity, may resolve if not complicated by other infections. However, severe, proliferative lesions on the birds' appendices could result in the loss of digits or at least prevent their routine use. Moreover, highly virulent avian pox virus may produce diphtheritic and cutaneous lesions around the eye and beak. This may result in limited survival due to impaired respiration, vision, and feeding ability.

In this study, characterization of the Hawaiian bird pox virus isolates can be considered to be the first step towards the development of vaccines against them. Although only a limited number of viruses have been examined and their immunological relationships have not been determined, the obvious genetic differences between those two originating from different species of birds would suggest that more than one vaccine will be needed. By analogy to current vaccines against FPV (Tripathy, 1993; Tripathy and Reed, 1997), attenuated strains of the various, distinct viruses can be envisioned. Rather than attempt to attenuate the various strains by passage in cell culture, a more effective approach would be to reduce their virulence by genetic manipulation of the viral genome. In this regard, one target would be the gene encoding thymidine kinase (TK). It should be noted that elimination of this non-essential enzyme by insertional inactivation of the TK gene of both an avian poxvirus and herpesvirus has resulted in an accompanying reduction in virulence (Tripathy and Schnitzlein, 1991; Schnitzlein et al., 1995). Such vaccines could be effective in reducing the morbidity and mortality of birds which are in captive propagation and translocation programs, especially after release into areas with active pox virus infections.

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