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## VIREMIA IN THREE ORDERS OF BIRDS (ANSERIFORMES, GALLIFORMES AND PASSERIFORMES) INOCULATED WITH OCKELBO VIRUS

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**ABSTRACT:** One-hundred six birds of 14 species were inoculated with approximately  $10^{2.7}$  plaque-forming units of Ockelbo virus and bled daily for 5 days to determine viremia levels. Virus was detected in birds of all 14 species tested (four Anseriformes, one Galliformes and nine Passeriformes). The onset of viremia occurred earlier and viral titers were higher in very young anseriforms and galliforms than in older birds. Adult passeriforms had Ockelbo viremias of higher titer and longer duration than did adult anseriforms. Viremia titers in adult birds of all three orders tested were sufficient to induce high transmission rates in enzootic mosquito vectors, and viremias in passeriforms could induce high transmission rates in bridging vectors as well. Passeriforms of the genera *Turdus* and *Fringilla* could serve as amplification hosts for Ockelbo virus based on the presently demonstrated viremia of high titer and long duration in these birds, and the previously demonstrated high prevalence of Ockelbo virus neutralizing antibodies in free-ranging individuals and great population size compared to birds of other taxa. Bird species of all three orders tested, however, could function as incidental hosts of the virus.

**Key words:** Alphavirus, Ockelbo virus, experimental viraemia, bird, Passeriformes, amplification host, incidental host.

### INTRODUCTION

Late summer outbreaks of arthralgia and rash in humans in northern Europe are associated with an *Alphavirus* closely related to Sindbis virus (Skogh and Espmark, 1982; Lvov et al., 1982; Brummer-Korvenkontio and Kuusisto, 1984). A candidate etiologic agent, designated Ockelbo virus, was isolated from mosquitoes collected in Sweden during an epidemic in 1982 (Niklasson et al., 1984). This viral strain was shown to be closely related to, but distinguishable from, Sindbis virus (Niklasson et al., 1984; Lvov et al., 1988).

Sindbis virus has a wide geographical distribution in the Old World, occurring in the tropical, subtropical and temperate climate zones (Karabatsos, 1985). It is transmitted by ornithophilic mosquitoes among bird populations (McIntosh et al., 1967; Jupp and McIntosh, 1970) and these mosquitoes also may act as bridging vectors between the enzootic cycle and human infection (Jupp, 1973; McIntosh et al.,

1976). Antibodies to Sindbis virus have been detected in bird species of several taxonomic orders, more commonly in Anseriformes than in Passeriformes (McIntosh et al., 1968; Ernek et al., 1975, 1977).

Based on field studies of Swedish bird and mosquito populations (Jaenson and Niklasson, 1986; Francy et al., 1989; Lundström et al., 1992) and from experimental vector competence studies (Lundström et al., 1990a, b; Turell et al., 1990), Ockelbo virus appears to be maintained in an enzootic cycle involving birds and ornithophilic mosquitoes, with *Culex torrentium* as the principal enzootic vector and several *Aedes* species as bridging vectors.

In contrast to South African and Czechoslovakian studies on Sindbis virus (McIntosh et al., 1968; Ernek et al., 1975, 1977), antibodies to Ockelbo virus occur more frequently in individual passeriforms than in anseriforms (Francy et al., 1989; Lundström et al., 1992). However, for birds to contribute to natural virus circulation, they



must produce a viremia of sufficient magnitude and duration to infect vector mosquitoes.

To assess the ability of birds present in Sweden to serve as amplification hosts of Ockelbo virus, we determined viremia titers in adult and immature birds of the orders Anseriformes, Galliformes and Passeriformes experimentally infected with Ockelbo virus.

## MATERIALS AND METHODS

### Virus

The Edsbyn 82/5 strain of Ockelbo virus, isolated from mosquitoes collected in central Sweden during the 1982 outbreak of Ockelbo disease (Niklasson et al., 1984), was used for infecting birds and also was used as the antigen in the antibody assays. Virus for bird inoculation were passed twice in Vero cell cultures (American Type Culture Collection, Rockville, Maryland, USA). The virus suspension was diluted in Hank's balanced salt solution to an estimated concentration of  $10^4$  plaque-forming units (PFU)/ml, fetal bovine sera was added to a 10% final concentration, and the diluted virus suspension divided into multiple 1.0 ml aliquots and stored at  $-70^\circ\text{C}$  until used. Virus used as antigen in neutralization tests also was passed twice in Vero cells but was stored undiluted.

