Experimental Evaluation of *Musca domestica* (Diptera: Muscidae) as a Vector of Newcastle Disease Virus

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J. Med. Entomol. 44(4): 666–671 (2007)

ABSTRACT House flies, *Musca domestica* L. (Diptera: Muscidae), were examined for their ability to harbor and transmit Newcastle disease virus (family *Paramyxoviridae*, genus *Avulavirus*, NDV) by using a mesogenic NDV strain. Laboratory-reared flies were experimentally exposed to NDV (Roakin strain) by allowing flies to imbibe an inoculum consisting of chicken embryo-propagated virus. NDV was detected in dissected crops and intestinal tissues from exposed flies for up to 96 and 24 h postexposure, respectively; no virus was detected in crops and intestines of sham-exposed flies. The potential of the house fly to directly transmit NDV to live chickens was examined by placing 14-d-old chickens in contact with NDV-exposed house flies 2 h after flies consumed NDV inoculum. NDV-exposed house flies contained $\approx 10^4$ 50% infectious doses (ID_{50}) per fly, but no transmission of NDV was observed in chickens placed in contact with exposed flies at densities as high as 25 flies per bird. Subsequent dose–response studies demonstrated that oral exposure, the most likely route for fly-to-chicken transmission, required an NDV (Roakin) dose $\geq 10^6$ ID_{50}. These results indicate that house flies are capable of harboring NDV (Roakin) but that they are poor vectors of the virus because they carry an insufficient virus titer to cause infection.

KEY WORDS Newcastle disease, house fly, disease vector, poultry

Newcastle disease (ND) is an emerging disease affecting the poultry industry worldwide. The nature of the international poultry industry facilitates the movement of poultry products, equipment, and people and the subsequent spread of disease. To monitor outbreaks and its potential spread, ND is included on the A-list of reportable diseases maintained by the Office International Epizooties (OIE 2006). In 2006, 15 countries reported active or resolved outbreaks of ND within their borders (OIE 2006). Recently, >117,000 chickens were destroyed on commercial broiler farms in Romania as a result of this disease (OIE 2006). In North America, ND was discovered in a California backyard chicken flock in fall 2002 (APHIS 2003). Within 2 mo, the disease had spread to area commercial poultry operations. Subsequently, Newcastle disease virus (family *Paramyxoviridae*, genus *Avulavirus*, NDV) showed up in Nevada, Arizona, New Mexico, and Texas. More >3 million birds were destroyed to keep the disease from spreading further with industry losses estimated at $5 billion (Anonymous 2003).

ND is an enveloped, negative-stranded RNA viral infection caused by avian paramyxovirus. Infected birds exhibit a variety of symptoms, including respiratory discomfort, sneezing, nasal discharge, coughing; nervous depression, drooping wings, tremors, paralysis, and gastrointestinal pathology, including greenish watery diarrhea (Alexander 1991, APHIS 2003). Migrating and exotic birds may be asymptomatic carriers and pose a risk to commercial flocks. The highly contagious and fatal disease is spread through direct contact with infected birds, their feces, and secretions (Alexander 1991). High virus titers in feces and secretions increase the potential of mechanical transmission of ND. Disease may be spread by exotic and feral birds, animals, poultry products, airborne, feed, water, and vaccines. Humans contribute significantly to the spread of ND, especially poultry workers, cleanout crews, rendering staff, feed delivery personnel, egg handlers, farmers, and vaccination crews (Alexander 1991).

The use of mesogenic and lentogenic live vaccines is common in vaccination programs (Alexander 1991). Lentogenic vaccines are generally reserved as primary vaccines and more virulent mesogenic vaccines are used as a secondary vaccine. The field-derived Roakin strain is a mesogenic vaccine. Roakin live vaccine may be administered by intramuscular or wing web inoculation (Alexander 1991). Vaccinated birds are not fully protected by immunity to ND, but mortality rates are lower and birds do not experience severe consequences of disease in the absence of vaccines.

