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Detection and Isolation of Exotic Newcastle Disease Virus from Field-Collected Flies

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ABSTRACT Flies were collected by sweep net from the vicinity of two small groups of "backyard" poultry (10–20 chickens per group) that had been identified as infected with exotic Newcastle disease virus (family *Paramyxoviridae*, genus *avulavirus*, ENDV) in Los Angeles County, CA, during the 2002–2003 END outbreak. Collected flies were subdivided into pools and homogenized in brain-heart infusion broth with antibiotics. The separated supernatant was tested for the presence of ENDV by inoculation into embryonated chicken eggs. Exotic Newcastle disease virus was isolated from pools of *Phaenicia cuprina* (Wiedemann), *Fannia canicularis* (L.), and *Musca domestica* L., and it was identified by hemagglutination inhibition with Newcastle disease virus antiserum. Viral concentration in positive pools was low (<1 egg infectious dose$_{50}$ per fly). Isolated virus demonstrated identical monoclonal antibody binding profiles as well as 99% sequence homology in the 635-bp fusion gene sequence compared with ENDV recovered from infected commercial egg layer poultry during the 2002 outbreak.

KEY WORDS flies, Newcastle disease virus, poultry, chickens, mechanical vector

Exotic Newcastle disease (END) is a contagious and fatal viral disease affecting the respiratory, nervous, and digestive systems, of poultry and other birds. END is so virulent that many birds may die without ever showing clinical signs of illness. There is a near 100% mortality in unvaccinated poultry, and even poultry vaccinated against the endemic low-virulence Newcastle disease virus strains are not adequately protected against END virus (family *Paramyxoviridae*, genus *avulavirus*, ENDV) (USDA–APHIS 2006).

Newcastle disease control in poultry has been ongoing in the United States since the first infections were identified in the early 1940s. Introductions of the more virulent strains of Newcastle disease virus, the cause of END, have occurred, and in most cases, the virus has been quickly eradicated (Utterback and Schwartz 1973). During two END outbreaks, virus was not quickly eradicated. The first occurred during 1971–1973, and this outbreak resulted in the quarantine of eight California counties, the destruction of 11.9 million birds, and eradication costs of $56 million (USDA–APHIS 1978). The second occurred during 2002–2003, and it resulted in the quarantine of 18,345 premises, the destruction of 3.2 million birds, and

eradication efforts cost of $170 million (Breitmeyer et al. 2003).

ENDV is primarily spread by direct contact between infected and healthy birds. However, it also can be transmitted indirectly via contaminated equipment and persons (Utterback and Schwartz 1973, Burridge et al. 1975). During the 1971–1973 California END outbreak, Rogoff et al. (1975) isolated virus from pools of *Fannia* spp. collected at commercial poultry operations with ENDV infected birds, implicating this species as a possible vector of ENDV.

Many insects, especially flies, are commonly associated with poultry operations, and they are known to disperse into surrounding areas (Lysyk and Axtell 1986, Axtell 1999, Sawabe et al. 2006). Wet manure is an excellent breeding environment for several fly species, and it also likely to support the survival of ENDV (Kinde et al. 2004). Flies are capable of harboring a diverse range of animal and human pathogens (Calibeo-Hayes et al. 2003, Graczyk et al. 2005, Sawabe et al. 2006), and they may be involved in transmission of these pathogens to otherwise healthy hosts (Shane et al. 1985, Calibeo-Hayes et al. 2003, Ahmad et al. 2007). However, the extent to which insects may be involved in the dispersal and transmission of ENDV to uninfected birds remains unknown.

In this study, fly species collected at two private homes with ENDV-infected poultry in Los Angeles County, CA, were examined for the presence of ENDV. Isolated ENDV was characterized by monoclonal antibody binding profile and sequence analysis of the fusion gene.

