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Diagnosis of Duck Plague in Waterfowl by Polymerase Chain Reaction

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SUMMARY. A recently developed polymerase chain reaction (PCR) assay was used for diagnosis of duck plague in waterfowl tissues from past and current cases of waterfowl mortality and to identify duck plague virus in combined cloacal/oral-pharyngeal swab samples from healthy mallards (*Anas platyrhynchos*) after a disease outbreak. The PCR was able to detect viral DNA from all the individual or pooled tissues assayed from 10 waterfowl, including liver and spleen samples from three Muscovy ducks (*Cairina moschata domesticus*) that did not yield virus isolates. The strong staining intensity of the PCR products from the waterfowl tissues indicated that large amounts of virus were present, even when virus was not isolated. Duck plague DNA was also detected in a cloacal swab sample from a wood duck (*Aix sponsa*) carcass submitted for diagnosis. The PCR assay identified duck plague DNA in 13 swab samples that produced virus isolates from carrier mallards sampled in 1981 after a duck plague die-off. The duck plague PCR clearly demonstrated the ability to quickly diagnose duck plague in suspect mortality cases and to detect virus shed by carrier waterfowl.

RESUMEN. Diagnóstico de la peste del pato en aves acuáticas mediante la reacción en cadena por la polimerasa.

Se utilizó la prueba de la reacción en cadena por la polimerasa desarrollada recientemente para el diagnóstico de la peste del pato en muestras de tejidos de aves acuáticas provenientes de casos de mortalidad de aves acuáticas pasados y presentes y para identificar el virus de la peste del pato en muestras combinadas de hisopos cloacales y orales (faringe) de patos reales (*Anas platyrhynchos*) después de un brote de la enfermedad. La prueba de la reacción en cadena por la polimerasa fue capaz de detectar el DNA viral en todos los tejidos individuales y mezclados examinados de 10 aves acuáticas, incluyendo muestras de hígado y bazo de patos almizcleros (*Cairina moschata domesticus*) de los que no se aisló virus. La intensidad de tinción fuerte de los productos de la reacción en cadena por la polimerasa de los tejidos de las aves acuáticas indicaron que cantidades grandes del virus estaban presentes aún cuando el virus no había sido aislado. El DNA del virus de la peste del pato se detectó también en muestras de hisopos cloacales de un pato maderero (*Aix sponsa*) enviado para diagnóstico. La prueba de la reacción en cadena por la polimerasa identificó el DNA del virus de la peste del pato en 13 hisopos en los cuales se había realizado aislamiento viral y habían sido tomados de patos reales portadores muestreados en 1981 después de que había pasado un brote de la enfermedad de la peste del pato. Se demostró claramente que la reacción en cadena por la polimerasa es una herramienta de diagnóstico rápida para el virus de la peste del pato en casos de mortalidad sospechosos y para detectar el estado portador de aves acuáticas que están eliminando el virus.

Key words: duck plague, duck virus enteritis, polymerase chain reaction, diagnostic test, field samples, avian herpesvirus

Abbreviations: BEH = bald eagle herpesvirus; CHV = pigeon herpesvirus; DP-VAC = duck plague vaccine; EDTA = ethylenediaminetetraacetic acid; FHV = prairie falcon herpesvirus; GEH = golden eagle herpesvirus; GHOH = great horned owl herpesvirus; HEPES = N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IBDC = inclusion body disease of cranes; ILTV = infectious laryngotracheitis virus; MEM = minimum essential medium;

NWHC = National Wildlife Health Center; PCR = polymerase chain reaction; PFH = peregrine falcon herpesvirus; PHV = psittacine herpesvirus; SOH = snowy owl herpesvirus

