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## Experimental infection of swine with a sandfly (*Lutzomyia shannoni*) isolate of vesicular stomatitis virus, New Jersey serotype

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The New Jersey serotype of vesicular stomatitis virus (VSV-NJ) has been the predominant serotype isolated from swine.<sup>18</sup> An enzootic focus of VSV-NJ exists on Ossabaw Island, a barrier island of the Georgia coast, where the virus has been repeatedly isolated from feral swine and the phlebotomine sandfly *Lutzomyia shannoni*,<sup>2,4,16</sup> a biological vector of the virus. Although infection of Ossabaw Island swine, as determined by a high annual rate of seroconversion, occurs, clinical disease (vesicle formation) is rarely seen.<sup>6,13-16</sup>

The source of a VSV-NJ isolate may determine its virulence in an animal species. When compared to bovine VSV-NJ isolates, lower titers of swine VSV-NJ isolates are required to produce experimental disease in swine,<sup>5</sup> the incubation period is shorter, and secondary vesicle formation is more likely to occur.<sup>5,8</sup> To our knowledge, the virulence of an Ossabaw Island sandfly-derived isolate of VSV-NJ has not been evaluated in swine or other mammalian species.

The intent of this study was 3-fold: 1) to assess the virulence of a 1991 Ossabaw sandfly isolate in swine and to compare the viral dosages used with those viral dosages that could potentially be delivered by a feeding sandfly; 2) to describe the progression of any subclinical or clinical disease; and 3) to determine if viral shedding occurs.

Seven 2-3-month-old crossbreed pigs were used. All pigs were negative for antibodies to VSV-NJ. Three pairs of pigs were inoculated with various dosages of virus, and 1 pig served as a control. Because vesicles on swine on Ossabaw Island have only been observed on the snout,<sup>16</sup> all of the pigs were injected intradermally in the apex of the snout with a single inoculum of 0.1 ml. The viral inocula were prepared from a 1991 Ossabaw Island sandfly isolate that had been passaged once in Vero cells<sup>a</sup> and diluted to the appropriate

titer with Dulbecco's phosphate-buffered saline (D-PBS).<sup>b</sup> Experimental oral infection of *Lutzomyia shannoni* has resulted in viral titers averaging  $10^{4.3}$  plaque-forming units in the heads and  $10^{5.3}$  plaque-forming units in the thoraces and abdomens of the flies.<sup>3</sup> In an attempt to approximate the viral dose an infected sandfly may be capable of delivering, the middle-dose pair of pigs (C, D) were inoculated with  $10^{4.5}$  median tissue culture infective doses (TCID<sub>50</sub>) of virus. The low-dose pair (A, B) and the high-dose pair (E, F) received  $10^{0.7}$  and  $10^{7.3}$  TCID<sub>50</sub> of virus, respectively. Viral doses were determined via end point titration in Vero cells. The control pig received an injection of D-PBS.

The pigs were restrained daily for physical examination and sample collection. Pigs were examined for the development of lesions, and rectal temperatures were recorded. Blood was collected daily via cranial vena caval puncture for serology, virus isolation, and complete blood counts. Hematologic values were compared with reference values for feeder pigs.<sup>7</sup> Nasal and tonsillar swabs were collected daily for virus isolation. The pigs were euthanized 10 days postinfection (PI) via intravenous sodium pentobarbital injection, and necropsies were performed. Tissue samples were collected from the snout, right periocular skin, right front leg interdigital skin and coronary band, perineal skin, dorsal lumbar skin, ventral abdominal skin, tonsil, mandibular lymph node, parotid lymph node, retropharyngeal lymph node, nasal mucosa, parotid salivary gland, lung, heart, liver, kidney, spleen, stomach, urinary bladder, and brain. A portion of each sample was placed in transport medium for virus isolation; the remainder of the sample was fixed in 10% buffered formalin for histologic examination.

Tissues and swabs were stored in 1.5 ml viral transport medium consisting of tryptose broth supplemented with antibiotics (1,000 U penicillin G/ml, 1 µg streptomycin/ml, 0.25 mg gentamycin/ml, 0.5 mg kanamycin/ml, 2.5 µg amphotericin B/ml)<sup>b</sup> and frozen at -70 C. Pharyngeal and nasal swabs were thawed, vortexed, and centrifuged at 1,500 x g for 10 minutes prior to inoculation on Vero cells. Blood

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**Table 1.** Serum virus neutralizing antibody titers of pigs inoculated with vesicular stomatitis virus, New Jersey serotype.

