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Authors: Lundström, Jan O., and Niklasson, Bo

OCKELBO VIRUS (TOGAVIRIDAE: *ALPHAVIRUS*) NEUTRALIZING ANTIBODIES IN EXPERIMENTALLY INFECTED SWEDISH BIRDS

Jan O. Lundström[1,2] and Bo Niklasson[3,4]

[1] Virology Unit, Swedish Institute for Infectious Disease Control, S-10521 Stockholm, Sweden
[2] Department of Zoology, Uppsala University, Villavägen 9, S-75236 Uppsala, Sweden
[3] Swedish Defense Research Establishment, FOA-4, S-90182 Umeå, Sweden
[4] Department of Defense Microbiology, Swedish Institute for Infectious Disease Control, S-10521 Stockholm, Sweden

ABSTRACT: The ability of native Swedish birds to produce and maintain production of Ockelbo virus neutralizing (Nt) antibodies were evaluated experimentally between 6 June 1990 and 27 July 1991. After experimental infection of 57 birds of the orders Anseriformes and Passeriformes with Ockelbo virus, these birds were examined for Ockelbo virus Nt antibodies at 5 days, and at 1, 3, 6, 9, and 12 mo after inoculation. One month after inoculation, Nt antibodies were more prevalent in birds with a detectable viremia (100%, n = 22) than in non-viremic birds (65%, n = 26). The Nt antibody prevalence varied over time among taxa; detectable antibodies occurred earlier after inoculation and for a longer time in Anseriformes than in Passeriformes. By 5 days after inoculation, antibodies could be detected in 22 (71%) of 31 Anseriformes but in none of 16 Passeriformes. However, at 1 mo the antibody prevalences at 1 mo were similar: 84% among the Anseriformes and 73% among the Passeriformes; at 3 mo the prevalences were 50% in Anseriformes and 15% in Passeriformes. Forty-two percent of the Anseriformes had detectable antibodies even 12 mo after inoculation.

Key words: Ockelbo virus, Sindbis virus, Alphavirus, neutralizing antibodies, immune response, birds, Anseriformes, Passeriformes.

INTRODUCTION

Ockelbo virus, a north European subtype of Sindbis virus (Togaviridae: *Alphavirus*) (Lundström et al., 1993a), is a mosquito-borne arbovirus infecting birds of the orders Passeriformes, Columbiformes (Francy et al., 1989), Anseriformes, and Galliformes (Lundström et al., 1992). The virus also infects humans causing Ockelbo disease with arthralgia, rash, and sometimes fever (Skogh and Espmark, 1982). Surveys for alphavirus infections in birds often are based on detection of specific antibodies. However, alphaviruses induced a weak and sometimes transient antibody response especially in passeriforms in some studies (McIntosh et al. 1969a; Main et al., 1988), but produced long lasting antibodies in other studies (Dalrymple et al., 1972). Differences in alphavirus antibody response among bird taxa may therefore influence the results of serological surveys of wild free ranging birds.

Our objective was to evaluate the induction and longevity of Ockelbo virus neutralizing (Nt) antibodies in native Swedish birds. Captive anseriforms, galliforms and passeriforms were examined for these antibodies during 1 yr after inoculation with virus.

MATERIALS AND METHODS

The second cell passage of the Ockelbo virus (strain Edsbyn 82/5) was used in this study (Niklasson et al., 1984). Vero cells (American Type Culture Collection, Rockville, Maryland, USA) were used for passage of virus as well as for the assay of Ockelbo Nt antibodies. Virus Nt antibodies were determined by plaque-reduction neutralization test in 24-well cell culture plates with 2 cm² per well (Costar, Cambridge, Massachusetts, USA) using heat-inactivated (56 C, 30 min) whole blood samples (Earley et al., 1967). Diluted blood samples were tested in serial four-fold dilutions, starting at 1:20 (sample diluted 1:10 and mixed with equal volume of virus solution), two wells per dilution, against 30 to 80 plaque forming units (PFU) of virus per well. After 1 hr incubation at 37 C, the blood-virus mixture was inoculated onto the confluent cell monolayers. Following 1 hr adsorption at 37 C in an atmosphere with 5% CO₂, the first agar overlay was added. Following a further 24 hr incubation, the second agar overlay with neutral red stain was added, and the plaques were counted at 45 hr. The Nt antibody titer was expressed as the reciprocal dilution giving ≥80% reduction in plaque num-

TABLE 1.   Prevalence of Ockelbo virus-neutralizing antibodies in birds one month after experimental infection, in relation to immune status prior to inoculation of virus and viremia status after infection.