Duck plague is a herpesvirus disease of waterfowl that has caused mortality in the United States since 1967 (7,11,12,13,14,15,18). Typical of herpesviruses, duck plague virus infection can result in healthy carrier waterfowl capable of shedding virus for years (3). Previous attempts to detect carrier waterfowl in migratory mallards or in waterfowl at sites of duck plague outbreaks have been mostly unsuccessful (1,2). Better disease and virus identification methods are required to more quickly identify duck plague-induced mortality and to detect healthy carrier waterfowl that are capable of transmitting duck plague virus to new locations. A recently developed polymerase chain reaction (PCR) assay that can detect duck plague DNA with more sensitivity than virus isolation procedures in tissue culture provides a new diagnostic method for this purpose (10). The objective of this work was to determine the ability of the duck plague PCR assay for rapidly identifying the disease in retrospective and concurrent cases of waterfowl mortality and to identify waterfowl that are duck plague virus carriers.

MATERIALS AND METHODS

Cell culture and sample preparation. Tissue culture was prepared from embryonated Muscovy duck eggs by the method of Docherty and Slota (6). Cells were grown to confluence in 25- or 75-cm^2 flasks at 37 C in 4% CO_2. The duck plague vaccine (DP-VAC) strain of virus (1.0 ml) and supernatant material (0.2 ml) from 10% tissue suspensions centrifuged at 800 × *g* were inoculated onto tissue culture monolayers and placed in the incubator for 1 hr. Growth medium containing M199-Earles supplemented with 10% fetal bovine serum, 2 mM L-glutamine, minimum essential medium (MEM) vitamins, MEM nonessential amino acids, antibiotics (100 IU/ml penicillin G. 100 μg/ml streptomycin sulfate, 100 IU/ml mycostatin, and 50 μg/ml gentamicin), and 50 mM HEPES buffer (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) was added to each flask before reincubation. Flasks were harvested at 90%–100% cytopathic effect by three freeze–thaw cycles (−85 C/+37 C) and centrifugation at 800 × *g* for 20 min, and the supernatant from each virus isolate was subdivided and stored at −85 C as a virus stock. Isolates were serologically identified as duck plague with specific antiserum in a neutralization test (20).

Samples for testing were obtained from waterfowl carcasses and field samples submitted to the Diagnostic Virology Laboratory of the National Wildlife Health Center (NWHC) (Table 1). Tissue samples were prepared as 10% suspensions and used for tissue culture inoculation, as described above, and DNA extraction. Samples for DNA extraction were centrifuged at 16,000 × *g* for 5 min and the pellet or the supernatant or both were processed for viral DNA without RNAase treatment as described previously (10). DNA pellets from tissues were suspended in 25–50 μl of sterile double distilled water and stored at 4 C. Tissues from an uninfected mallard (liver and spleen) and chicken liver were processed for virus isolation and DNA as described above and used as negative tissue controls.

Thirteen cloacal/oral-pharyngeal samples (nos. 513, 519, 520, 521, 533, 535, 537, 538, 539, 541, 542, 550, and 553) that were virus isolation positive for duck plague had been collected in a gelatin-based virus transport medium in 1981 as reported previously (2). The wood duck cloacal swab was collected in a tube containing 2.0 ml Hanks balanced salt solution with 5% glycerine plus antibiotics (1500 IU/ml penicillin G, 1500 μg/ml streptomycin sulfate, 100 μg/ml gentamicin, and 100 IU/ml mycostatin) adjusted to pH 7.6 with sodium bicarbonate. A 400-μl sample from each swab suspension was pelleted at 16,000 × *g* for 30 min, and the pellets were resuspended in 200 μl Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris-HCl, pH 8.0, with 1 mM EDTA) and processed for DNA as described above. DNA extracts were suspended in 30 μl of sterile water and stored at 4 C. DP-VAC virus-infected tissue culture was extracted and DNA quantity determined by spectrophotometer (Model DU65; Beckman Instruments, Inc, Fullerton, CA) and used as a duck plague-positive DNA control in each PCR assay (10).