Fig id.	Days postinfection										
	0	1	2	3	4	5	6	7	8	9	10
A	<1:8	<1:8	<1:8	<1:8	<1:8	1:8	<1:8	<1:8	1:8	1:16	1:16
B	<1:8	1:8	<1:8	<1:8	<1:8	1:8	<1:8	<1:8	1:8	<1:8	1:16
C	<1:8	<1:8	<1:8	<1:8	<1:8	1:8	<1:8	1:8	1:16	1:16	1:64
D	1:16	<1:8	1:16	<1:8	1:16	1:16	1:16	1:16	1:32	1:128	≥1:512
E	<1:8	1:8	1:8	1:8	1:32	1:256	≥1:512	≥1:512	≥1:512	≥1:512	≥1:512
F	<1:8	<1:8	<1:8	1:8	1:64	1:128	≥1:512	≥1:512	≥1:512	≥1:512	≥1:512
Control	1:8	<1:8	<1:8	<1:8	<1:8	<1:8	1:8	<1:8	1:16	<1:8	1:8

samples were collected in heparinized tubes and frozen at -70 C. Once thawed, blood samples were vortexed and diluted 1:10 in VSV isolation medium (minimal essential medium<sup>b</sup> supplemented with 3% heat-inactivated fetal bovine serum,<sup>b</sup> 2 mM L-glutamine,<sup>b</sup> 100 U penicillin/ml, 0.1 mg streptomycin/ml, and 0.25 µg amphotericin B/ml), sonicated, and centrifuged at 1,500 x g for 10 minutes. Tissue samples were thawed, and approximately 2- x 2- x 2-mm portions were removed and placed in 1.0 ml of VSV isolation medium where they were mechanically disrupted, sonicated, and centrifuged at 1,500 x g for 15 minutes. Prepared swab, blood, and tissue samples were inoculated (100 µl) into individual wells of a 12-well tissue culture plate containing a confluent monolayer of Vero cells and allowed to adsorb for 1 hour at 37 C. Monolayers were then washed with D-PBS, and 2 ml of VSV isolation medium was added. Tissue cultures were evaluated for cytopathic effect every 24 hours for 72 hours. Virus isolation attempts were determined to be negative if no cytopathic effect was apparent after 72 hours. All isolates were verified via reisolation from frozen stock, and virus was quantified via end point titration. Isolate identification was confirmed with an indirect fluorescent antibody technique. For the indirect fluorescent antibody technique, isolates were inoculated onto Vero cells in 2 wells of an 8-well chamber slide and incubated for 24 hours. The slides were then fixed in cold acetone, rinsed in PBS, and flooded with VSV-NJ mouse hyperimmune ascitic fluid<sup>d</sup> diluted 1:500 with PBS containing 3% bovine serum albumin.<sup>b</sup> Slides were incubated at room temperature for 30 minutes and then rinsed in PBS. Slides then were flooded with fluorescein-conjugated anti-mouse IgG<sup>b</sup> diluted 1:128 with PBS containing 3% bovine serum albumin and incubated at room temperature for 30 minutes. After a final PBS rinse, the slides were examined immediately by epifluorescence microscopy.

Serum neutralization assays were performed as previously described.<sup>9</sup> A titer of 1:32 was considered positive.

The apices of the snouts of the control pig and both low-dose pigs were mildly swollen on day 1 PI. This swelling lasted only 1 day and was the only sign observed in these pigs.

The middle-dose pigs developed a serous nasal discharge on day 3 PI, which persisted for 2 days in pig D and 3 days in pig C. The nasal plana of both these pigs were swollen on days 4 and 5 PI. A small ulcer was found on pig D on day 6 PI at the approximate site of inoculation.

The high-dose pigs developed marked clinical disease. The apex of the snout of pig E was swollen on days 1 and 2 PI.

This pig was depressed on day 3 PI and had developed a scab over the previously swollen snout site. Severe diarrhea developed the following day. The interdigital portions of both hind feet were swollen and reddened on day 5 PI; this swelling had subsided by the following day. Pig F developed the most severe clinical signs and lesions. This pig developed a large vesicle on its snout and was severely lame and febrile (105.5 F [40.8 C]) on day 2 PI. The coronary bands of the lateral claws of both hind feet were severely swollen and blanched on day 3 PI, and vesicles developed along the coronary bands of the lateral claws and in the interdigital spaces on days 4 and 5 PI. Lameness had subsided by day 4 PI. By day 6 PI, the crust that had formed over the snout vesicle was beginning to peel, exposing a bed of granulation tissue. On day 9 PI, the proximal portions of the hooves of both hind feet were beginning to separate from the coronary bands and underlying hoof wall.