### Birds

Twelve bird species present in Sweden were selected for the study based either on natural occurrence of Ockelbo virus neutralizing antibodies (capercaillie, *Tetrao urogallus*; Canada goose, *Branta canadensis*; bean goose, *Anser fabalis*; fieldfare, *Turdus pilaris*; redwing, *T. iliacus*; song thrush, *T. philomelos*; house sparrow, *Passer domesticus*; chaffinch, *Fringilla coelebs*; greenfinch, *Carduelis chloris*; yellowhammer, *Emberiza citrinella*), or absence of these antibodies among all of >40 birds tested (mallard, *Anas platyrhynchos*; great tit, *Parus major*) (Francy et al., 1989; Lundström et al., 1992; Lundström and Niklasson, unpubl.). Two species (goldeneye, *Bucephala clangula*; blackcap, *Sylvia atricapilla*) were included in the study because they occur commonly in the endemic area (Ulfstrand and Högstedt, 1976; Bruun et al., 1986; Lundström et al., 1991).

All birds originated from or near the Boda Wildlife Research Station (Boda)  $61^\circ32'\text{N}$ ,  $17^\circ52'\text{E}$  situated within the Ockelbo disease endemic area in central Sweden (Lundström et al., 1991). We used offspring of captive capercaillies, Canada geese, bean geese and mallards held

at Boda. Goldeneyes were hatched from eggs collected from the nests of free-ranging wild birds. Adult passeriforms were captured in Japanese mist nets and nestlings were collected by hand.

The age of each bird was determined on the basis of hatching protocols for the anseriforms and galliforms reared at Boda, while the age of wild passeriforms was estimated to be either 1 wk, 2 to 4 wk, or adults on the basis of the birds' size and plumage. Capercaillies, the single galliform species tested, were either 2 days old or adults ( $\geq 1$  yr old). The anseriforms were either 2 to 4 days old, 14 to 21 days old, or adults. The birds were held at Boda in an indoor aviary with a 20:4 light:dark cycle to simulate Swedish summer illumination. The aviary was protected against insects by double curtains of fine meshed insect net over the only entrance. Insect nets, walls and airspace were treated daily with Radar insecticide (Nobel Consumer Goods AB, Bromma, Sweden). Cages with young birds were heated with an infrared lamp to keep the birds warm. Nestling passeriforms were held in an incubator at approximately  $35^\circ\text{C}$ .

Geese, mallards, adult goldeneyes and capercaillies were given a mixture of seeds and dried Allfoder commercial bird food (Foderinger HB FORI, Västerås, Sweden) supplemented with fresh herbs. Young goldeneyes and capercaillies were given live ants (*Formica* spp. pupae and imagoes and *Tenebrio molitor* larvae). Adult house sparrows, chaffinches, green finches, yellow hammers and great tits were given seeds and fresh fruits. Adult fieldfares, redwings and song thrushes were given live worms *Lumbricus* spp. and fresh fruits. Nestling blackcaps, fieldfares and great tits were fed a slurry of triturated moistened Triumph commercial dog-food (Canicum AB, Bro, Sweden), that contains several essential vitamins and minerals.

### Laboratory procedures

Immature birds of all three taxonomic orders and adult galliforms and passeriforms were bled from the jugular vein, while adult anseriforms were bled from the tarsal vein. A 0.1-ml blood sample was obtained from each bird using a 27-gauge needle and a 1.0-ml syringe, and the sample was diluted in 0.9 ml of the diluent, Hanks' balanced salt solution supplemented with 10% heat-inactivated fetal bovine serum, antibiotics and HEPES buffer (Lundström et al., 1990a, 1992). Diluted blood samples were held on wet ice  $<3$  hr, held on dry ice  $\leq 2$  wk, and then stored at  $-70^\circ\text{C}$  until tested for Ockelbo virus and for Ockelbo virus neutralizing antibodies.

Blood samples were assayed for virus content at 10-fold dilutions by a plaque assay (Lundström et al., 1990a). The assay was conducted

on confluent Vero cell monolayers in 24-well cell culture plates with an area of 2.0 cm<sup>2</sup> per well (Costar, Cambridge, Massachusetts, USA).

A plaque-reduction neutralization test (PRNT) on heat-inactivated (30 min, 56 C) blood samples diluted fourfold starting at 1:20 was used to examine birds for virus-neutralizing antibodies (Earley et al., 1967; Franczy et al., 1989). The PRNT was conducted on Vero cells in 24-well plates, two wells per dilution. Each well received 30 to 80 PFU of Ockelbo virus and the neutralizing antibody titers were expressed as the reciprocal of the dilution giving  $\geq 80\%$  reduction in plaque numbers.