Common and scientific name	Non-immune[a]		Immune
	Viremia positive[b]	Viremia negative	Viremia negative
Canada goose (*Branta canadensis*)	100 (2)[c]	80 (5)	100 (1)
Bean goose (*Anser fabalis*)	NT	100 (1)	100 (1)
Mallard (*Anas plathyrhynchos*)	100 (7)	60 (10)	NT
Goldeneye (*Bucephalus clangula*)	100 (5)	100 (1)	NT
Subtotal Anseriformes	100 (14)	71 (17)	100 (2)
Capercaillie (*Tetrao urogallus*)	100 (2)	NT	NT
Subtotal Galliformes	100 (2)	NT	NT
European blackbird (*Turdus merula*)	NT	NT	100 (1)
Fieldfare (*Turdus pilaris*)	NT	NT	100 (2)
Great tit (*Parus major*)	NT	100 (2)	NT
House sparrow (*Passer domesticus*)	100 (1)	ND (1)	NT
Chaffinch (*Fringilla coelebs*)	NT	NT	100 (1)
Greenfinch (*Carduelis chloris*)	100 (5)	ND (1)	100 (3)
Yellowhammer (*Emberiza citrinella*)	NT	60 (5)	NT
Subtotal Passeriformes	100 (6)	56 (9)	100 (7)
Grand total	100 (22)	65 (26)	100 (9)

[a] Pre-inoculation blood was tested for Ockelbo virus neutralizing antibodies in a plaque-reduction neutralization test that could detect antibody titers ≥1:20.

[b] Blood samples from days 1 to 5 after inoculation of virus were tested for Ockelbo viremia in a plaque-test that could detect infective virus at a concentration ≥100 PFU/ml.

[c] Percent antibody prevalence (number of birds tested); NT = not tested; ND = not detected.

bers as compared to the average counts of control wells.

Fifty-seven birds of 12 species were included in the study (Table 1). The bird species were selected based either on the natural occurrence of Ockelbo virus neutralizing antibodies (capercaillie, *Tetrao urogallus*; Canada goose, *Branta canadensis*; bean goose, *Anser fabalis*; fieldfare, *Turdus pilaris*; house sparrow, *Passer domesticus*; chaffinch, *Fringilla coelebs*; and yellowhammer, *Emberiza cintrinella*), absence of these antibodies although several birds have been tested (mallard, *Anas plathyrhynchos*; great tit, *Parus major*) (Francy et al., 1989; Lundström et al., 1992), or that the species (goldeneye, *Bucephalus clangula*; European blackbird, *Turdus merula*; greenfinch, *Carduelis chloris*) occurred in central Sweden (Bruun et al., 1986). All birds originated from or near the Boda Wildlife Research Station (Boda) (61°32'N, 17°52'E), situated within the Ockelbo disease enzootic area in central Sweden (Lundström et al., 1991). Anseriforms and galliforms were from a stock of indigenous Swedish birds hatched in captivity at Boda, whereas passeriforms were captured from wild bird populations in the Boda area. All birds were held in a mosquito-proof aviary for 3 wk after inoculation with virus, to prevent accidental viral transmission during the viremic period. The primaries on the right wing of anseriforms (Canada goose, bean goose, mallard, and goldeneye) were cut to prevent escape and these birds then were held together in a dam surrounded by a fence. The capercaillies were held in an outdoor cage. The passeriforms (European blackbird, fieldfare, great tit, house sparrow, chaffinch, greenfinch, and yellowhammer) were held in an indoor aviary. The experiment was started 6 June 1990, when pre-inoculation blood samples were taken, and was terminated 27 July 1991, when blood samples were obtained from the remaining birds.

Birds were inoculated subcutaneously with $10^{2.7}$ plaque-forming units (PFU) of Ockelbo virus 1 to 4 days after arrival at the aviary. Sequential blood samples were taken daily for the first 5 days and then at 1, 3, 6, 9, and 12 mo after inoculation. Specimens collected at arrival and on days 1 to 5 after inoculation were tested for viremia by plaque assay (Lundström et al., 1993b). Up to 49 birds per day (Fig. 1) were tested during the six sampling periods for serology (at day 5, and at 1, 3, 6, 9 and 12 mo), but only 26 anseriforms, one galliform, and three passeriforms were followed during the entire 12-mo period. Blood samples (0.1 ml) were diluted in 0.9 ml of Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, Missouri, USA) supplemented with 10% heat-in-

activated fetal bovine serum, penicillin 100 units/ml and streptomycin 100 μg/ml (Sigma Chemical Co.), and Hepes buffer (Sigma Chemical Co.). Diluted blood samples were held at approximately 18 C for less than 3 hr and then stored at −20 C until tested. The offspring from experimentally infected mallards were bled the day after hatching for detection of passively-acquired Nt antibodies.