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Synanthropic flies are thought to have contributed to the transmission and maintenance of ND in California in the 1970s, and fly management was considered a factor in eradication (Bram et al. 1974). The little house fly, *Fannia canicularis* (L.), was incriminated in the transmission of ND in the California outbreak (Rogoff et al. 1975). Seven species of flies [*Musca domestica* L., *F. canicularis*, *Fannia femoralis* (Stein), *Muscina stabulans* (Fallen), *Stomoxys calcitrans* L., *Ophyra* (*Hydrotaea*) spp., and *Phaenicia* spp.] were captured on ND-positive farms. Collections were made <2 d and >2 d of depopulation. NDV was found in two pools of *F. canicularis* and one pool of *F. femoralis* (Rogoff et al. 1975). Interestingly, house fly pools were negative for NDV in this study. *F. canicularis* was proven a vector of NDV in subsequent laboratory studies (Rogoff et al. 1977). Unfortunately, the proposed evaluation of the house fly under similar laboratory conditions was never completed. The only report of the house fly being involved in the transmission of NDV was published in Russia (Milushev et al. 1977). Under laboratory conditions, the house fly carried virus on the body and was capable of transmitting NDV virus up to 96 h.

The feeding habits of little house fly and house fly are similar. Adult house flies feed on semisolid and liquid diets, often in the form of manure and excreted bodily fluids. The adult fly, using sponging mouthparts, consumes these fluids that are stored temporally in the insect crop. The crop holds ≈2.3 ± 0.03 μl of fluid (unpublished data). Generally, quantities of crop fluids are mixed with semisolid foods that pass into the insect midgut over 5 h for digestion (Hainsworth et al. 1990). The fly may use crop fluids to help liquefy food solids before ingestion and regurgitate crop contents onto foods and surfaces producing "fly specks" (West 1951). Similarly, flies frequently defecate while feeding and resting on surfaces. As a result of such feeding habits, the house fly have been implicated in the transmission of >30 bacterial, protozoan, and viral diseases (Greenberg 1973, Graczyk et al. 1999, Gough and Jorgenson 1983, Calibeo-Hayes et al. 2003). With flights up to 12 km within 24 h (Greenberg 1973), house flies are capable of disseminating pathogens within and between poultry houses (Lysyk and Axtell 1986).

Our goal was to determine the potential role of the house fly as a vector of NDV by using the mesogenic Roakin strain of the virus. Specifically, our objectives were to 1) establish whether NDV (Roakin) survives in the crop and intestinal tract of the house fly through a series of time course experiments; 2) examine the longevity of NDV in fly tissues; 3) determine whether NDV replicated in these tissues, increasing the potential risk of fly transmission; and 4) determine whether NDV-exposed house flies were capable of transmitting disease to naïve chicks.

Materials and Methods

ND Virus Culture. NDV (Roakin strain) was obtained from Dr. D. J. King, Southeast Poultry Research Laboratory, Athens, GA. The virus was propagated by inoculation of 10-d-old embryonated chicken eggs by the allantoic route, 0.1 ml per egg (SPAFAS, Charles River Laboratories Inc., Wilmington, MA). Allantoic fluid was harvested from eggs that died 3–4 d postinoculation. Titration of the virus was accomplished by preparation of 10-fold dilutions of allantoic fluid in Dulbecco's minimal essential medium (DMEM) and inoculation of 0.2 ml of each dilution onto confluent monolayers of chicken embryo fibroblast (CEF) cells in 48-well tissue culture plates, four wells per dilution. Five days after inoculation, contents of wells were decanted into a container containing sodium hypochlorite to inactivate live virus, and cells were stained with 0.1% crystal violet. Titer was determined by calculation of the 50% infectious doses (ID_{50}) as described by Reed and Muench (1938). An inoculum was prepared to contain $\approx 10^7$ ID_{50}/0.1 ml, and it was stored at −70°C.

NDV Serology. Serum samples were assayed for presence of NDV-specific antibody by using a hemagglutination inhibition test as described previously (Beard 1989).

House Fly Culture. House flies used in this study have been maintained in culture at North Carolina State University since 1997. The colony was established from wild-captured flies from a dairy. Collected house fly eggs were added to a prepared house fly larval medium. Fly larvae were reared in sweater boxes at constant temperature (27°C) until pupation. The fly pupae were harvested by flotation 8 d after egg hatch. House fly pupae were stored at room temperature until adult emergence 3 d later. All flies emerging within a 24-h period represent day 0. Adult flies were provided food and water. Once the flies were 3–5 d old, they were separated for use in these experiments.