PCR. The PCR assay used a hot start method with wax (Ampliwax beads; Perkin-Elmer Applied Biosystems, Foster City, CA) and reagents from a commercial PCR kit (GeneAmp PCR Reagent Kit; Perkin-Elmer Applied Biosystems) according to their setup recommendations as previously described (10). Duck plague-specific oligonucleotide primers used were 5′ to 3′ 5F-GGCTGGTATGCGTGACAT and 5R-GTATTGGTTTCTGAGTTGGC reported previously (10), and a new primer set called number 7 (7F-GAAGGCGGGTATGTAATGTA and 7R-CAAGGCTCTATTCGGTAATG) was constructed from the dve_p481 clone sequence with Primer 0.5 soft-

Table 1. History of waterfowl and samples submitted to the NWHC for duck plague diagnostic testing.

Year collected	Case no.	Location	Species	Number and type of submission
1981	PR2450	Illinois (2)	Mallard (*Anas platyrhynchos*)	13 cloacal swabs
1996	14245	Virginia	Muscovy duck (*Cairina moshata domesticus*)	3 whole birds
	14254	Pennsylvania	Muscovy duck	1 pooled tissues from same bird
	14297	Wisconsin	Muscovy duck	5 whole birds
1997	14794	Virginia	Wood duck (*Aix sponsa*)	1 whole bird

ware (4). Custom primer sets were purchased commercially from BRL Life Technologies (Grand Island, NY). Primer set 7 was tested for duck plague genome specificity and sensitivity by the methods of Hansen *et al.* (10). Avian herpesvirus genomes used for specificity testing included inclusion body disease of cranes (IBDC) virus, golden eagle herpesvirus (GEH), bald eagle herpesvirus (BEH), great horned owl herpesvirus (GHOH), snowy owl herpesvirus (SOH), peregrine falcon herpesvirus (PFH), prairie falcon herpesvirus (FHV), pigeon herpesvirus (CHV), psittacine herpesvirus (PHV), and infectious laryngotracheitis viruses (ILTVs) as previously described (10).

Primer sets were prepared according to the manufacturer's recommendation and optimized with 1 ng/10 μl of DP-VAC DNA (5). DP-VAC DNA was used at 1 pg/10 μl as a specific DNA control. A lambda DNA fragment (500 bp) and primers supplied with the commercial PCR kit were used as known positive and negative DNA controls in each PCR assay.

Extracted samples (10 μl) from diagnostic tissues and uninfected mallard (liver and spleen) and chicken (liver) tissues were used undiluted in the PCR assay. Uninfected tissues served as negative tissue controls, and a PCR reaction tube without DNA was used to verify that non-specific amplification did not occur.

PCR product from the wood duck swab and liver samples for primer set 7 were sequenced by the University of Wisconsin, Biotechnology Center, Madison, WI, with dye terminator cycle sequencing on an ABI 377 automated DNA sequencer as described (16,19). The sequences were compared with the known primer set 7 target region of the dve_p481 clone sequence.

Samples were cycled for PCR in a model 480 thermal cycler (Perkin-Elmer, Norwalk, CT) with an initial cycle of 94 C for 2 min, 37 C for 1 min, and 72 C for 3 min. Samples from tissues and the wood duck cloacal swab were cycled 35 times and the mallard cloacal swabs 40 cycles at 94 C for 1 min, 55 C for 1 min, and 72 C for 2 min. A final extension step was at 72 C for 7 min.

PCR products were visualized by ultraviolet illumination of ethidium bromide-stained 1% agarose gels run for 1 hr at 120 V with 1× Tris acetate–EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) and photographed with Polaroid (type 655) film (17). The molecular sizes of PCR products were compared with a 1-kbp DNA ladder and/or a low molecular weight 100-bp DNA ladder from BRL Life Technologies.

