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REVIEW ARTICLE

Risk of resistant avian influenza A virus in wild waterfowl as a result of environmental release of oseltamivir

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Oseltamivir is the best available anti-influenza drug and has therefore been stockpiled worldwide in large quantities as part of influenza pandemic preparedness planning. The active metabolite oseltamivir carboxylate (OC) is stable and is not removed by conventional sewage treatment. Active OC has been detected in river water at concentrations up to 0.86 μg/L. Although the natural reservoir hosts of influenza A virus (IAV) are wild waterfowl that reside in aquatic environments, the ecologic risks associated with environmental OC release and its potential to generate resistant viral variants among wild birds has largely been unknown. However, in recent years a number of *in vivo* mallard (*Anas platyrhynchos*) studies have been conducted regarding the potential of avian IAVs to become resistant to OC in natural reservoir birds if these are drug exposed. Development of resistance to OC was observed both in Group 1 (N1) and Group 2 (N2, N9) neuraminidase subtypes, when infected ducks were exposed to OC at concentrations between 0.95 and 12 μg/L in their water. All resistant variants maintained replication and transmission between ducks during drug exposure. In an A(H1N1)/H274Y virus, the OC resistance mutation persisted without selective drug pressure, demonstrating the potential of an IAV with a permissive genetic background to acquire and maintain OC resistance, potentially allowing circulation of the resistant variant among wild birds. The experimental studies have improved the appreciation of the risks associated with the environmental release of OC related to resistance development of avian IAVs among wild birds. Combined with knowledge of efficient methods for improved sewage treatment, the observations warrant implementation of novel efficient wastewater treatment methods, rational use of anti-influenza drugs, and improved surveillance of IAV resistance in wild birds.

Keywords: *influenza A*; *avian influenza*; *resistance*; *oseltamivir*; *environmental*; *mallard*; *antiviral resistance*; *sewage treatment*

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Influenza A virus (IAV) belongs to the Orthomyxoviridae family and is an enveloped, negative-sense single-stranded RNA virus with a segmented genome (1) that can infect many animal species, including humans, although the major natural reservoir hosts are wild waterfowl (2, 3).

All known human IAVs contain genetic segments that originate from IAVs of wild birds (4, 5). If an avian (or swine) origin IAV with new antigenic properties and with the ability to efficiently replicate and transmit between humans is introduced to the human population, a rapid and widespread pandemic disease can occur, as was the case with the Spanish flu (H1N1) in 1918–1919, the Asian flu (H2N2) in 1957, the Hong Kong flu (H3N2) in 1968, and the swine flu (H1N1) in 2009 (4, 5). Pandemic viruses often give rise to severe disease with high mortality (2, 6), but as a result of acquired immunity in the population and antigenic drift of the virus the severity is then gradually reduced to a moderate airway disease when pandemic viruses continue to circulate as new seasonal IAV variants (1, 5). Thus far only the H1N1, H2N2, and H3N2 subtypes have caused widespread human disease and become human-adapted viruses (2, 4, 5). However, aside from human-adapted viruses, avian IAVs may occasionally infect humans and can sometimes give rise to very severe generalized disease with high mortality, as is the case with the highly pathogenic avian influenza (HPAI) A(H5N1) virus and the Chinese influenza A(H7N9) virus (7–9).

The ideal means of prevention of human IAV infections is vaccination, which reduces morbidity and mortality (10). However, a disadvantage with influenza vaccines for the prevention of seasonal influenza is that they are made well in advance of the influenza season and the actual

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circulating variants may not perfectly match the antigenicity of the chosen vaccine strains. Regarding pandemic vaccines, a major drawback is that they cannot be made in advance as the antigenicity remains unknown until the virus emerges and thus the final distribution of a new vaccine can only be achieved after several months (11, 12). Therefore, a cornerstone in the treatment and prevention of new human pathogenic influenza viruses, as well as of severe seasonal cases, is direct anti-influenza drugs.

