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Serologic Evidence for Rabbit Syncytium Virus in Eastern Cottontail Rabbits (*Sylvilagus floridanus*) in Ohio

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ABSTRACT: Thirteen of 20 eastern cottontail rabbit (*Sylvilagus floridanus*) sera collected near Delaware, Ohio (USA) in 1991 were positive by indirect immunofluorescent antibody test (IFAT) for antibody to rabbit syncytium virus (RSV), a Kemerovo serogroup orbivirus. In addition, two of 10 domestic bovine sera and three of 30 sheep sera collected in southeastern Ohio gave weak positive IFAT reactions to RSV.

Key words: Cottontail rabbits, *Sylvilagus floridanus*, rabbit syncytium virus, orbivirus, Kemerovo serogroup, serology, prevalence.

The Kemerovo serogroup comprises a complex group of globally distributed, tick-borne orbiviruses whose vertebrate hosts include avian and mammalian species (Gorman et al., 1983). Some members of this serogroup infect humans and may cause febrile illness or neurological disease (Chumakov et al., 1963; Libikova et al., 1978). Moreover, several Kemerovo serogroup viruses induce encephalitis or meningitis in intracerebrally inoculated newborn mice, rats, and hamsters and young rhesus monkeys (Chumakov et al., 1963; Libikova et al., 1965, 1970).

Until lately North American isolates of this serogroup were exclusively from argasid or ixodid ticks infesting relatively inaccessible nesting sites of cliff swallows or seabirds (Yunker, 1975). Recently, however, Theil and McCloskey (1991) demonstrated that rabbit syncytium virus (RSV) was antigenically related to Tribec virus, a Kemerovo serogroup member. The RSV was isolated in 1962 from an eastern cottontail rabbit (*Sylvilagus floridanus*) trapped in the Delmarva peninsular region of Virginia (USA) (Morris et al., 1965); this virus induced paralysis, meningitis, and death when inoculated intracerebrally into suckling mice. Further, Morris et al. (1965) found that a third of the cottontails trapped in Virginia had complement-fixing antibodies to RSV. Their serologic survey represents the only data on RSV prevalence in cottontails.

Eastern cottontails often live in proximity to humans and to domesticated animals. Given the pathogenicity of some Kemerovo serogroup viruses, our objective was to determine if RSV is present in Ohio (USA). Accordingly, we tested sera from cottontails and selected livestock in Ohio for RSV antibodies. In addition, clinical and serum antibody responses of domestic rabbits, *Oryctolagus cuniculus*, inoculated with RSV were determined.

Sera were collected from the ear vein of 20 adult eastern cottontails (12 males and 8 females) live-trapped at the Delaware Wildlife Area, near Delaware, Ohio (40°18′N, 83°06′W) between 23 September and 8 October 1991. This 1,943 ha area is in a rural agricultural region comprising meadows, grain crops, and brushy cover; it has been described by Boyd and Henry (1991). Weights of 17 cottontails were determined and ranged from 830 to 1,500 g; 13 cottontails that weighed <1,325 g were assumed to be born during 1991 (Boyd and Henry, 1991). Cottontails were ear-tagged, bled, and then released at the site of capture.

Sera were treated at 56 C for 30 min and then tested for RSV antibody by a virus neutralization test (VNT) and by an indirect immunofluorescent antibody test (IFAT) (Theil and McCloskey, 1991). For the VNT, each serum was diluted 1:5 and incubated one hr at 37 C with 30 to 100 median tissue culture infective doses ($TCID_{50}$) of RSV. Virus–serum mixtures then were each inoculated onto three BHK-21 cell monolayers prepared in roller tubes