RESULTS

The PCR assay of waterfowl tissues clearly identified specific duck plague DNA in all the

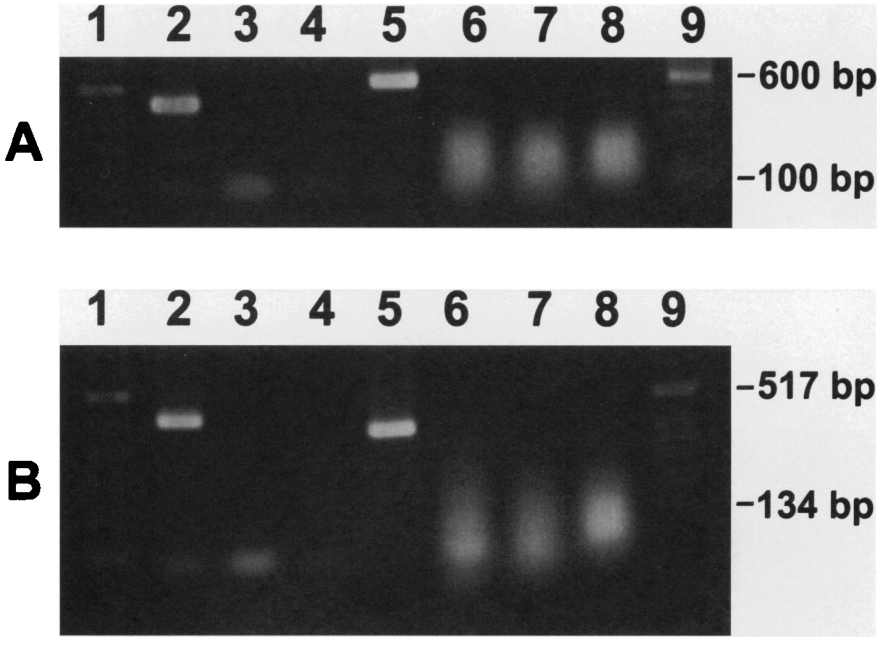

Fig. 1. Comparison of PCR products from uninfected bird tissues. (A) Primer set 5: mallard spleen (lane 6), mallard liver (lane 7), and chicken liver (lane 8). (B) Primer set 7: mallard spleen (lane 6), mallard liver (lane 7), and chicken liver (lane 8). A positive lambda template (A and B, lane 2), a negative lambda template (A and B, lane 3), a 1-pg DP-VAC DNA (A and B, lane 5), and negative DNA control (A and B, lane 4) were used as controls. A 100-bp DNA ladder was used in A, lanes 1 and 9, and B, lane 1, and a 1-kbp DNA ladder was used as a molecular size control in B, lane 9.

tissue samples from diagnostic cases but not in uninfected bird tissues (Figs. 1–3). Sample preparations with the whole tissue suspension, from uninfected and some suspect cases, produced a mass of low molecular weight material in the gels that would have obscured PCR product less than 400 bp (Figs. 1,3). Both primer sets 5 and 7 produced amplicons of expected molecular size, 603 and 446 bp, respectively, with DP-VAC DNA that was above the unknown material in the product (Fig. 1). The individual or pooled tissue samples from duck plague cases were strongly positive for primer set 5 (Fig. 2) and primer sets 5 and 7 (Fig. 3). PCR results for case no. 14297 are shown only in Table 2.

Primer set 7 was sensitive and specific for DP-VAC DNA. The primers targeted a DNA segment nested within the genome region identified by primer set 5. Primer set 7 was able to detect as little as 1 fg of specific duck plague DNA but did not produce PCR product when assayed with nine other avian herpesvirus genomes including IBDC virus, GEH, BEH, GHOH, SOH, PFH, FHV, CHV, PHV, and ILTV (data not shown) as reported previously for primer set 5 (10).

PCR products from ground tissue supernatant extracts of the spleen and liver from bird no. 14245-1 and wood duck liver no. 14794-1 were equally intense with the processed whole tissues from other birds (Figs. 2,3). The PCR products from tissue supernatant extracts were generally cleaner than whole tissue extracts. The PCR results were available within 24 to 36 hr of sample collection.