The control pig and the low-dose pair failed to seroconvert by day 10 PI (Table 1). Pigs C and D became seropositive on days 10 and 8 PI, respectively. The high-dose pair seroconverted by day 4 PI.

VSV-NJ was isolated from tonsillar swabs of both high-dose pigs and from the snout vesicle and the vesicles and ulcers that developed on the hind feet of pig F (Table 2). Virus was not isolated from the blood or nasal swabs from any of the pigs or from the tonsillar swabs of the medium-dose, low-dose, or control pigs. VSV-NJ was only isolated from necropsy tissues of the high-dose pigs. These isolates were restricted to tonsillar tissue.

No histologic lesions were apparent in the tissues of the experimentally infected pigs. Except for a slight neutrophilia of 20,770/µl and 19,772/µl (normal range, 300-15,200/µl)<sup>7</sup> observed in pig C on days 6 and 7 PI, respectively, the only abnormal hematologic values found were sporadic lymphocytosis in several of the pigs: 18,333 lymphocytes/µl in pig A on the day of inoculation; 19,188/µl and 19,988/µl in pig B on days 2 and 8 PI, respectively; 17,941/µl, 20,046/µl and 39,185/µl in pig D on days 2, 6, and 7 PI, respectively; and 18,785/µl in pig F on the day of inoculation (normal range, 3,600-18,500/µl).<sup>7</sup>

The sandfly isolate used in this study was capable of producing subclinical and severe clinical disease in swine. The rate of seroconversion and the development of clinical disease were dose dependent. The dose that produced clinical disease exceeded the reported viral titers found in experimentally infected sandflies.<sup>2</sup> As expected, the middle dose, which could probably be delivered by a sandfly, produced

**Table 2.** Viral titers\* of samples collected from pigs inoculated with vesicular stomatitis virus, New Jersey serotype.

Fig id.	Days postinfection									
	1	2	3	4	5	6	7	8	9	10
A	...	...	...	...	...	...	...	...	...	...
B	...	...	...	...	...	...	...	...	...	...
C	...	...	...	...	...	...	...	...	...	...
D	...	...	...	...	...	...	...	...	...	...
E	...	T ≤ 2.7	T = 3.8	T ≤ 2.3	...	...	...	...	...	TT < 2.0
F	T < 2.0	T = 4.5 SV = 8.9	T = 3.5	T < 2.0 RRU ≤ 2.1 LRV = 4.9	...	LRC = 3.5	...	...	...	TT = 5.0

\* Median tissue culture infective doses (TCID<sub>50</sub>). T = tonsillar swab (TCID<sub>50</sub>/swab); SV = snout vesicular fluid (TCID<sub>50</sub>/ml); RRU = right rear foot ulcer swab (TCID<sub>50</sub>/swab); LRV = left rear foot vesicular fluid (TCID<sub>50</sub>/swab); LRC = crust from left rear foot ulcer (TCID<sub>50</sub>/tissue volume not determined); TT = tonsillar tissue harvested at necropsy (TCID<sub>50</sub>/2-x2-x2 mm tissue).

subclinical disease. However, the possibility that the sandfly isolate may have been more virulent if it had not been passaged once in Vero cells cannot be discounted.

Although seroconversion indicated viral replication in both middle- and high-dose groups, virus was only recovered from the high-dose pigs. Virus was recoverable from the tonsillar swabs of both high-dose pigs during a period roughly corresponding to the onset and duration of the snout lesions and mostly preceding seroconversion. Virus was recovered from the tonsillar tissues of the high-dose pigs 10 days PI, which was 6 days after seroconversion and several days after the clinical recovery of pig E. Ten days PI is also several days later than previously reported for swine with experimental vesicular stomatitis infection.<sup>11,12,17</sup> Thus, tonsillar tissue may serve as a source of virus for contact transmission from clinically normal seropositive pigs for an extended period of time. The greatest amounts of virus were found in vesicular fluid, which therefore represents a tremendous although transient source of virus for contact transmission.

Our inability to isolate virus from blood was consistent with the findings of other investigators.<sup>1</sup> The secondary development of lesions in 1 pig's feet following snout inoculation is suggestive of a viremia, although infection of abraded areas by environmental contaminants cannot be discounted. If a viremia occurred, it may have persisted such a short time or have been of such a low level that it was not detected.