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Materials and Methods

Flies were captured by sweep net from the immediate vicinity of two private homes in Los Angeles Co., southern California. Both locations, one location in the city of Compton (home A), and the other location in Inglewood (home B), contained chicken flocks (<20 birds) that had been identified by the California Department of Food and Agriculture (CDFA) as having one or more ENDV-infected birds within the preceding 5 d. Flies were collected before poultry were euthanized and removed from the private homes by the END Task Force (a joint United States Department of Agriculture and CDFA organization) responsible for efforts to eradicate END in California. Fly collectors followed all biosecurity precautions recommended by the CDFA while sampling at these homes. Multiple 5-min sweep collections were made at each home, taking care not to touch poultry cages or the ground with the net while sweeping. Sweep net collections continued at each home until flies were no longer readily captured. Captured insects were killed on dry ice, placed into labeled vials by using sterilized forceps, and held on dry ice until they could be returned to the laboratory to be identified and counted. Sweep nets and forceps were sterilized between each 5-min collection by submergence in 10% Lysol (Reckitt Benckiser Inc., Wayne, NJ) after which nets were allowed to dry before reuse.

Flies were kept frozen on dry ice during identification in a biological safety cabinet, and then they were pooled into groups of five or fewer flies of the same species from the same home. Fly pools were stored at −80°C until shipment on dry ice to the USDA Southeast Poultry Research Laboratory (SEPRL), in Athens, GA, where they were again placed at −80°C until tested for the presence of ENDV. Pools were homogenized in 1.5 ml of brain-heart infusion (BHI) broth with antibiotics (200 μg of gentamicin/ml, 2000 U of penicillin/ml, and 4 μg of amphotericin B/ml, Sigma-Aldrich, St. Louis, MO) by using a tissue grinder with sterile plastic pestles in microfuge tubes and centrifuged at 16,000 × g for 10 min. Virus isolation was performed by inoculating 100 μl of the supernatant into the allantois of each of three 9- or 10-d-old embryonated chicken eggs (ECE). Eggs were incubated at 37°C in a standard humidified incubator. The embryonated chicken eggs were obtained from the SEPRL specific-pathogen-free White Leghorn flock. Eggs were candled to determine embryo death each 24 h through 7 d postinoculation. Embryos that died within the first 24 h were discarded. Embryos that died between 24 h and 7 d as well as all survivors at 7 d were chilled at 4°C. Amnio-allantoic fluid (AAF) harvested from chilled eggs was tested for hemagglutination (HA) activity to detect ENDV. Virus presence in HA-positive samples was confirmed by hemagglutination-inhibition (HI) with Newcastle disease virus (family *Paramyxoviridae,* genus *Avulavirus,* NDV)-specific antiserum (King 1996a). Amnio-allantoic fluids from HA negative dead embryos and embryos alive at 7 d postinoculation were subjected to a second serial pas-

sage by inoculation of 100 μl of the AAF into each of three additional embryonated chicken eggs. Eggs were candled, and killed embryos were handled as before. If by day 7 postinoculation there was no HA activity in the AAF of the second passage dead or surviving embryos, the specimen was regarded as negative for ENDV.

A monoclonal antibody profile was determined for each isolated ENDV using nine monoclonal antibodies (mAbs) with different Newcastle disease virus HI specificities by using previously described methods (King 1996a, Kommers et al. 2001). The mAbs included 15C4, AVS, B79, 161/167, P11C9, P3A11, 10D11, P15D7, and P10B8 (Kommers et al. 2003).