Virus was isolated from most of the tissues tested with the exception of livers and spleens from the Virginia Muscovy ducks (Table 2). Virus isolates from all of the tissues were identified serologically as duck plague. Sequence data for the PCR products for primer set 7 from the wood duck liver and cloacal swab were identical to the known duck plague sequence in clone dve_p481 (data not shown).

All 13 cloacal samples from the mallard ducks and one wood duck were positive by PCR assay, producing amplicons for both prim-

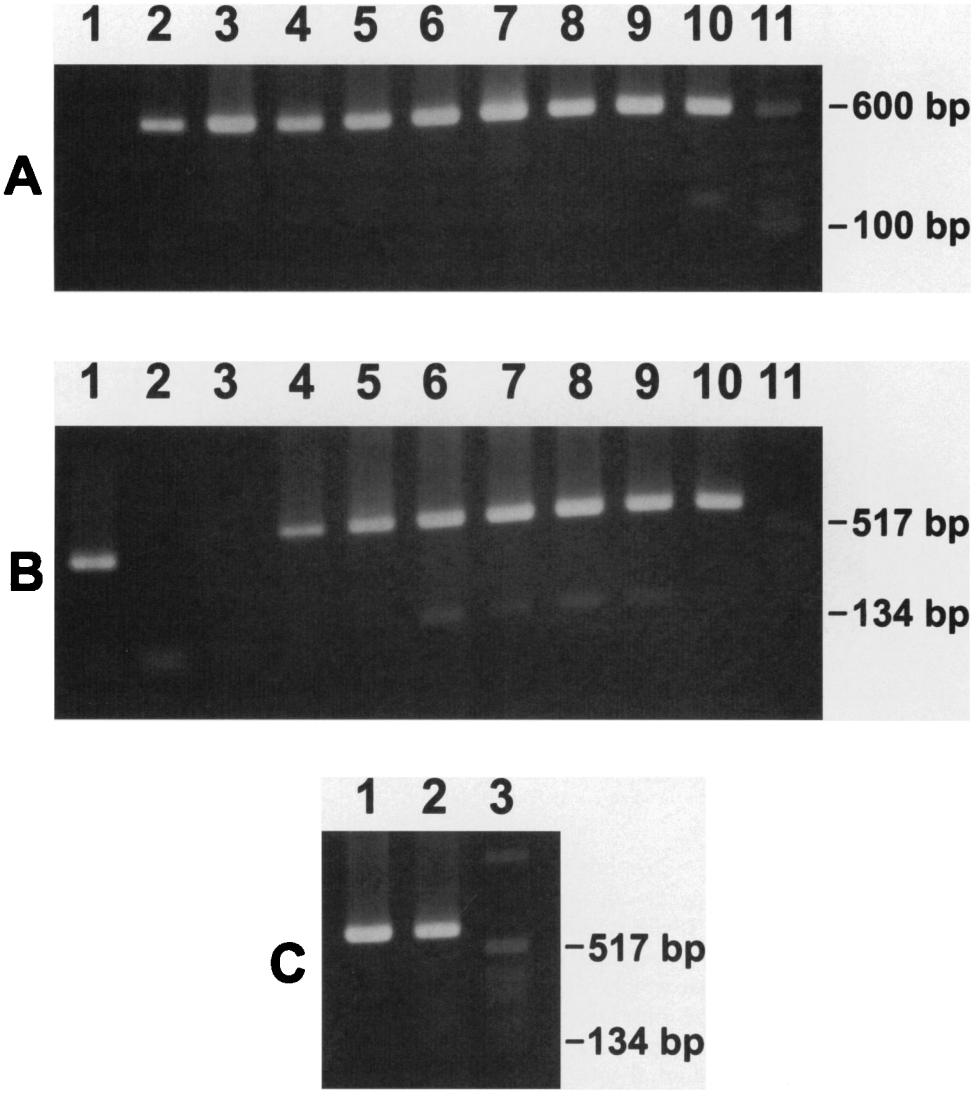

Fig. 2. Comparison of primer set 5 PCR results for pooled and individual tissues from Muscovy ducks where virus was either isolated (A, lanes 3–10; C, lanes 1 and 2) or not isolated (B, lanes 5–10). Muscovy ducks: no. 14245-1 esophagus (A, lane 3), colon (A, lane 4), spleen (B, lane 5), and liver (B, lane 6); no. 14245-2 esophagus (A, lane 5), intestine (A, lane 6), spleen (B, lane 7), and liver (B, lane 8); no. 14245-3 esophagus and proventriculus (A, lane 7), intestine (A, lane 8), colon and cloaca (A, lane 9), spleen (B, lane 9), and liver (B, lane 10); no. 14254-1 tissue pool (Table 2) (A, lane 10). The tissue supernatant results for no. 14245-1 spleen (C, lane 1) and liver (C, lane 2) were included for comparison. A positive lambda template (B, lane 1), a negative lambda template (B, lane 2), a 1-pg DP-VAC DNA (A, lane 2, and B, lane 4), and negative DNA control (A, lane 1, and B, lane 3) were used as controls. A 100-bp DNA ladder was used in A, lane 11, and a 1-kbp DNA ladder was used in B, lane 11, and C, lane 3, as molecular size controls.