This sandfly isolate is capable of producing severe clinical disease and lesions comparable to those described with swine isolates. The development of distant secondary vesicles in pigs following intradermal inoculation, such as those seen in this study, is rare when nonswine VSV-NJ isolates have been used to produce experimental disease.<sup>8</sup> Because partial genomic sequencing of numerous VSV-NJ swine and sandfly isolates from Ossabaw have revealed a high degree of conservation,<sup>10</sup> the ability of this sandfly isolate to produce disease in swine is not surprising.

### Sources and manufacturers

- American Type Culture Collection, Rockville, MD.
- Sigma Chemical Co., St. Louis, MO.
- Difco Laboratories, Detroit, MI.
- Dr. R. B. Tesh. Yale University, New Haven, CT.

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## Identification of parvovirus-like particles associated with three outbreaks of mortality in young pheasants (*Phasianus colchicus*)

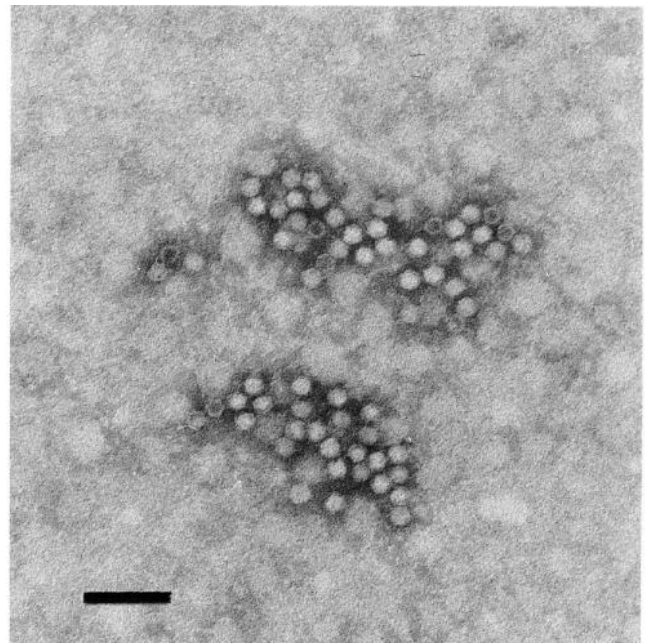
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During the 1994 hatching season, a pathologic syndrome was observed in 3 different rearing facilities of pheasants (*Phasianus colchicus*) in northern Italy, all located within a 20-km radius. The disease was characterized by depression, anorexia, mild enteritis, and sudden and high mortality in 15-25-day-old pheasants. The 3 outbreaks had identical clinical signs and lesions. The disease was not similar to any other previously reported in pheasants.<sup>11</sup> In this report, we describe the results of laboratory examinations undertaken to identify the nature and causes of this disease.

Farm 1 consisted of 1,000 breeders, predominantly Mongolian. The total production was about 20,000 birds/year, without forced laying. The poults were kept in cages and were fed a commercial starter feed. The disease appeared in May, beginning from the sixth hatch, and involved all the following hatches. The eggs of the sixth, seventh, and eighth hatches were incubated in another farm. Only young pheasants 15-25 days of age became ill; mortality was about 50% and remained constant in all the flocks. Farm 2 consisted of 650 breeders, predominantly Mongolian, kept in a colony without forced laying with a production of 15,000 birds/year. The young pheasants were bred in cages in brooding barns. A different firm provided the starter feed. The first hatch involved was the fourth, and all the following hatches were affected. The disease appeared in 20-25-day-old birds, and the mortality was 50-60%. Farm 3 was made up of 1,200 breeders, predominantly Mongolian, with a yearly production of 28,000 birds, in part by forced laying. The young poults were put on litter at 10 days of age and were fed with a commercial feed provided by a third source. Birds from 5

of 12 hatches (from sixth to tenth) were affected by the disease at 15-25 days of age. The mortality was around 50%. The clinical course, similar in all outbreaks, was characterized by anorexia, depression, diarrhea, and sudden death.

Several birds from each outbreak were referred to the diagnostic laboratory for necropsy at different times. Grossly, blood suffusions or small subcutaneous hemorrhagic areas and muscular scattered pinpoint hemorrhages were observed. The liver was normal in volume and consistency but was pale in color and mottled due to hemorrhagic suffusions.



**Figure 1.** Electron micrograph of liver homogenate of 20-day-old pheasant showing numerous icosahedral nonenveloped viral particles, 18-22 nm in diameter, morphologically corresponding to parvovirus-like virus. Bar = 100 nm.

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