Differences in Nt antibody prevalence between bird groups were evaluated by the Fisher exact probability test for 2 × 2 tables (Siegel and Castellan, 1989).

RESULTS

One bean goose, one Canada goose, one European blackbird, two fieldfares, one chaffinch, and three greenfinches had detectable Ockelbo virus Nt antibodies prior to virus inoculation (Table 1). No significant rises in titer were recorded in any of these nine birds. Due to prior Ockelbo virus infection, they were excluded from further analysis.

All birds with a detectable Ockelbo viremia of ≥100 PFU (the lower limit for the plaque assay) within 5 days post inoculation developed detectable Nt antibodies 4 wk later, whereas 12 of 17 anseriforms and five of nine passeriforms without detectable viremia seroconverted at 1 mo (Table 1). The antibody prevalence was significantly (P = 0.002) higher in birds that produced a viremia than in those without detectable viremia.

Ockelbo virus Nt antibodies occurred significantly (P < 0.0001) earlier in anseriforms than in passeriforms. Five days after inoculation, 22 of 31 anseriforms but none of the 16 passeriforms had detectable antibodies.

Eleven (69%) of 16 anseriforms with detectable Nt antibodies to Ockelbo virus at 1 mo post-inoculation, and tested at 12 mo, maintained detectable antibodies for the whole study period (Fig. 1). In contrast, the antibody prevalence in passeriforms decreased from 11 (73%) of 15 birds at 1 mo to two (15%) of 13 birds at 3 mo after inoculation. Thus, one greenfinch and one yellowhammer with Ockelbo virus Nt antibodies at 1 mo maintained signifi-

cant antibody titers for another 2 mo, while one house sparrow, three greenfinches, and two yellowhammers reverted from seropositive to negative.

One of nine newly hatched mallards contained Nt antibodies 1 day after hatching; thus these were maternal antibodies. The efficiency of transfer of maternal antibodies could not be determined because the eggs were not from identified females.

DISCUSSION

The best single indicator of active participation of a vertebrate species in an arbovirus transmission cycle is frequent isolation of virus from free-ranging individuals (Scott, 1988). Many alphaviruses seldom are recovered from wild vertebrates, however, because viremias usually are of brief duration. Consequently, the classification of vertebrate species into categories of varying host potential often is dependent upon the detection of virus-specific antibodies.