er sets (Figs 3,4). Six of the mallard samples (nos. 513, 521, 537, 542, 550, and 553) are presented in Fig. 4. The intensity of the stained PCR product for three of the samples (nos. 521, 550, and 553) was stronger for primer set 7 than 5. The amount of swab material remaining in these samples was insufficient to determine whether the duck plague virus was still viable. The staining intensities of the PCR products for the wood duck cloacal sample and

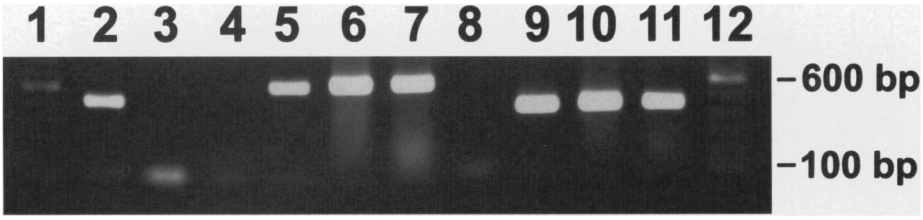

Fig. 3. Comparison of PCR results for primer set 5 cloacal swab (lane 6) and liver (lane 7) and primer set 7 cloacal swab (lane 10) and liver (lane 11) samples from wood duck no. 14794-1. A positive lambda template (lane 2), a negative lambda template (lane 3), a 1-pg DP-VAC DNA (lanes 5 and 9), and negative DNA control (lanes 4 and 8) were used as controls. A 1-kbp DNA ladder was used as a molecular size control (lanes 1 and 12).

liver extract were similar (Fig. 3) and comparable with the tissue amplicons from the other waterfowl (Fig. 2).

DISCUSSION

The duck plague PCR assay has been shown to be a rapid molecular diagnostic method for identifying the presence of virus in tissues from suspect duck plague cases. The PCR was able to detect DNA in tissue samples that did not yield a virus isolate (Fig. 2; Table 2). Viral DNA was detectable by PCR in ground tissue supernatant as easily as in whole processed tissue (Fig. 2). The PCR amplicon from the wood duck cloacal swab extract produced an equally intense gel band when compared with the liver sample (Fig. 3), providing a more convenient sample source without waiting for a necropsy. Both the ground tissue supernatant and the cloacal sample were easier to process and produced a cleaner PCR product, indicating that these samples, especially the cloacal swab, would be preferred over whole tissue extracts for the diagnostic PCR assay.