RNA was extracted from AAF by using TRIzol LS according to manufacturer's instructions (Invitrogen, Carlsbad, CA); 750 μl of TRIzol LS reagent was added to 250 μl of allantoic fluid. The fluid was vortexed and incubated at room temperature for 7 min. RNA was separated into the aqueous phase with the addition of 200 μl of chloroform, followed by precipitation with isopropanol. After one wash with 70% ethanol, RNA was dried and resuspended in RNase-free water. The 5′ end of the ENDV fusion gene was amplified by polymerase chain reaction (PCR) and sequenced using primers targeting a 635-bp fragment that included the fusion protein cleavage site (forward primer, 5′-GAG GTT ACC TCY ACY AAG CTR GAG A-3′; reverse primer, 5′-TCA TTA ACA AAY TGC TGC ATC TTC CCW AC-3′). These primers amplify the NDV genomic region between positions 4317 and 5084. Standard 50 μl reverse transcription (RT)-PCR reactions were carried out using a kit (SuperScript III One Step RT-PCR, Invitrogen) with annealing temperature at 56°C. PCR-amplified samples were separated on a 1% agarose gel, and the bands were excised and eluted using the QuickClean 5M gel extraction kit (GenScript Corp., Piscataway, NJ). Once the PCR products were cleaned, samples were quantified using a standard spectrophotomer and sequenced. All double-stranded nucleotide-sequencing reactions were performed with fluorescent dideoxynucleotide terminators in an automated sequencer (ABI 3700 automated sequencer, Applied Biosystems, Foster City, CA). Nucleotide sequence editing and analysis were conducted with the LaserGene sequence analysis software package (LaserGene, version 5.07, DNAStar, Inc., Madison, WI). The virus was sequenced and compared by Blast analysis (Altschul et al. 1990) to an END viral sequence [chicken/U.S.(AZ)/236498/03] obtained from commercial poultry infected with ENDV during the 2002 outbreak. It also was compared with other available GenBank ENDV sequences. Genomic sequences from viruses recovered from flies at each collection site were deposited in GenBank (accession nos. EF424375 and EF424376).

Results

Overall, 87 flies composing nine species were collected from the area surrounding the two private homes housing ENDV-infected birds (Table 1). Most

Table 1. Detection of ENDV from flies collected at two homes (A and B) in Los Angeles County with ENDV-infected poultry during 2002–2003 ENDV outbreak

Fly species	No. flies per species		No. pools per species		Positive pools	
	A	B	A	B	A	B
Calliphora spp.	1		1			
Fannia canicularis	10	10	2	2		1 (n = 5)
Fannia femoralis	1		1			
Musca domestica	8	15	2	3		1 (n = 5)
Muscina stabulans	2	1	1	1		
Phaenicia sericata	2		1			
Phaenicia cuprina	14	21	3	5	2 (n = 10)	1 (n = 5)
Phormia regina	1		1			
Stomoxys calcitrans	1		1			

of the collected flies were *Phaenicia cuprina* (Wiedemann), *Musca domestica* L., or *Fannia canicularis* (L.). There were 24 fly pools representing groups of ≤5 flies of the same species from the same home. Five pools of flies contained ENDV; three pools of *P. cuprina* (two pools from home A and the other pools from home B) and one each of *F. canicularis* and *M. domestica* (both from home B). The amount of virus recovered from each of the fly pools was very low (<1 egg infectious dose [EID]$_{50}$ per fly) and required a second serial passage in ECE for detection.

The mAb binding profile (Table 2) of the virus isolated from each of the fly pools was identical to an ENDV strain obtained from infected commercial egg-layer poultry during the 2002–2003 END outbreak [chicken/U.S.(CA)/12430/02] and different than the profile of the heterologous NDV B1 strain included as a control. The NDV B1 strain is commonly used as a live virus vaccine in commercial poultry. The partial sequence of the fusion gene of the virus isolated from the flies showed 99% homology (634/635 bp) to a 2002–2003 ENDV isolate [chicken/U.S.(AZ)/236498/03] and 87% homology (552/635 bp) to a 1971–1973 ENDV isolate [chicken/U.S.(CA)/1083(Fontana)/72], both obtained from commercial egg-layer poultry during ENDV outbreaks. At the amino acid level, the partial sequence of the fly isolate was 100% identical to the 2002–2003 outbreak strain and 92% identical to the 1971–1973 outbreak strain. Viruses recovered from flies at both collection sites were 100% identical to each other.