Based on our results, we propose that interspecific variation in the duration of detectable Ockelbo Nt antibodies can cause variation in the antibody prevalence among bird taxa. Similarly, McIntosh et al. (1969a) observed that Nt antibodies to Sindbis virus were undetectable in six of 10 passeriforms (red bishop, *Euplectes orix*) at 12 mo after infection, and in three of five passeriforms (masked weaver, *Ploceus velatus*) at 24 mo, while these antibodies were detectable for 12 mo in two passeriforms (one house sparrow and one cape sparrow *Passer melanurus*) and for 18 mo in the 11 columbiforms (five Cape turtle dove, *Streptopelia capiola,* and six laughing doves *Streptopelia senegalensis*) tested. A variable duration of Nt antibodies in passeriforms and a generally longer duration of Nt antibodies in non-passeriforms also have been shown for New World alphaviruses. Main et al. (1988) demonstrated variability in the duration of Nt antibodies against eastern equine encephalomyelitis (EEE) and Highlands J (HJ) viruses in repeatedly sampled free-
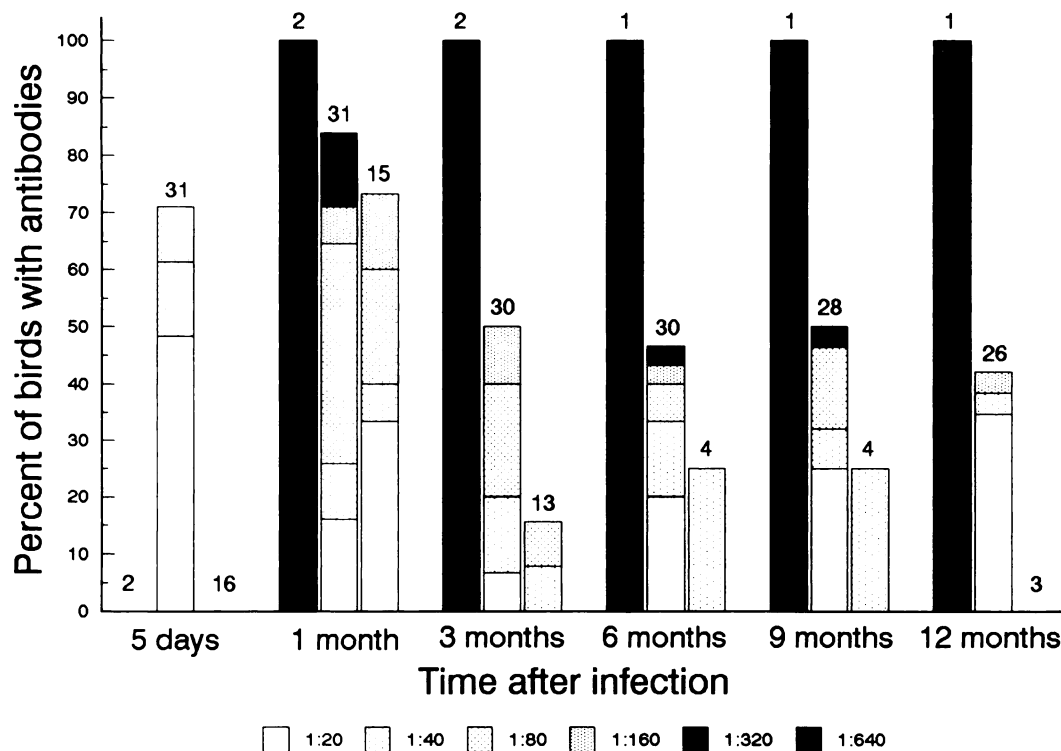
FIGURE 1.   Prevalence of Ockelbo virus-neutralizing antibodies and antibody titer distribution in experimentally infected birds at intervals up to 12 months after infection. For each day, the left bar indicates Galliformes, the middle bar Anseriformes, and the right bar Passeriformes. The number of birds tested each day, which are combined figures for viremic and non-viremic birds, is given at the top of the bars.

ranging North American passeriforms. They found that 18 of 22 recaptured and naturally infected black-capped chickadee's *Parus atricapillus*, lost detectable Nt antibodies to EEE virus in 1 to 44 wk (mean 10 wk between a positive and a subsequent seronegative sample) after a positive sample, and 11 of 16 chickadee's lost detectable Nt antibodies to HJ virus in 1 to 48 wk (mean 18 wk). Reversions from Nt antibody positive to seronegative against either EEE or HJ virus after an ultimate Nt antibody positive sample also occurred in recaptured grey catbirds (*Dumetella carolinensis*), swamp sparrows (*Melospiza georgiana*), and veeries (*Catharus fuscescens*), while the duration of Nt antibodies to EEE or HJ virus often exceeded 1 yr in blue jays (*Cyanocitta cristata*), and brown thrashers (*Toxostoma rufum*). Production of western equine en-

cephalomyelitis (WEE) virus Nt antibodies was maintained for 2 yr in nine naturally immunized domestic sparrows (Holden et al., 1973). However, in non-passeriforms the Nt antibodies to alphaviruses generally are detectable at least 6 to 12 mo as shown for EEE virus Nt in a white ibis (*Guara alba*) (Kissling et al., 1954a), in a bobwhite quail (*Colinus virginianus*) (Dalrymple et al., 1972), and in 11 whooping cranes (*Grus americana*) (Dein et al., 1986); it also was observed for WEE Nt antibodies in five bobwhite quail (Dalrymple et al., 1972) and four domestic pigeons (*Columba livia*) (Reisen et al., 1992). Obviously, the variable longevity of alphavirus Nt antibody among bird taxa, especially in passeriforms could influence the reliability of results from serological surveys to determine infection prevalences in wild bird populations. The short dura-

tion of Nt antibodies to Sindbis, Ockelbo, EEE, HJ, and WEE viruses in some passeriform species is evidence that negative antibody results in this group of birds should be interpreted with caution.