This PCR assay was clearly superior to virus isolation for diagnosing duck plague in waterfowl. Virus isolates were not recovered from the liver and spleen tissues from three Muscovy ducks that are required to make a traditional diagnosis of duck plague by serologic identification. However, the diagnosis of duck plague positive by PCR was supported by isolation and serologic identification of duck plague virus from other tissues in these same birds (Table 2). The inability to isolate duck plague virus from the tissues of suspect cases is not unusual (9), even though these PCR results indicated clearly that large quantities of duck plague virus were present in those tissues (Fig. 2). For comparison, the amount of DP-VAC DNA used in the PCR positive control reaction was 1 pg, which is approximately 5000 virus particles on the basis of the known size of the duck plague genome (8). Because the extraction methods used for processing these samples target whole virus particles as the method for separating viral DNA from cellular genetic material, the PCR results are indicating that intact virus was present in these tissues (10). However, the viability of these virus particles is unknown. These results indicate that attempts to isolate virus should include tissues other than or in addition to liver or spleen to increase the chances for a successful diagnosis.

The detection of duck plague in swab samples from asymptomatic mallards 16 yr after collection demonstrates the usefulness of the PCR technology for identifying healthy virus carriers after a disease outbreak. These virus-shedding mallards were housed in a pen adjacent to black ducks (*Anas rubripes*) that died in 1981, but they shared a common water pool that was separated in the middle by a fence (NWHC records). Although the duck plague PCR cannot distinguish between a field and a vaccine strain of duck plague virus, no vaccine had ever been used at this site. Therefore, the mallards could not have been shedding the vaccine virus.

The different staining intensities for three of the mallard samples (nos. 521, 550, and 553) suggested that the two primer sets used may have different affinities for the duck plague genome (Fig. 4). Even though the two primer sets have the same sensitivities for DP-VAC DNA (1 fg) in controlled tests, primer set 7 appeared to detect the DNA in the swab samples better

Table 2. Summary of duck plague test results for waterfowl tissues.

Case no.	Die-off location	Species	Bird no.	Tissue	Test results[A]	
					Tissue culture	PCR
14245	Virginia	Muscovy duck	1	Esophagus	+	+
			1	Colon	+	+
			1	Liver	−	+
			1	Spleen	−	+
			1	Liver[B]	−	+
			1	Spleen[B]	−	+
			2	Esophagus	+	+
			2	Intestine	+	+
			2	Liver	−	+
			2	Spleen	−	+
			3	Esophagus/proventriculus	+	+
			3	Intestine	+	+
			3	Colon/cloaca	+	+
			3	Liver	−	+
			3	Spleen	−	+
14254	Pennsylvania	Muscovy duck	1	Tissue pool	+	+
14297	Wisconsin	Muscovy duck	1	Liver/intestine/pharynx/cloaca[B]	+	+
			2	Liver/spleen/esophagus/jejunum/intestine/pharynx[B]	+	+
			3	Liver/spleen/intestine/cloaca[B]	+	+
			4	Liver/spleen/intestine/cloaca[B]	+	+
			5	Liver/spleen/esophagus/cloaca[B]	+	+
14794	Virginia	Wood duck	1	Liver[B]/spleen/esophageal–proventricular junction/cecum/cloaca	+	+[C]
Normal mallard tissue				Liver[B]	−	−
				Spleen[B]	−	−
Normal chicken tissue				Liver[B]	−	−

[A]+ = virus isolation on initial inoculation or identification by PCR; − = virus not isolated or detected by PCR.
[B]Supernatant of tissue suspension was used for PCR.
[C]Only liver was tested by PCR.