Discussion

Although all fly species were collected in small numbers, ENDV was isolated from the three most abundant species collected during this study. This is the first report of ENDV isolated from field-collected *P. cuprina* and *M. domestica*; ENDV had previously been isolated from *F. canicularis* and *Fannia femoralis* (Stein) from a commercial poultry operation during the 1971 END outbreak (Rogoff et al. 1975). Given the small numbers of flies collected, the prevalence of ENDV-infected flies was high with ≈30% of the pools containing the three species mentioned above having one or more flies per pool infected with virus. The virus prevalence in *Fannia* spp. collected from commercial poultry operations during the 1971 END outbreak was far lower with only two of 78 pools of *Fannia* spp. (3,926 total flies) collected before or within 2 d of poultry removal containing ENDV, and no virus was recovered from other common fly species (Rogoff et al. 1975). It is unclear to what extent Rogoff et al. (1975) may have collected flies from poultry facilities where poultry or even manure had already been removed from the site, perhaps resulting in the much lower ENDV infection prevalence in flies relative to this study. The higher ENDV prevalence in this study also may be due to differences between commercial poultry and backyard poultry in animal housing, manure handling, or vaccination status of the poultry against Newcastle disease virus. Backyard poultry are often housed together on the ground or in wooden boxes, giving them greater access to an infected bird and its manure relative to commercial egg-layer poultry that are separated into cages suspended above the ground where the manure is allowed to accumulate. Additionally, backyard poultry are usually not vaccinated against Newcastle disease virus and infected

Table 2. Hemagglutination-inhibition test results of ENDV isolated from field-collected flies against NDV-specific mAbs

Viral isolate	mAbs								
	15C4	AVS	B79	161/617	P11C9	P3A11	10D11	P15D7	P10B8
P. cuprina (A1)	+	−	+	−	+	+	−	+	+
P. cuprina (A2)	+	−	+	−	+	+	−	+	+
P. cuprina (B1)	+	−	+	−	+	+	−	+	+
F. canicularis (B1)	+	−	+	−	+	+	−	+	+
M. domestica (B1)	+	−	+	−	+	+	−	+	+
CK/CA/12430/02 (outbreak strain)	+	−	+	−	+	+	−	+	+
CK/US/B1/48 (vaccine strain)	+	+	+	−	+	+	−	+	−

Antibody-inhibited HA (+); no HA inhibition (−). 15C4 inhibits all NDV but pigeon PMV-1. AVS inhibits lentogenic strains like the B1 and LaSota ND vaccine virus. B79 inhibits most NDV including most pigeon PMV-1. 161/617 inhibits only pigeon PMV-1. P11C9, P3A11, P15D7, and P10B8 inhibit various NDV strains. 10D11 inhibits neurotropic NDV mesogens and velogens such as virulent Texas GB. CK/CA/12430/02 is an ENDV strain recovered from commercial poultry during the 2002–2003 outbreak in California. CK/US/B1/48 is a strain widely used as a Newcastle disease vaccine in the United States. The antibody inhibition profile of the viruses tested indicates the ENDV isolates from three fly species at premises A and B are antigenically similar as well as being similar to the CK/CA/12430/02 ENDV isolate from chickens but are different than the vaccine strain B1.

birds would be expected to shed a greater concentration of ENDV in their manure relative to vaccinated commercial poultry (Kapczynski and King 2005).

The mAb profile and sequence analysis of the virus recovered from each of the fly pools indicated that these viruses were the same ENDV strain circulating in commercial and backyard poultry during this same period. Viruses recovered during 2002–2003 were genotypically different from viruses recovered during the earlier 1971–1973 ENDV outbreak (genotypes V and VI, respectively) (Czegledi et al. 2006) with virus isolates from the 2002–2003 ENDV outbreak being most similar to ENDV isolated from chickens in Mexico during 1998–2000 (Pedersen et al. 2004).

Flies are thus contacting infectious ENDV in the environment and are capable of harboring at least low levels of this virus. The ENDV infective dose for a chicken is reported to range from $\approx 10^{3.0}$ (King 1996b) to $10^{4.0}$ EID_{50} (Alexander et al. 2006), far greater than the <1 EID_{50} per fly found in our field-collected fly pools. However, given the small number of flies collected, it would be premature to assume flies are not capable of carrying substantially higher viral loads. It is entirely possible that these flies had contacted a source of ENDV several days before our sampling and the low viral load represented residual virus still associated with the flies at the time of their capture.