#### Experimental protocol

Birds were bled immediately upon their arrival at the aviary. Blood samples were tested for virus and for neutralizing antibodies to determine previous Ockelbo virus infections. One to 4 days after the pre-inoculation sample, each of the 127 birds was inoculated subcutaneously in the loose skin anterior to the right knee with 0.1 ml of a suspension containing approximately  $10^{2.7}$  PFU of virus; three ampules were assayed for virus content and all contained  $10^{2.7}$  PFU/0.1 ml (Lundström et al., 1990a). To minimize variation in infective dose each virus aliquot was thawed only once and inoculated into a maximum of five birds within 5 min after thawing. Groups of 8 to 22 birds were inoculated with virus on 8, 10, 15, 17, 24 through 28, and 30 June, and on 1 July. Each bird then was bled daily for 5 days, and the diluted blood samples were assayed for infectious virus. Differences in viremia titers between bird groups were tested with the Wilcoxon-Mann-Whitney test (Siegel and Castellan, 1989). Differences in viremia length were tested with Fisher's exact test (Brownlee, 1965).

#### RESULTS

Data from 21 of the 127 birds tested were excluded from the study due to presence of either a pre-inoculation viremia (one adult male mallard had a natural Ockelbo viremia of  $10^{3.7}$  PFU/ml when bled upon its arrival at the laboratory June 11, 1990) or neutralizing antibodies (20 birds) (data not shown).

Birds of all 14 species tested produced viremia after inoculation of Ockelbo virus (Table 1). Viremia titers were significantly ( $P < 0.005$ ) higher in 2- to 4-day-old anseriforms than in 14- to 21-day-old or adult anseriforms during the first 2 days after

TABLE 1. Proportion of birds viremic and peak mean daily viremia titers per species and age-group for indigenous Swedish birds after inoculation of approximately  $10^{2.7}$  plaque-forming units (PFU) of Ockelbo virus.

Order Species	Age at the time of infection			
	≤1 wk		≥2 wk	
Anseriformes				
Canada goose	25 (4) <sup>a</sup>	2.0 <sup>b</sup>	50 (2)	3.7
Bean goose	100 (6)	6.2	0 (1)	
Mallard	50 (8)	5.0	58 (12)	4.5
Goldeneye	100 (2)	6.3	83 (6)	4.5
Passeriformes				
Blackcap	100 (4)	6.2	NT	
Redwing	NT		100 (4)	6.2
Song thrush	NT		100 (1)	6.6
Fieldfare	100 (2)	5.0	57 (7)	6.1
Great tit	100 (3)	4.8	0 (3)	
House sparrow	NT		67 (3)	5.8
Chaffinch	NT		83 (6)	6.9
Greenfinch	NT		83 (12)	7.5
Yellowhammer	NT		44 (16)	6.3
Galliformes				
Capercaillie	100 (2)	8.2	100 (2)	6.4

<sup>a</sup> Percent viremic (number tested), NT = not tested.

<sup>b</sup> Peak mean daily viremia in log<sub>10</sub> PFU/ml blood.

infection (Fig. 1, and data not shown). Similarly, viremia titers were higher in 2-day-old capercaillies than in adults (Table 1). However, viremia titers in young passeriforms, approximately 1 wk old, were not significantly different from titers in older birds ( $\geq 2$  wk old) (Table 1, Fig. 1). For older birds, the viremia titers were significantly ( $P < 0.005$ ) higher in passeriforms than in anseriforms during days 3, 4 and 5 after infection (Fig. 1). The proportion of infected birds that developed detectable viremia also was inversely related to the birds' age at the time of infection.

The daily proportions of birds viremic and the average daily viremia titers over a 5-day period were compared among older birds of the seven species for which at least four birds were tested (Table 2). During the first 2 days after inoculation, the proportion of birds viremic were higher in the anseriform species (mallard and goldeneye) than in the passeriform species