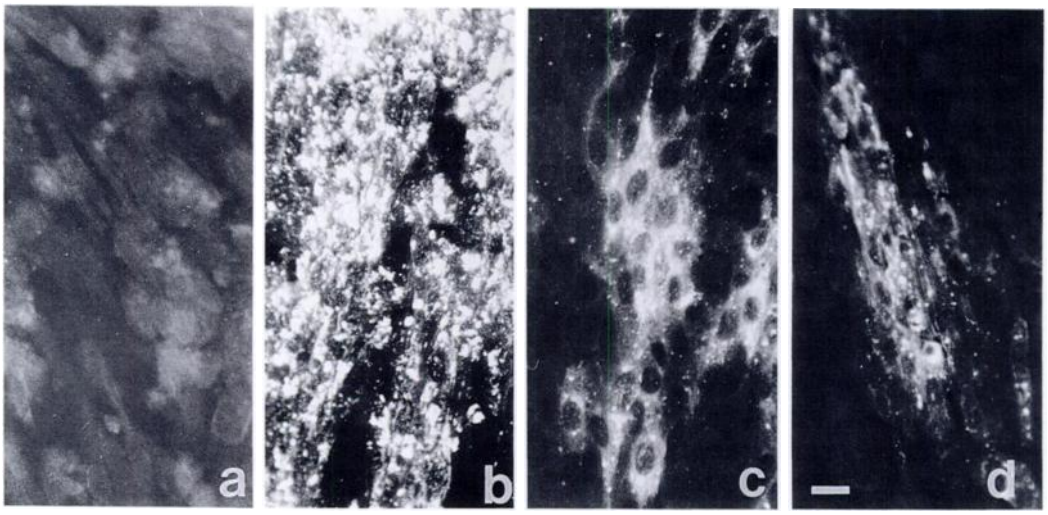

FIGURE 1. Indirect immunofluorescent antibody test (IFAT) reactions of rabbit sera with BHK-21 cell monolayers infected with rabbit syncytium virus (RSV). a, pre-inoculation serum from a laboratory rabbit; b, post-inoculation serum from a laboratory rabbit given RSV intravenously; c and d, positive sera from two eastern cottontail rabbits. Bar = 50 μm.

(Theil and McCloskey, 1991). Inoculated monolayers were observed five days for cytopathic effect (CPE) and a serum was considered to contain neutralizing antibody if it prevented the appearance of CPE in one or more inoculated monolayers. In the IFAT, RSV-infected BHK-21 cell monolayers prepared on coverslips (Theil and McCloskey, 1991), were reacted first with sera diluted 1:10 followed by a 1:15 dilution of fluorescein-conjugated antibody (FCA) to domestic rabbit IgG heavy and light chains (ICN Biomedicals Inc., Costa Mesa, California, USA). This FCA was suitable for the IFAT since domestic rabbit and cottontail IgG's share many common antigens (Rodkey and Conrad, 1972; Conrad and Rodkey, 1973; Teherani and Mandy, 1976). Stained monolayers were examined by fluorescent microscopy and sera producing specific reactions characterized by immunofluorescence of discrete intracytoplasmic inclusions were considered positive for RSV antibodies.

All cottontails were seronegative for neutralizing antibodies to RSV. In contrast, however, 13 (seven males and six females) of 20 were seropositive for RSV antibodies by IFAT (Fig. 1), including three of the four born before 1991. In addition, at least seven cottontails born in 1991 were RSV seropositive and these must have been infected during the spring or summer of that year. Based on our findings, we suggest that RSV, or a virus antigenically related to it, is widespread since the seropositive Ohio cottontails were geographically and temporally removed from the seropositive cottontails trapped in Virginia >30 yr ago.

Twenty normal female domestic rabbits, allotted into groups of five by age, were used; the age of the rabbits in the four groups at inoculation were 7, 8, 16, and 21 wk, respectively. Rabbits were caged individually and serum was collected from each before the experiment. Each group, comprised of a non-inoculated control and four principals, was kept in a separate room. Principals each were inoculated via the medial vein near the ear tip with 5×10^5 $TCID_{50}$ of RSV that had been passed in cell culture 16 to 19 times; intravenous inoculation was used since it simulates the probable natural route of RSV infection. Rabbits then were observed daily for clinical signs.

Before the experiment all rabbits were

seronegative for RSV antibody by both assays. After inoculation all rabbits, except two in the 8-wk-old age group, remained normal. On post-inoculation (PI) day 1, these two rabbits developed ear droop associated with ruffled pelage on the neck and shoulders. Both ears of one rabbit were affected whereas only the inoculated ear of the other rabbit was affected. The drooping ears had no apparent lesions and were not edematous. This condition persisted, with gradual improvement, until the rabbits were killed 3 wk later by exsanguination via cardiac puncture while they were under sedation with 25 mg/kg ketamine hydrochloride (Ketaset, Aveco Company, Inc., Fort Dodge, Iowa, USA) administered intramuscularly. Daily rectal temperatures of all rabbits were unremarkable. Sera were collected from each rabbit between PI days 19 and 21 and tested for RSV antibodies as above; all inoculated rabbits had RSV antibodies demonstrable only by IFAT. Furthermore, the immunofluorescent reactions of these PI sera were of comparable intensity to those observed with IFAT positive cottontail sera (Fig. 1). Controls remained normal throughout the test period and seronegative by both tests. Thus, intravenously inoculated cell culture-passed RSV has little or no pathogenicity for recently weaned and young adult domestic rabbits; but based on the seroconversion of inoculated rabbits detected by IFAT they became infected. These findings extend those of Morris et al. (1965), although the inoculation route and the age and number of rabbits used in their study were not specified.