The mechanism by which the flies might be acquiring virus has not been determined. It has been shown that ENDV-infected poultry shed a considerable amount of virus in their feces (Kapczynski and King 2005) and that virus can persist in the manure for up to 16 d (Kinde et al. 2004). Wet manure provides an excellent developmental environment for several fly species as well as a substrate on which adult flies feed. Manure also provides an excellent media for maintaining and transmitting infectious agents to flies as the viscosity of the manure enhances manure attachment to fly tarsi and cuticular bristles (Tan et al. 1997). In addition, flies feeding on bacteria in the manure would be expected to consume some amount of the infectious agent. Further studies are needed to determine whether ENDV is associated with these fly species simply as an external contaminant or if the virus is protected or even amplified within the gut or other tissues of the fly.

Shane et al. (1985) demonstrated the potential for house flies to transmit pathogens by placing flies into cages with *Campylobacter jejuni*-infected chickens and subsequently moving the flies to cages with pathogen-free chickens, resulting in *C. jejuni* infection in the previously uninfected chickens. Similarly, turkey poults housed in isolation units became infected with turkey corona virus (family *Coronaviridae*, genus *Coronavirus*, TCV) when house flies orally inoculated with TCV were released into the isolation units for 24 h (Calibeo-Hayes et al. 2003), and cattle held in confined pens became infected with *Escherichia coli* O157:H7 after house flies orally inoculated with the bacteria were released into the pens for 48 h (Ahmad et al. 2007). However, the actual mechanism of bacterial transmission from the flies to the chickens or cattle in the studies mentioned above remains unknown.

Poultry are known to consume adult flies in laboratory studies (Calibeo-Hayes et al. 2003) as well as in the field (A.C.G., unpublished observation). Additionally, flies may regurgitate and defecate virus while feeding or resting on the surface of foods (Greenberg 1973). It is possible that chickens and other birds can consume virus with recently infected flies or contaminated fly feces and regurgitated matter (Sawabe et al. 2006). The consumption of many adult flies in a short period might result in infection even if the viral load of each individual fly is lower than the required infectious dose.

Fly larvae developing in infected manure also might serve as a source of infection when consumed by poultry having access to the manure (typical of backyard poultry and some commercial poultry), although this has not been evaluated. Additional studies are required to evaluate the vector competence of flies and other poultry associated arthropods.

Dispersal of infectious virus by flies moving from premises with infected poultry to nearby premises with uninfected poultry is an important biosecurity concern for END and other avian diseases. Greenberg (1973) reported that house flies were capable of flights ranging from 2.3 to 11.8 km within 24 h. Although fly involvement in virus movement between commercial poultry facilities is probably minor relative to other means of virus movement (e.g., on personnel, shared equipment, or manure hauling vehicles), fly involvement in virus movement among poultry houses at a single commercial facility or among backyard poultry may be significant given the short distances flies would need to move between infected and uninfected poultry. Backyard poultry are geographically clumped in southern California with small numbers of poultry at many adjacent homes and only a few meters often separating poultry at one home from the next. During the 2002–2003 END outbreak, homes with ENDV-infected poultry were often very close to each other, forming a cluster that was distinct within the somewhat larger geographic clumping of backyard poultry (C.J.C., unpublished data). In addition to fly dispersal, movement of birds or personnel between these clustered homes also may have occurred.

Although this study has not shown flies to be competent vectors of ENDV, a conservative approach would indicate that biosecurity measures should include fly control on and near premises with END-infected poultry as also suggested by Bram et al. (1974). Fly control measures would be especially important for a quarantined poultry facility (to include backyard flocks) before removal of birds and their manure by a state and/or federal task force as part of the disease eradication effort. Removal of birds and manure would be expected to encourage fly dispersal into the surrounding area. It is recommended that future END task force organizations consider the use of insecticides providing rapid knockdown of adult flies immediately before initiating eradication efforts at commercial and backyard poultry facilities and that

control measures continue throughout the period of manure removal and site disinfection.

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