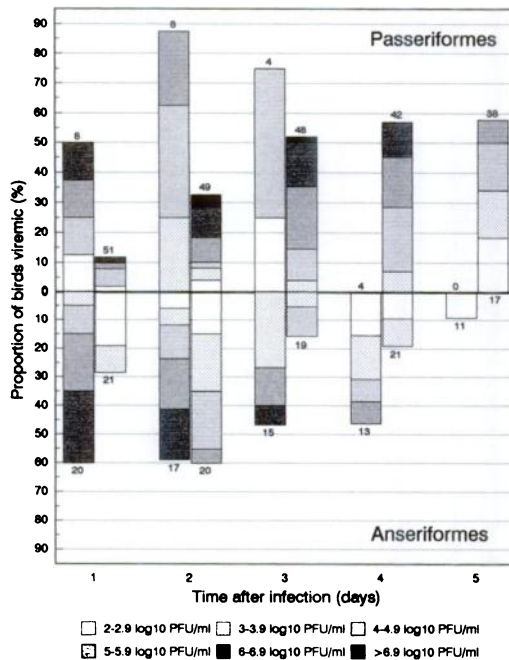


FIGURE 1. Proportion of birds viremic and viremia titer distribution by day after infection for Swedish passeriforms and anseriforms inoculated with approximately  $10^{2.7}$  PFU of Ockelbo virus. For each day, the left bar indicates juvenile and the right bar adult birds, respectively. The number of birds tested each day is given at the end of the bars.

(redwing, fieldfare, chaffinch and greenfinch). However, during days 3 to 5 the proportions of birds viremic were higher in the passeriforms than in the anseriforms. The viremia titers in redwings, chaffinches and greenfinches were significantly ( $P <$

0.03) higher than in both mallards and goldeneyes during days 3, 4 and 5 after infection.

Adult passeriforms also had a significantly ( $P < 0.001$ ) longer duration of viremia than adult anseriforms. Most (24 of 27) viremic passeriforms had a viremia for  $\geq 3$  days. However, the maximum duration could not be determined because passeriforms were often viremic during the fifth and last sampling day (Table 2, Fig. 1). In contrast, nine of twelve anseriforms had a viremia of only 1 to 2 days.

The timing of the first detectable viremia varied from 1 to 5 days after inoculation. Onset of viremia in 12 adult anseriforms was either on day 1 (50%) or day 2 (50%). In contrast, onset of viremia in 33 adult passeriforms was less synchronized, with onset occurring on each day tested; day 1 (18%), day 2 (33%), day 3 (42%), day 4 (3%) or day 5 (3%). Likewise in young birds, onset of viremia occurred earlier in 13 young anseriforms (day 1, 92%; day 2, 8%) than in 9 young passeriforms (day 1, 44%; day 2, 44%; day 3, 11%).

## DISCUSSION

All 14 avian species inoculated with Ockelbo virus developed peak average daily viremias  $\geq 10^{3.7}$  PFU/ml. This is sufficient to infect nearly all blood feeding *Cx. torrentium*, the main enzootic vector,

TABLE 2. Proportion of birds viremic and average viremia titers per day post-infection in Swedish birds older than two weeks, after subcutaneous inoculation with  $10^{2.7}$  plaque-forming units (PFU) of Ockelbo virus. Only species for which  $\geq 4$  individuals are tested are included in the table.

Species	Number of days after inoculation									
	1		2		3		4		5	
Mallard	17 (12)	2.0 <sup>a</sup>	55 (11)	4.0	20 (10)	4.5	33 (12)	3.0	0 (8)	
Goldeneye	67 (6)	3.2	83 (6)	4.5	0 (6)		0 (6)		0 (6)	
Redwing	25 (4)	4.8	50 (4)	6.2	100 (4)	6.0 <sup>b</sup>	100 (3)	4.8 <sup>b</sup>	100 (3)	3.4 <sup>b</sup>
Fieldfare	0 (7) <sup>c</sup>		29 (7)	5.5	50 (6)	6.1	80 (5)	5.6	80 (5)	4.8 <sup>b</sup>
Chaffinch	33 (6)	5.4	67 (6)	6.9	75 (4)	6.1 <sup>b</sup>	100 (3)	5.6 <sup>b</sup>	100 (3)	4.5 <sup>b</sup>
Greenfinch	0 (12) <sup>c</sup>		27 (11)	5.6	83 (12)	7.5 <sup>b</sup>	82 (11)	5.6 <sup>b</sup>	64 (11)	4.9 <sup>b</sup>
Yellowhammer	12 (16)	3.8	29 (14)	6.3	20 (15)	6.2	21 (14)	5.5	42 (12)	4.6

<sup>a</sup> Percent with viremia (number tested) mean viremia titer presented as  $\log_{10}$  PFU/ml blood.

<sup>b</sup> Viremia titers significantly ( $P < 0.03$ , Wilcoxon-Mann-Whitney test) higher than in mallards and in goldeneyes.