Sera from 10 bovine bulls pastured at Coshocton, Ohio (40°16′N, 81°53′W), 10 and 20 sheep pastured at Wooster (40°46′N, 81°57′W), and Caldwell (39°44′N, 81°32′W), Ohio, respectively, and nine turkeys raised on the range during summer and autumn in Wooster, Ohio, were tested for RSV antibodies at 1:5 and 1:10 dilutions by VNT and IFAT, respectively; these animals all were normal and none had traveled outside Ohio. The VNT and IFAT were performed as above except that FCA's to bovine IgG (ICN Biomedicals Inc.), ovine IgG (ICN Biomedicals Inc.), and turkey IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, USA) were used at 1:15 dilutions in the IFAT with appropriate sera. All sera were negative by VNT but two bull sera and three sheep sera collected at Caldwell gave weak positive IFAT reactions. Thirty-one sera from suckling pigs farrowed in northeast Ohio between August and October during the years 1987 through 1991 also were tested at a 1:10 dilution for RSV antibodies by IFAT using FCA to porcine IgG (ICN Biomedicals Inc.) and were negative. This was not unexpected as these young pigs and their dams did not have access to pastures.

Several explanations exist for the absence of RSV-neutralizing antibody in cottontail sera positive by IFAT. First, Kemerovo serogroup orbiviruses are differentiated into four antigenic subgroups, or complexes, comprising 20 serotypes (Gorman, 1992). Of the serologic assays used to characterize Kemerovo serogroup isolates, immunofluorescent stains are the most broadly reactive, detecting a specific intergroup antigen shared by members of all subgroups (Carey and Nuttall, 1989). Thus, Ohio cottontails may have been infected with a Kemerovo serogroup virus belonging to a different serotype than RSV. Second, the VNT may be too insensitive to detect the low levels of neutralizing antibody commonly induced by Kemerovo serogroup isolates (Libikova et al., 1965; Yunker, 1975). This may be the case since IFAT-positive PI sera from RSV-inoculated domestic rabbits also were seronegative by VNT even though the intensity of their IFAT reactions were comparable to those produced by IFAT-positive cottontail sera. A plaque reduction assay, such as that used to detect low levels of serum neutralizing antibody to Kemerovo

virus (Mayer and Kozuch, 1964), may help resolve this discrepancy.

Many aspects concerning the ecology of RSV infections await elucidation including their medical or veterinary importance. In Czechoslovakia many sheep, goats, cattle, and horses have antibodies to Kemerovo serogroup orbiviruses (Libikova et al., 1964; Ernek et al., 1966; Hubalek et al., 1986). In the current study, we found that a few cattle and sheep pastured in the unglaciated Allegheny plateau of southeastern Ohio, where cottontails abound, were apparently exposed to a virus antigenically related to RSV. The significance of livestock exposure to this virus requires further assessment. Elsewhere, however, domesticated animals infected with Kemerovo or Tribec virus were asymptomatic (Libikova et al., 1965; Ernek et al., 1966).

The presumed tick vector for RSV should be identified since it will largely determine the vertebrate host range of this orbivirus. The ticks most commonly found on eastern cottontails—*Haemaphysalis leporispalustris* and *Ixodes dentatus*—usually attach only to lagomorphs and ground foraging birds (Bishopp and Trembley, 1945). This perhaps explains why, of eight wildlife species tested in Virginia, only cottontails had RSV antibodies (Morris et al., 1965).

Finally, the relationship of RSV to other Kemerovo serogroup members, particularly those recovered from seabird rookeries, should be determined. Lagomorphs and seabirds can have close ecological interactions. For instance, the European rabbit, *Oryctolagus cuniculus*, has been released onto islands for centuries and now inhabits nearly 600 islands (Flux and Fullagar, 1983). Thus many islands serving as seabird rookeries are also populated with rabbits. Moreover, some seabirds, such as puffins, shearwaters, and petrels, may nest in rabbit burrows (Lockley, 1938; Warham, 1967; Freethy, 1987). Kemerovo serogroup viruses often are recovered from *Ixodes uriae* (Gorman et al., 1983), a tick that engorges on many seabird species and often infests seabird burrows (Murray and Vestjens, 1967; Mehl and Traavik, 1983); moreover, this tick also bites mammals, including humans (Mehl and Traavik, 1983). Many of these virus isolates were from ticks gathered on islands inhabited by rabbits. Whether interactions between lagomorphs and seabirds provide a mechanism whereby an orbivirus that infects mammalian hosts can achieve global distribution remains to be determined.

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