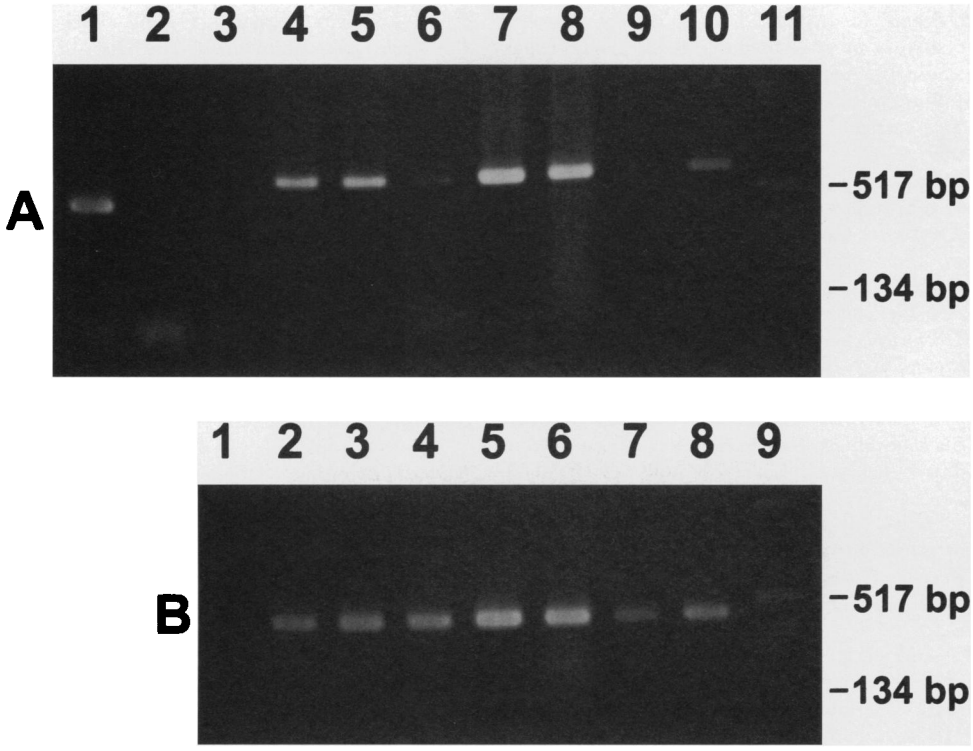

Fig. 4. PCR results of cloacal swab samples. (A) Primer set 5: no. 513 (lane 5), no. 521 (lane 6), no. 537 (lane 7), no. 542 (lane 8), no. 550 (lane 9), and no. 553 (lane 10). (B) Primer set 7: no. 513 (lane 3), no. 521 (lane 4), no. 537 (lane 5), no. 542 (lane 6), no. 550 (lane 7), and no. 553 (lane 8). A positive lambda template (A, lane 1), a negative lambda template (A, lane 2), a 1-pg DP-VAC DNA (A, lane 4, and B, lane 2), and negative DNA control (A, lane 3, and B, lane 1) were used as controls. A 1-kbp DNA ladder was used as a molecular size control (A, lane 11, and B, lane 9).

than primer set 5. Products from primer set 7 were consistently more intense than those from primer set 5. This difference probably reflects the smaller and more variable number of viral genome templates available in some samples and that primer set 7 was better optimized for amplification than primer set 5 under the PCR conditions used. The 13 samples tested were the only ones remaining from the original 18 with sufficient material to test by PCR but insufficient to repeat virus isolation attempts. Duck plague virus had been previously isolated and identified serologically from all of these samples (2). Which swab sample, the cloacal, the oral-pharyngeal, or both, contributed the virus identified is unknown. However, the use of the swab samples for PCR assays, whether from a carcass or living bird, simplifies field collection methods.

The duck plague PCR does not detect other avian herpesviruses (10), and no other waterfowl herpesvirus is known that could confuse the detection of duck plague virus. Because of the range of samples that can be rapidly tested, the diagnostic PCR assay also provides a new research tool for studying the epizootiology of duck plague under natural conditions. Work is in progress to survey captive-raised and free-flying nonmigratory waterfowl in suspected disease endemic areas for the presence of duck plague virus by PCR.

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