Ockelbo Nt antibodies at titers ≤1:20 were not detectable by the present technique, and therefore birds with a 1:10 titer of antibodies would have been considered antibody negative. Virus Nt antibody at a titer of 1:10, however, was reported for EEE virus in whooping cranes (Dein et al., 1986), VEE virus in common egrets (*Casmerodius albus*) (Bowen and McLean, 1977), and for Ockelbo virus in Canada geese (Lundström et al., 1992). However, of 26 Swedish birds with naturally acquired Ockelbo virus Nt antibodies, the titer distribution was 1:10 for one bird, 1:20 for two birds, 1:40 to 1:320 for 21 birds, and ≥1:640 for two birds (Lundström et al., 1992). Thus, Ockelbo virus Nt antibodies at a 1:10 titer occur infrequently in naturally infected free-ranging Swedish birds.

We found that all birds producing viremia after experimental Ockelbo virus inoculation also produced Nt antibodies detectable at 4 wk after inoculation. Similarly, all domestic fowl (*Gallus gallus*) that became viremic after inoculation with EEE virus (Calisher et al., 1986), and all domestic pigeons that became viremic after inoculation with WEE virus (Winn et al., 1957), produced Nt antibodies detectable 1 mo after inoculation. In other experimental studies the alphavirus Nt antibody response in birds were more variable (McIntosh et al., 1969b; Bowen and McLean, 1977), and ranged from 17 to 58% in passeriforms inoculated with Sindbis or Venezuelan equine encephalomyelitis (VEE) viruses, 55% in anseriforms, 90 to 100% in columbiforms inoculated with Sindbis virus, and 95% in ciconiiforms inoculated with VEE virus. The reported variance in antibody response may have been caused by failure of non-replicating virus to induce an immune response that could be detected by the methods employed. However, McIntosh et al. (1969a)

claimed that only one of four masked weavers that became viremic after inoculation of Sindbis virus produced Nt antibodies detectable 1 mo after infection. Thus, it appears that in some combinations of birds and alphaviruses, even a detectable viremia may be insufficient to stimulate a detectable Nt antibody response.

A high proportion of anseriforms and passeriforms developed Ockelbo Nt antibodies without a detectable viremia. The antibody production may either have represented an anamnestic immune response or could have been induced by viruses replicating at low titers. However, the experimental infection did not induce increased production of Ockelbo Nt antibodies in the birds containing detectable concentration of Nt antibodies. Reisen et al. (1992) found an insignificant increase of Nt antibody titers in pigeons rechallenged with a low dose of WEE virus at 16 wk after the first infection. Further experimental studies are needed to quantify the anamnestic immune response in birds with undetectable antibodies.

The occurrence of Ockelbo virus Nt antibodies in a hatchling mallard was not unexpected. Kissling et al., (1954b) and Holden et al., (1973) found that birds could transfer alphavirus Nt antibodies to their progeny. Interestingly, nestling house sparrow progeny from WEE virus immune mothers were susceptible to challenge with the same virus (Holden et al., 1973). These nestlings produced WEE viremia of high titer, although they contained WEE virus Nt antibodies derived from their mothers.

Lundström et al. (1993b) showed that experimentally infected passeriforms produced Ockelbo viremia of higher titer and longer duration than did similarly infected anseriforms. Peak mean daily viremia titers were $10^{5.8}$ to $10^{7.5}$ PFU/ml blood in the passeriforms, and only $10^{3.7}$ to $10^{4.5}$ PFU/ml blood in the anseriforms. In the present study, we examined a portion of these birds for Ockelbo virus Nt antibodies and observed that none of the passeriforms but

almost all the anseriforms had antibodies at 5 days after inoculation of virus. The slower immune response in passeriforms was correlated positively with prolonged viremia. Thus, the higher experimental viremia response of passeriforms versus anseriforms, could in part be explained by the significantly slower Nt antibody response in passeriforms.

In conclusion, the rapid and relatively long lasting Ockelbo virus Nt antibody response in anseriforms makes detection of these antibodies relatively reliable markers of previous infection, whereas the short duration of Nt antibodies in passeriforms makes detection of antibodies useful mainly for determination of recent virus exposure. Passeriforms without detectable Ockelbo virus Nt antibodies may either never have been infected, have been infected a few days ago, or were infected several months ago; but antibodies were undetectable by the present technique. The present results provides additional evidence that passeriforms naturally are infected more frequently with Ockelbo virus than are anseriforms. The previously shown high Ockelbo Nt antibody prevalence in passeriforms probably represents a high frequency of recently acquired infections, whereas the low prevalence in anseriforms may represent the accumulated risk for Ockelbo infections over more than one season.

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