Chickens. Fertile specific-pathogen-free (SPF) chicken eggs were obtained from SPAFAS, Charles River Laboratories, Inc. Eggs were incubated and hatched at North Carolina State University. One-day-old chickens were housed in electrically heated brooders in an isolation room until chickens were 2 wk of age. Chickens were provided nonmedicated game bird starter and water ad libitum.

Experimental Design. Four experiments were conducted to examine the survival of NDV in the digestive tract tissues, crops, and intestines (mid- and hindgut) of flies after exposure to NDV (Roakin) and the ability of NDV-exposed flies to transmit the virus to susceptible chicks.

Adult flies of mixed sex were separated into two groups of 350 flies each. Adult flies were held for 18 h at 22°C in screened containers without food or water. Flies were anesthetized with cold by placement in a −20°C freezer for 5 min. Small plastic reservoirs containing ≈2,000 μl of NDV (Roakin) inoculum was placed in each fly cage. The control group ($n = 350$) was administered DMEM without NDV. The flies were given 30 min to fully recover from the cold and to consume the treated and untreated medium.

Experiment 1. Survival of NDV in the fly crop, a storage site for liquids before passage to the midgut,

was evaluated. After ingestion of the medium, each treatment group was provided food and water and held at room temperature (≈22°C). At 0.5, 1, 3, 6, 9, 12, 24, and 96 h postfeeding, the flies were temporarily chilled, and 40 flies were removed from each treatment group. These flies were placed in sterile petri dishes, the dish was labeled for treatment and time interval, and frozen at −70°C.

Frozen flies were surface sterilized by immersing the flies in 95% ethanol (EtOH), transferred to 10% bleach (NaOCl) a minimum of 3 min, and rinsed in distilled sterile water. Whole crops ($n = 40$) were aseptically removed from the flies, pooled, and placed in 2 ml of chilled DMEM plus 1% fetal bovine serum, 1% gentamicin, and amphotericin B. Dissection instruments were sterilized in dilute bleach and EtOH after each dissection. Pooled fly crops were frozen at −70°C. The experiment was replicated three times.

Pooled fly crops in DMEM with antibiotics were homogenized and clarified by centrifugation at 1500 × *g* for 20 min at 4°C. Clarified homogenates were prepared as 10-fold dilutions and then assayed for NDV as described above. Presence of virus in samples was determined by titration in CEF cells as described above.

Experiment 2. Survival of NDV in house fly intestines was evaluated. A time-delineated experiment was conducted to examine the presence of NDV in the digestive tract of the house fly. Flies were separated into two groups of 350 flies and administered NDV as described above. Groups of 40 flies were examined for the presence of virus at 0.5, 1, 3, 6, 9, 12, 24, and 96 h postfeeding. Target tissues for experiment 2 were fly mid- and hindgut. After surface sterilization, the terminal abdominal plate was excised and the intestinal tract was gently pulled through the opening. Separating at the proventriculus, the mid- and hindgut tissues remained intact. If gut or crop was compromised, the fly was rejected. Gut tissues were excised from flies ($n = 40$), pooled, and placed in 2 ml of chilled DMEM supplemented with antibiotics. Pooled fly gut tissues were stored frozen at −70°C. Gut tissues were homogenized and assayed for the presence of virus as described above.

For analysis, Mean NDV titers were calculated from each replicate time interval for crop and gut tissue. We used correlation to test the relationship between live virus titer and time. Regression analysis was used to plot the relationship of titer decay against time. The slope of the curve was calculated using a logarithmic regression model (Minitab, Inc. 1997).

Experiment 3. The potential of the house fly to directly transmit NDV to live chicks was investigated. Fourteen-day-old chickens were divided into groups of 10 birds and placed in Horsfall isolation units with negative-pressure ventilation. Chicks were exposed to fly densities of 10, 100, and 250 house flies per isolation unit, and a no fly control for four treatments and replicated twice within experiment and the experiment was conducted twice.