Neuraminidase inhibitors and resistance

Since the widespread global resistance to the adamantane M2 ion channel inhibitors in the mid-2000s (13, 14), the neuraminidase inhibitors (NAIs) have been the best drugs available to treat severe influenza infections (15). Zanamivir (ZA) and oseltamivir have been globally approved since 1999, whereas peramivir and laninamivir have been approved in Japan and a few other countries since 2010 (13, 16).

Among the NAIs the orally available oseltamivir (Tamiflu®) is the most used and is the primary drug that has been stockpiled worldwide as part of pandemic preparedness planning (17, 18). After oral administration of the ethyl ester prodrug oseltamivir phosphate, which is converted by liver esterase, 75% of an oral dose reaches the plasma as active oseltamivir carboxylate (OC), which is then eliminated unchanged in the urine (19). OC and all other NAIs act by competitive binding to the extracellular enzymatic site of the neuraminidase (NA) protein. Thereby, the NA binding and the catalytic destruction of its sialic acid targets is inhibited and the release of newly formed virions from infected cells and viral spread through respiratory secretions is reduced (16). NAI binding to the enzymatic target differs slightly between the structurally different neuraminidase (NA) proteins (20), which are phylogenetically grouped to Group 1 (including subtypes N1, N4, N5, N8) and Group 2 (including subtypes N2, N3, N6, N7 and N9) (1, 21).

The NA group–specific differences also have implications for which resistance-related amino acid substitutions may evolve under drug pressure (22, 23). Resistance to NAIs is primarily caused by amino acid substitutions in the NA protein leading to reduced binding of the drugs, either by substitution of active site residues or of framework residues (22, 24). In IAVs containing N1 NA proteins the most common resistance-related change seen *in vivo* is the framework substitution H274Y (N2 numbering, used hereafter), whereas in N2-containing viruses the framework E119V and active-site R292K substitutions are most commonly described (22, 25).

Resistance to OC and ZA was described *in vitro* during the drug development phases and was accompanied by the demonstration of reduced viral fitness of resistant variants (26–28). NAI treatment of IAV-infected humans may also generate resistant viruses and is primarily described following treatment with oseltamivir and occurs both in human (29–31) and avian viruses (22, 32, 33). Clinically, resistance to NAIs is associated with prolonged infections in children and in immunosuppressed patients (29, 34); and in A(H7N9) and HPAI A(H5N1) cases with high viral loads and severe clinical outcomes (32, 33). Following market introduction, the overall OC resistance in clinical settings was reported to be less than 1% in adults and 4% in children under 12 years of age but higher in hospitalized children, immunocompromised individuals, and in HPAI A(H5N1) infected patients (22). Although much less common than OC resistance (35), ZA resistance in ZA treated patients has also been described (22, 36).

However, raising more concern than selection for resistant variants by clinical treatment is the circulation of NAI-resistant human strains in the absence of selective drug pressure. This feature was primarily described in the seasonal A(H1N1) virus 2007–2009 when the circulating strain was OC resistant due to an H274Y substitution in NA, without selective drug exposure (37). In 2009, the resistant seasonal A(H1N1) virus was entirely replaced by the NAI-susceptible (though adamantane-resistant) pandemic A(H1N1)/pdm09 virus. However, since 2010/2011, an increasing number of community cases with OC-resistant A(H1N1)/pdm09 viruses have been reported without previous oseltamivir exposure; in nearly all instances the resistance has been conferred by the H274Y substitution in NA (38–41).

Avian IAVs of wild waterfowl have been tested for NAI susceptibility only to a very limited extent compared to human viruses, especially regarding subtypes other than those infecting humans (42). Two screening studies on avian N1 and N6 subtypes, each containing fewer than 100 samples mainly collected from North America between 1976 and 2010, as well as a European study with 21 samples collected between 2002 and 2005, did not detect naturally occurring high-level resistance among wild bird avian IAVs (42–44).

