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Research paper

Urbanization and the dynamics of RNA viruses in Mallards (*Anas platyrhynchos*)

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ABSTRACT

Urbanization is intensifying worldwide, and affects the epidemiology of infectious diseases. However, the effect of urbanization on natural host-pathogen systems remains poorly understood. Urban ducks occupy an interesting niche in that they directly interact with both humans and wild migratory birds, and either directly or indirectly with food production birds. Here we have collected samples from Mallards (*Anas platyrhynchos*) residing in a pond in central Uppsala, Sweden, from January 2013 to January 2014. This artificial pond is kept ice-free during the winter months, and is a popular location where the ducks are fed, resulting in a resident population of ducks year-round. Nine hundred and seventy seven (977) fecal samples were screened for RNA viruses including: influenza A virus (IAV), avian paramyxovirus 1, avian coronavirus (CoV), and avian astrovirus (AstroV). This intra-annual dataset illustrates that these RNA viruses exhibit similar annual patterns to IAV, suggesting similar ecological factors are at play. Furthermore, in comparison to wild ducks, autumnal prevalence of IAV and CoV are lower in this urban population. We also demonstrate that AstroV might be a larger burden to urban ducks than IAV, and should be better assessed to demonstrate the degree to which wild birds contribute to the epidemiology of these viruses. The presence of economically relevant viruses in urban Mallards highlights the importance of elucidating the ecology of wildlife pathogens in urban environments, which will become increasingly important for managing disease risks to wildlife, food production animals, and humans.

1. Introduction

Urbanization is intensifying worldwide; most humans live in urbanized areas, and the urban human population is expected to continue to grow (United Nations Population Fund, 2007). Within the global growth of cities, urbanization increasingly shapes the emergence and trajectory of infectious disease, both human disease and disease and parasitism in wild animals (Alirol et al., 2011; Neiderud, 2015). In association with urbanization, factors affecting pathogen (and parasite) transmission in wild animals include an increase in aggregation and resource availability resulting in increased contact rates, decrease in biodiversity, modulation in host immunity and stress levels (Becker and Hall, 2014; Becker et al., 2015; Bradley and Altizer, 2006; Delgado and French, 2012; Patz et al., 2004; Penczykowski et al., 2014). Furthermore, in cities, increased contact among humans, domestic animals and wild animals may facilitate cross species spillover of (vertebrate) pathogens, with consequences for wildlife conservation, agriculture, and human health (Becker et al., 2015; Bradley and Altizer, 2006; Delgado and French, 2012; Patz et al., 2004).

Influenza A virus (IAV) is a multi-host virus, wherein spillover between birds, humans and agricultural animals does occur, and dabbling ducks, such as those found in city parks, constitute the main reservoir host for these viruses (Olsen et al., 2006; Webster et al., 1992). Indeed, highly urbanized areas may contain canals and large city parks with ponds housing a wide variety of wild and semi-domestic birds. RNA viruses such as IAV have a low pathogenicity phenotype in their natural hosts (Olsen et al., 2006), but have large negative socioeconomic consequences when they spillover into food production animals and humans (Alexander and Brown, 2009; FAO, 2005, 2012). For example, the most recent remerging highly pathogenic IAV H5N8, which was transported globally by waterfowl, resulted in the culling of hundreds of thousands of chickens and turkeys, and is a risk to human health given the reassortment potential (European Food Safety Authority, 2014; Lee et al., 2014; Pasick et al., 2015; Verhagen et al., 2015; Wu et al., 2014). Dabbling ducks are a host for a number of RNA viruses, including avian

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coronavirus [CoV], avian paramyxovirus type 1 [APMV-1], and emerging evidence suggests they may also be hosts for an array of avian astroviruses [AstroV] (e.g. Chu et al., 2012; Ramey et al., 2013; Tolf et al., 2013b; Wille et al., 2015; Wille et al., 2016b). These viruses do not cause signs of disease in their wildlife hosts, but have closely related forms causing morbidity and mortality in poultry, such as infectious bronchitis (CoV) (e.g. Domanska-Blicharz et al., 2014; Jackwood et al., 2012; Zhuang et al., 2015), Newcastle disease (APMV-1) (e.g. Alexander, 2011; Jindal et al., 2009; Ramey et al., 2013; Snoeck et al., 2013; Tolf et al., 2013b), duck hepatitis (AstroV) or avian nephritis (AstroV) (e.g. Chu et al., 2012; Pantin-Jackwood et al., 2011). These viruses have been assessed, to various degrees, in wild migrating waterfowl. In Sweden, and globally, the ecology of IAV is well described in wild waterfowl, where up to 30% of Mallards (*Anas platyrhynchos*) are infected during the autumn migration (Latorre-Margalef et al., 2014; Olsen et al., 2006). Recent studies have been instrumental in starting to describe dynamics and ecology of AMPV-1 and CoV in wild birds; 9–12% of migrating Mallards have CoV infections, compared to a lower prevalence (2%) of AMPV-1 towards the end of the migratory season in Sweden (Tolf et al., 2013b; Wille et al., 2015). Most APMV-1 is detected during IAV studies where agglutinating agents are detected after culture that are not IAV (e.g. Jindal et al., 2009; Ramey et al., 2013), so few true prevalence estimates exist. Beyond these viruses, we have a limited understanding of the virodiversity in waterfowl; astroviruses for example have only recently been assessed in wild birds, and the results of a single study suggest that waterfowl may be important in the epidemiology of these viruses (Chu et al., 2012). Given that waterfowl are hosts for both multi-host viruses and viruses that cause morbidity and mortality in food production birds, combined with the increased contact between waterfowl and humans in urban areas, dynamics of these viruses in urban bird populations should be explored.