Approximately 800 adult house flies (3–5 d old) were divided into three feeding chambers, each holding ≈265 flies. Starved flies were provided NDV-inoculated medium in a reservoir as described above. After the inoculum was consumed, the flies were cold anesthetized and further sorted into two groups of 10 flies, two groups of 100 flies, and two groups of 250 flies. Two hours postfeeding, the flies were released in the isolation chambers, and the bird behavior was observed. One day after the flies were released in the brooders, the fresh air intake was sprayed with 0.30% pyrethrin insecticide synergized with 2.40% piperonyl butoxide (CB-38 ExtraTM, Waterbury Companies Inc., Waterbury, CT) to kill the flies. Dead flies were removed from the chambers.

A portion of the NDV-exposed flies was held in reserve to determine NDV titers in whole flies. Immediately after placement of other NDV-exposed flies in Horsfal units, these flies were counted, pooled, homogenized, clarified by centrifugation, and NDV titers were determined as described above. Mean NDV titers per fly were determined by dividing the titer for pooled flies by total number of flies within the homogenate.

The birds were observed daily for clinical signs of infection. At 3-d postexposure, tracheal swabs were collected from each of five birds in each group. Swabs were placed in 1 ml of DMEM, placed on ice, and stored at −70°C. Presence of NDV in tracheal swabs was determined by inoculation of chicken embryo fibroblasts as described above. Chicks were bled for NDV serology on day 21 and euthanatized.

Experiment 4. Dose–response study to evaluate effect of dose and inoculation site for NDV (Roakin) infectivity. Fourteen-day-old chickens were divided into eight groups of 10 birds each and placed in separate Horsfall isolation units. Chicks were intranasally inoculated by squirting 100 μl containing a 10^2, 10^3, and 10^4 ID_{50} of NDV (Roakin) into the left nasal passage. Chicks were inoculated orally by passing a no. 5 French catheter into the crop and delivering 1.0 ml containing the calculated dose of 10^4, 10^6, and 10^8 infectious units NDV (Roakin). Two control groups of 10 birds were sham inoculated. Birds were observed daily for clinical signs of disease. At day 21 postexposure, blood was collected from six birds in each group; all birds then were humanely euthanatized. Serum was evaluated by hemagglutination inhibition assays for presence of NDV-specific antibodies. The experiment was conducted once.

Results

Experiment 1. Adult house flies harbored Newcastle Disease virus in both crop and gut tissues. Live NDV was detected in crops tissues of house flies for 96 h postfeeding in two of three replicated experiments (Table 1). In the one replicate, NDV was detectable at 24 h postexposure, but not at 96 h. Virus titers were greatest within 30 min of a single feeding of the flies. NDV titer decay was predictably time dependent from 0.5 to 24 h following the logistic model [$y = -0.4306\ln(x) + 2.0187$; $R^2 = 0.7329$] (Fig. 1). Virus titers would be expected to remain stable or

Table 1. NDV titers (log_{10} ID_{50}/0.2 ml specimen) in pooled crops of flies (n = 40) after experimental exposure to virus containing media[a]

Replicate	Postexposure (h)							
	0.5	1	3	6	9	12	24	96
1	3.5	1.3	1.5	<1[b]	<1	<1	<1	<1
2	2.4	1.0	<1	<1	<1	<1	<1	0[c]
3	2.8	2.4	1.5	<1	<1	<1	<1	<1

[a] NDV was not detected in the tissues of sham-exposed flies collected at the same time intervals.
[b] Virus detected only after second passage in cell culture.
[c] Virus not detected.

Table 2. NDV titers (log_{10} ID_{50}/0.2 ml specimen) in pooled gut tissues of flies (n = 40) after experimental exposure to virus containing media[a]

Replicate	Postexposure (h)							
	0.5	1	3	6	9	12	24	96
1	5.4	5.4	5.3	2.3	<1[b]	0	<1	0[c]
2	7.0	5.3	4.0	2.0	<1	<1	<1	0
3	3.0	2.5	2.0	1.5	<1	<1	<1	0

[a] NDV was not detected in the tissues of sham-exposed flies collected at the same time intervals.
[b] Virus detected only after second passage in cell culture.
[c] Virus not detected.

increase if NDV survived or was replicated in the fly tissues. NDV was not detected in the tissues of sham-exposed flies collected at the same time intervals.