<sup>c</sup> Viremia titers significantly ( $P < 0.03$ ) lower than in mallards and in goldeneyes.

which has an  $ID_{50} = 10^{3.0}$  PFU/ml (Lundström et al., 1990a). Most passeriform species had viremias  $\geq 10^5$  PFU/ml, sufficient to infect all *Cx. torrentium* and all *Aedes* spp. previously identified as potential bridging vectors (Lundström et al., 1990a; Turell et al., 1990). Viremia profiles depended on both the age of the bird at the time of inoculation and on its taxonomic order. For anseriform and galliform species tested, viremia titers were inversely related to the bird's age at the time of infection with Ockelbo virus, and viremia titers were higher in adult passeriforms than in adult anseriforms. In previous work, alphaviruses also produced viremia in almost all avian species tested (Taylor et al., 1955; McIntosh et al., 1969; Whitehead, 1969; Dickerman et al., 1980).

Young anseriforms and galliforms in the present study developed Ockelbo viremias of higher titers than did their older conspecifics. Higher viremia titers in very young birds than in older birds appear to be a general feature of alphavirus infections (Taylor et al., 1955; Whitehead, 1969; Scott et al., 1988); thus, viremic nestlings may function as sources of infective blood meals for vector mosquitoes. The involvement of nestling birds as hosts of mosquito-borne arboviruses also depends on the frequency of mosquito feedings on this part of the population. Blackmore and Dow (1958) found that *Culex* mosquitoes fed more often on nestlings than on older birds of three altricial and one precocial species. Increased mosquito feeding on newly hatched altricial birds has been attributed to reduced antimosquito behavior in nestlings as compared to adults (Kale et al., 1972; Edman and Scott, 1987). Frequent isolation of mosquito-borne arboviruses from nestling and fledgling passeriforms in North America indicates that vector mosquitoes successfully feed on young birds of at least some altricial species (Dalrymple et al., 1972; Holden et al., 1973). These results also may be applicable to the Ockelbo virus ecosystem in northern Europe.

There is a positive correlation between the ability of vector mosquitoes to transmit Ockelbo virus upon refeeding on uninfected hosts and the viremia titer in the infective blood meal from the first host (Lundström et al., 1990a, b; Turell and Lundström, 1990). An infective blood meal containing  $10^{3.4}$  PFU/ml of Ockelbo virus resulted in nearly 100% transmission by *Cx. torrentium* (Lundström et al., 1990a), while a viremia of  $10^{5.7}$  PFU/ml yielded a transmission rate of about 50% in *Aedes* spp., which may serve as bridging vectors (Turell et al., 1990). In the present study, birds of all three orders developed viremias of sufficient titer to induce high transmission rates in *Cx. torrentium*. Thus, anseriform, galliform and passeriform species could infect this efficient enzootic vector. However, based on the high titer and long duration of viremia in several passeriform species and the single galliform species, these species probably are more efficient amplification hosts than anseriforms, both in the enzootic circulation of virus and as a source of infective blood meals for the bridging vectors.

In contrast to the viremia onset in galliforms and anseriforms, the onset of Ockelbo viremias in passeriforms was slower than the onset of most other alphavirus viremias in birds (Taylor et al., 1955; McIntosh et al., 1968, 1969; Whitehead, 1969; Scott et al., 1988). However, Dickerman et al. (1980) reported a similar delay of another alphavirus (Venezuelan equine encephalitis virus) viremia onset in semipalmated sandpipers (*Charadrius semipalmatus*).

We have shown that Ockelbo virus could produce viremias of sufficient titer in several avian species to infect the enzootic vectors. However, for these birds to play a major role in the maintenance cycle, they must occur in high numbers and have frequent contact with vector mosquitoes. Ockelbo virus-neutralizing antibodies, indicative of a previous Ockelbo virus infection, have been detected most frequently in four species of passeriforms; chaffinch

(8 to 44%), fieldfare (7 to 35%), redwing (13 to 50%) and song thrush (67%) (Francy et al., 1989; Lundström et al., 1992; Lundström and Niklasson, unpubl.). The individuals of these four species comprise 18% of the bird population breeding in Sweden (Ulfstrand and Högstedt, 1976), and they commonly breed in the forests of northern Europe (Bruun et al., 1986), including the Ockelbo endemic areas in Sweden, Finland and Russia (Lvov et al., 1982; Brummer-Korvenkontio and Kuusisto, 1984; Lundström et al., 1991).

In conclusion, several passeriform species of the genera *Turdus* and *Fringilla* are the most likely amplification hosts for Ockelbo virus. This is based on Ockelbo viremia of high titer and long duration in experimentally infected birds, high antibody prevalence in wild populations of these birds, and high absolute numbers and relative abundance of these passeriforms compared to other bird taxons in Ockelbo endemic areas during the summer season. Other birds, including both passeriform and non-passeriform species, may be involved as incidental hosts in the Ockelbo virus transmission cycle.

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