Environmental pollution with NAIs

After administration of both oseltamivir and ZA, 75–80% of the active OC and ZA are excreted by urine or feces (19, 45). Both components are poorly removed by conventional sewage treatment and therefore end up in aquatic environments (46, 47). OC is the most studied substance, both regarding drug measurements in aquatic environments and regarding experimental studies on degradation and removal of the metabolite from water.

There is a correlation between the amount of oseltamivir that is prescribed to patients and the OC concentrations detected in effluents of sewage treatment plants (STPs) and in river water, with higher drug concentrations in STP effluents (48–53). It is however the presence of

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active drugs in aquatic environments that can be expected to have an ecological effect.

In Japan, which accounts for over 70% of the global oseltamivir prescription (54, 55), numerous environmental measurements over the last 5–10 years have detected OC in river water in the range of a few 100 ng/L up to 865 ng/L (48, 51, 56–60). Studies in a number of European countries have detected OC in river water at average concentrations of approximately 50 ng/L, with a range up to 200 ng/L (49, 50, 52, 61, 62). Samples from the Rhine River at the border between Germany, France, and Switzerland, contained high concentrations of non-metabolized OP relative to OC (OP/OC ratio 13.1 as compared to <1 in STP effluents), indicating release from drug manufacturing (in Switzerland) in addition to sewage discharge (52).

The lower use of ZA, peramivir, and laninamivir as compared to OC is reflected in lower levels of active drugs released to aquatic environments. In Japan, drug concentrations of up to 59 ng/L of ZA, 11 ng/L of peramivir, and 9 ng/L of laninamivir have been measured in river water, with dynamics that are correlated with the number of influenza cases during the influenza season (53, 58, 60).

Environmental risk assessment of NAIs in aquatic systems includes evaluation of eco-toxicological effects and of direct antiviral effects on naturally circulating IAVs, including the potential for resistance development. There is a lack of knowledge regarding the eco-toxicological effects by OC, and prediction studies by mathematical modeling have led to varying conclusions regarding toxic effects on algae and fish (63–66).

Removal of oseltamivir by sewage water treatment

Conventional STPs use different techniques to remove waste products, usually a combination of mechanical treatment followed by chemical and biological (active sludge) treatment (46, 52, 59). Measurements of pharmaceuticals from influents and effluents of conventional STPs have demonstrated between 0% (49, 59) and 59% OC removal by the treatment (52). Experimental studies have demonstrated that OC is not removed by conventional sewage treatment (46) nor degraded by UV light exposure (46, 67), which often is an important degradation mechanism of drug metabolites in the environment (68).

In studies investigating other potential degradation methods, bacterial strains able to degrade and use OC as their sole carbon and energy source (a *Nocardioides* sp. and a *Flavobacterium* sp.) were isolated from environmental water sediments, suggesting biological degradation pathways of OC (69). Accordingly, in several degradation studies in water, microbial processes were demonstrated to be important for the dissipation of OC from waste water. OC removal can be increased by addition of active microbial sludge (70, 71), active sediments from natural waters (69, 72), and by fungal (*Phanerochaete chrysosporium*) exposure (73). The results of biodegradation studies have led to suggestions for bioremediation approaches in sewage treatment (69, 72), though their efficiency might be questioned if the OC load were very high, that is in a pandemic situation (71).

Adding ozone treatment to the conventional methods of wastewater treatment has proven very efficient in removing OC and other NAIs experimentally (53, 74). Confirming the experimental data, drug measurements from STP influents and effluents at units that use ozone treatment in addition to conventional techniques have repeatedly demonstrated significantly lower concentrations of released drugs as compared to conventional STPs. Over 85% of all NAIs are removed by adding a tertiary sewage treatment with ozone (51, 53, 59, 60, 75).