Experiment 2. NDV was detected in the gut tissues of the house fly for 24 h postfeeding (Table 2.) Titration of NDV in gut tissues at the 30-min interval was greater than that found in the crop tissues. The 24-h rate of decay in virus titer was time dependent in the gut tissues [$y = -1.2677\ln(x) + 4.3786$; $R^2 = 0.914$] (Fig. 1). Little virus as detected 9 h after the initial exposure to NDV. Virus was not detected in the tissues of sham-exposed flies collected at the same time intervals.

Experiment 3. NDV-infected house flies were confined to isolation units containing 2-wk-old chicks. Attracted by the movement of the flies within the units, chicks readily consumed all but a few house flies within 24 h.

No virus was isolated from tracheal swabs collected at 3 d postexposure (five birds per group examined); and at day 21, no NDV-specific antibodies were detected (six birds per group examined). NDV titer in pooled NDV-exposed flies was determined to be $\approx 3 \times 10^4$ ID_{50} per fly.

Experiment 4. NDV Roakin was infectious to all chickens (100%) when introduced intranasally at doses $>10^3$ ID_{50} (Table 3). When NDV Roakin was administered to chickens by oral gavage, all chicks (100%) were seropositive when inoculated with 10^8 ID_{50} of virus. No chicks became infected when orally inoculated with 10^4 ID_{50} of virus.

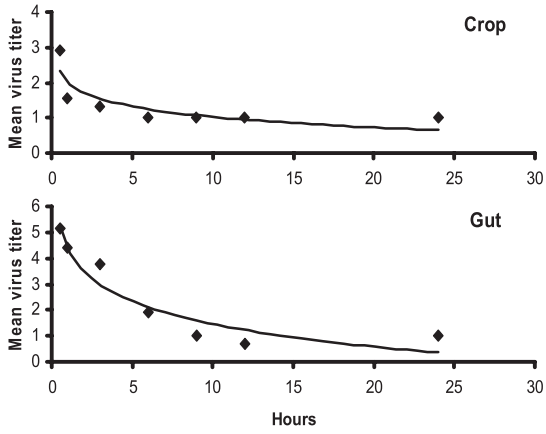

Fig. 1. Decrease in Newcastle disease virus titer (log_{10} ID_{50}/0.2 ml specimen) in house fly crop [$y = -0.4306\ln(x) + 2.0187$; $R^2 = 0.7329$] and gut [$y = -1.2677\ln(x) + 4.3786$; $R^2 = 0.914$] tissues 0.5–24 h postfeeding. The 96-h observation is not shown.

Discussion

The house fly satisfies many requirements of an efficient vector: abundance, mobility, tendency to feed on manure, and ability to harbor pathogenic agents. The current study demonstrates the potential of the house fly to harbor NDV in the crop and gut tissues, and it confirms the findings of Milushev et al. (1977). Virus was detectible in the fly crop for 96 h after a single feeding event. Virus titers in pooled fly crops ranged from 10^3 ID_{50} measured within 30 min of feeding on NDV inoculum to $<10^1$ ID_{50} at 96 h. Although NDV titers in pooled mid- and hindgut tissues were higher (10^7 ID_{50}/0.2 ml) immediately after a single feeding, live virus was only detectable for 24 h. The starting NDV dose was $\approx 10^7$ ID_{50} for both experiment 1 and 2. It is interesting that the crop had much less virus at 30 min than the gut, suggesting that the flies directed a significant portion of the virus containing medium directly to the gut in response to periods of food deprivation. In a previous study, we found turkey coronavirus (family *Coronaviridae*, genus *Coronavirus*, TCV) remained active in the crop of

Table 3. Dose response of SPF chickens experimentally inoculated with NDV (Roakin) by intranasal and oral gavage routes

Dose (ID_{50})	No. infected/no. tested
Intranasal exposure[a]	
Sham	0/6
10^2	0/6
10^3	2/6
10^4	6/6
Oral exposure[b]	
Sham	0/6
10^4	0/6
10^6	3/6
10^8	6/6

[a] Chickens were intranasally inoculated by squirting 0.1 ml containing the calculated dose of NDV (Roakin) into the left nasal passage.
[b] Orally inoculated by passing a no. 5 French catheter into the crop and delivering 1.0 ml containing the calculated dose of NDV (Roakin).

the house fly for 9 h, but it was inactivated in the gut (Calibeo-Hayes et al. 2003). It is likely that midgut proteolytic enzymes inactivate NDV within 24 h and similar enzymes are not present in the crop where the virus may be active longer. As with TCV, the current study indicates that the house fly is a mechanical vector of NDV. The decrease in virus titers indicates that the house fly is not a propagative vector of NDV.

Presumably, the potential of the house fly to transmit a disease agent is enhanced by the relative abundance of the vector. In our study, flies were released in isolation units at rates of 10, 100, and 250 flies per unit, simulating conditions that may be found in the field. Although the chicks were observed eating flies that each carried $\approx 3 \times 10^4$ ID_{50} virus, none of the susceptible chicks became infected. These findings suggested a possible dose effect.

Subsequent experimentation to establish an infectious dose necessary for oral exposure indicated that NDV Roakin, when administered by oral gavage at 10^4 ID_{50} of virus, was insufficient to cause infection (Table 3); only those birds inoculated with a dose $\geq 10^6$ ID_{50} became infected. These findings suggest that NDV transmission, in the current study, failed because chicks failed to ingest an adequate quantity of virus-laden flies; based on calculations chicks would have needed to consume >33 flies to reach a sufficient dose for infection by the oral route. However, intranasal inoculation at 10^4 ID_{50} infected 100% of the chicks. Although the house fly did not transmit NDV in our study, the potential to mechanically transmit NDV remains a possibility because NDV was detectable in both crop and gut tissues for up to 96 and 24 h, respectively, postfeeding (Tables 1 and 2).

Newcastle disease outbreaks vary significantly in virulence, transmissibility, and persistence (Feener et al. 1993, Kinde et al. 2004). Velogenic forms of the virus are most virulent and cause the greatest mortality, whereas virulent mesogenic forms may cause mortality, and lentogenic forms are generally nonfatal. Live vaccines are usually developed from lentogenic and mesogenic strains and include Mukteswar (mesogenic), Roakin (mesogenic), and La Sota (lentogenic), among others (Alexander 1991). Accordingly, lentogenic vaccines may be administered intranasally and mesogenic vaccines intramuscularly. As with field strains of avian viruses, the virulence of NDV viruses may have bearing on vector competency studies, and virulent velogenic ND may be more amenable to fly transmission at lower doses.

In our study, NDV Roakin was infectious at a dose of 10^3 ID_{50} after 100-μl intranasal inoculation. Under epizootic conditions, NDV replicates in the upper respiratory and intestinal tract of the avian host (Feener et al. 1993). Respiratory tract receptor sites may be the initial foci for NDV attachment and becoming systemic as virus spreads to other target organs. Respiratory tract inoculation by house flies is not likely to occur.

In summary, after a single feeding, the house fly harbored live NDV Roakin in both crop and gut tissues 96 and 24 h, respectively. NDV did not replicate in fly crop or digestive tract tissues. Mechanical transmission of NDV by the house fly was not demonstrated in the current study. In controlled experiments, NDV-exposed house flies did not transmit NDV even though each fly carried $\approx 10^4$ ID_{50} of virus when placed in contact with susceptible chickens. This was unexpected, because NDV-exposed flies were eaten by contact chickens soon after placement in isolation units. However, subsequent experiments demonstrated a dose effect for NDV Roakin: an $ID_{50} \geq 10^6$ was required to infect chickens by the oral route, the likely route of transmission for NDV by flies. These studies indicate that at least for NDV Roakin, house flies did not carry sufficient quantities of virus to infect chickens and are unlikely vectors. Additional studies using other NDV strains, particularly velogenic strains, are needed to definitively determine the role of house flies as vectors of NDV.

Acknowledgments

This project was funded by grant 525 from U.S. Poultry and Egg Association, Athens GA. This study was conducted under North Carolina State University Institutional Animal Care and Use Guidelines protocol 04-021A.

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Received 19 January 2007; accepted 11 April 2007.