Influenza A in the natural hosts

The natural reservoir host of IAV is wild waterfowl, primarily Charadriiformes (in particular gulls, terns, and waders) and Anseriformes (in particular ducks, geese, and swans) (2, 3). Most subtype combinations of the 16 hemagglutinin (HA) and 9 NA surface proteins can be detected in wild waterfowl. Despite more pronounced subtype diversity among shorebird viruses, viral prevalence is highest in dabbling ducks (76, 77), among which the mallard (*Anas platyrhynchos*) is considered to be the most common IAV host species (78). During the autumn migration of birds in the Northern Hemisphere, the IAV prevalence typically peaks in dabbling ducks at up to 60% compared to 0.4–2% at wintering grounds (2, 3, 79). On the contrary, in the Delaware Bay on the North American east coast IAV prevalence is exceptionally high in shorebirds during the spring migration with over 10% prevalence, which is much higher than elsewhere and coincides with the congregation of waders and gulls foraging for horseshoe crab eggs (3, 76, 77, 80, 81).

In wild waterfowl IAVs cause an intestinal tract infection, and although large amounts of virus are shed in feces the infection is relatively asymptomatic (2, 82, 83). The temporal and spatial dynamics, as well as the evolution of IAVs in the natural hosts, are closely related to the ecology, immunology, and migration of the birds (3). As dabbling ducks switch breeding grounds between years, there are opportunities for viral transmission to different subpopulations over wide geographical areas (3, 78, 84). Perpetuation of low pathogenic avian influenza (LPAI) viruses in wild waterfowl year round is suggested to be a combination of 1) continuous transmission to juvenile and non-immune ducks at breeding and pre-migration congregation areas; 2) spread of virus with migrating birds; and 3) low prevalence circulation in resident ducks during the winter season in temperate locations (2, 85, 86). In addition, IAVs are known to stay infectious for a long time in lake water (2); freezing of viruses in lakes at breeding areas with

reinfection of birds the following season is suggested to be another mechanism for viral perpetuation (78).

The genetic variability of wild waterfowl viruses is much greater than that of IAVs in other hosts, including combinations of most HAs and NAs without persisting sublineages (2) and a high diversity between HA and NA subtypes as well as of the nonstructural (NS) gene (87, 88). There is a continuous emergence of new viral variants, achieved both by frequent point mutations (genetic drift) (89, 90) and, typical for the segmented IAV genome, by reassortment events (genetic shift), which occur at a high frequency as a result of very common coinfections with more than one IAV in wild waterfowl (86, 87, 91).

Risk for resistant IAV of wild waterfowl

Despite the fact that natural IAV hosts reside in aquatic environments potentially polluted by NAIs, resistance screening of avian IAVs carried by wild waterfowl has to date been limited, as discussed above. Experimental *in vivo* systems testing the hypothesis that OC exposure of IAV-infected natural host birds leads to resistance development have confirmed that avian IAVs containing both Group 1 and Group 2 NAs become resistant when infected mallards are exposed to OC in their water (92–95). When mallards experimentally infected with an avian A(H1N1) virus were exposed to 0.95 μg/L of OC in their water, OC resistance conferred by the H274Y substitution in NA evolved. Despite the H274Y resistance substitution, leading to highly reduced OC susceptibility, infectivity and transmissibility between mallards was maintained (92). In a subsequent study in the same experimental model with the resistant A(H1N1)/H274Y variant in which drug exposure was removed from infected mallards, the resistant variant persisted without drug pressure (96). Maintenance of a resistant genotype and phenotype without selective drug pressure suggests a maintained viral fitness as compared to wild-type virus. In the same *in vivo* mallard model an A(H6N2) virus acquired high-level OC resistance conferred by the R292K substitution when ducks were exposed to 12 μg/L of OC (94), while a low pathogenic A(H7N9) virus acquired the resistance-related I222T framework substitution at 2.5 μg/L of OC exposure (95). Infectivity and transmissibility between mallards was maintained during drug pressure by both of the viruses, but when the resistant A(H6N2)/R292K variant was allowed to replicate in mallards without drug pressure it reverted to wild type, confirming a reduced viral fitness of the resistant variant (97). In another *in vivo* mallard model, selection for the framework NA substitution E119V in a low pathogenic A(H5N2) virus was demonstrated when infected ducks were exposed to 1 μg/L of OC in water. The resistant A(H5N2)/E119V variant dominated the viral population and was transmissible between mallards, but it was outcompeted by wild-type virus when drug exposure was removed (93).

The available results from experimental OC exposure studies of avian IAVs demonstrate that both Group 1 and Group 2 viruses may acquire resistance if the natural host birds are exposed to low levels of OC in their water. The propensity to maintain the acquired resistance without drug pressure varies between NA groups, subtypes, and strains. The different propensity is influenced by the subtype-specific resistance substitutions, as a framework substitution like H274Y may be compensated for more easily without compromising viral fitness than an active site residue, like R292K. Clearly, the exact genetic context in which a resistance mutation is induced is paramount for the potential of its persistence without drug pressure and for the potential of the resistant viral variant to circulate among wild birds.

In human IAVs, resistance to OC occurs in clinical settings both in A(H3N2) and in A(H1N1) viruses (22), but thus far only resistant A(H1N1) strains have circulated in the community without drug pressure. The dependence on the genetic context in which a resistance mutation is acquired is demonstrated by the permissive mutations compensating for reduced viral fitness. Different permissive mutations have been identified for OC-resistant human A(H1N1) viruses in which the resistance is conferred by the H274Y substitution, including the seasonal virus in 2007–2009 (37, 98, 99) and OC-resistant A(H1N1)/2009pdm/H274Y community clusters in Australia and Japan (40, 41, 100).

In the experimental OC-exposure mallard studies, the drug concentrations at which resistant variants emerged varied between studies. In the environment, numerous measurements of OC in the main river systems in Japan have confirmed concentrations up to 0.86 μg/L (57), while European studies at several river sites have detected OC in the range of 0.02–0.2 μg/L (50, 52, 62). The drug concentrations at which avian IAVs experimentally developed resistance in mallards were thus above the environmental ones detected to date. However, they are in the same magnitude, and as the OC levels in river water vary with oseltamivir consumption (48, 61) and with the quality of sewage treatment (51) higher environmental concentrations may occasionally occur. Although the exposure of aquatic birds may be lower at STP effluents, it should be noted that OC concentrations in these locations are multiples higher than in river water (48, 49, 51).

As IAVs acquire resistance mutations at various drug concentrations, predictions on the amount of pharmaceuticals in the environment that constitute a risk for resistance induction are uncertain. The high genetic variability of avian IAVs (87) provides the opportunity for genetic contexts to be permissive if selective pressure for resistance occurs. The experimental results from OC exposure studies over the last couple of years (92–97) have contributed to the estimations of the ecologic risks related to the release of active NAIs to the environment.

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The results indicate that there is a risk for the evolution of resistant avian IAVs in the natural host birds.

Knowledge on the levels of OC released to aquatic environments (50, 57), combined with the observations of IAV resistance development in experimentally exposed host birds, warrants broad implementation of new efficient sewage water treatment techniques (59) and rational use of available NAIs.

It remains an area of research whether an OC-resistant waterfowl-adapted IAV may maintain a resistance trait through the complex evolutionary process to a new resistant human pathogenic virus. If such a virus were to emerge and be highly pathogenic to humans, the current stockpiles of oseltamivir would be of no use and the public health impact of a pandemic would be substantially worsened. Other future areas of research include increased OC resistance screening of avian IAVs of wild waterfowl, both regarding the number of tested samples and geographic regions, as well as the evaluation of the potential for resistance development to other NAIs in natural host birds. A number of new anti-influenza drugs are expected to be introduced to the market within the coming years (13). The development of new influenza drugs needs to include studies on their ecologic and environmental impact, including their potential to generate resistant IAVs in wild birds.

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