In this study, we followed the dynamics of RNA viruses at a pond utilized year round by Mallards, located in the centre of Sweden's fourth largest city. This pond is on the same migratory route as wild Mallards assessed for these viruses in southern Sweden, allowing a comparison between urban and wild ducks on a limited spatial scale (Latorre-Margalef et al., 2014; Tolf et al., 2013a; Tolf et al., 2013b; Wille et al., 2015; Wille et al., 2016b). Thus, this intra-annual dataset allows us to add to the natural history of IAV, CoV, AMPV-1, and the rarely assessed AstroV. Furthermore, we aim to elucidate if less frequently studied RNA viruses follow intra-annual cycles similar to that of the intensively studied IAV. In context of IAV, and to a lesser degree CoV and APMV-1, an assessment of virus prevalence and diversity in an urban population will further allow us to assess if dynamics in wild birds are reflected in an urban setting.

2. Methods

2.1. Sample collection

An urban population of Mallards residing in the artificial pond "Svandammen" in the centre of the city of Uppsala, Sweden (59°51′16″N, 17°38′25″E) were sampled. The pond is kept ice-free during the winter months, and is a popular location where the ducks are fed, resulting in a resident population of ducks year-round. This pond has a largely constant population size between 300 and 600 individuals through the autumn and winter, with fewer birds occupying the pond during breeding in the summer months (Fig. A.1). The low population count in May is likely the result of unfavorable conditions on the day of the count and sampling. Slightly higher population counts in the winter, when most of the city ponds are frozen, likely represent the congregation of birds from ponds across Uppsala to utilize this ice-free habitat (Fig. A.1).

Two sampling strategies were employed: following capture, freshly deposited feces were collected from a single-use cardboard box, or, due to difficulties in capturing birds, freshly deposited feces were collected from the ground around the perimeter of the pond. Samples were collected with a sterile tipped applicator, and were placed in virus transport media (VTM) and stored at −70 °C within 2–6 h of collection.

Ethical approval for trapping and sampling was obtained from the Uppsala animal ethical committee (Reference Number C228/12), a permit was obtained from the City of Uppsala to capture, and a permit from Swedish Museum of Natural History to ring birds.

2.2. Sample screening

Viral RNA was extracted from pooled VTM samples, containing 4 samples per pool, with the Magnatrix 8000 extraction robot (Magnetic Biosolutions, Sweden) and Vet Viral NA kit (NorDiag ASA, Oslo, Norway). The RNA extraction was performed by the Molecular Diagnostics Department at the Swedish National Veterinary Institute. Positive pools were re-extractioned individually using the Maxwell 16 Instrument and Viral Total Nucleic Acid Purification Kit (Promega, Madison, USA). Following extraction, samples were assayed by real time reverse transcriptase PCR (rRT-PCR) for IAV, CoV, and AMPV-1 using previously published methods. Briefly, IAV was screened using a rRT-PCR assay targeting a short region of the matrix gene (Spackman et al., 2002) and a pan-coronavirus rRT-PCR assay targeting the RNA-dependant RNA polymerase (RdRp) gene (Muradrasoli et al., 2009) using the iScript One Step RT-PCR Kit (BioRad, Hercules, USA). A rRT-PCR targeting the matrix (M) gene (Tolf et al., 2013b; Wise et al., 2004) with the One Step RT-PCR Kit (Qiagen, Hilden, Germany) was employed to screen for APMV-1. A cycle threshold (Ct) cutoff of 40 was used for all screens. To screen for AstroV, cDNA was synthesized using Superscript III (Invitrogen) and random hexamers (Invitrogen) followed by a nested PCR targeting the RdRp (Chu et al., 2012; Chu et al., 2008) using Taq polymerase (Qiagen).

2.3. Virus characterization

Samples positive for IAV were propagated in 10–11 day old embryonated chicken eggs. Eggs were inoculated via the allantoic route, and allantoic fluid was harvested two days following inoculation. The fluid was assayed for the presence of IAV using a haemagglutination assay. RNA was extracted from positive samples as previously described. Egg isolation and extractions from allantoic fluid were performed by the Molecular Diagnostics Department at the Swedish National Veterinary Institute. Full length HA, NA, and M sequences were generated as described in Wille et al. (2013), and two samples were additionally deep sequenced in-house at the Swedish National Veterinary Institute (Virus 540/H10N3 and 816/H1N1).

A fragment of the CoV RdRp was sequenced as described in Wille et al. (2016b). The RdRp fragment generated during screening of AstroV was used and subsequently cloned with pGEM-T easy vector system (Promega). All PCR products were purified by the Wizard Clean-Up System (Promega) and all sequencing was completed at Macrogen (The Netherlands). In the case of astroviruses, 3–5 clones of each sample were sequenced.

Resulting sequences were aligned using the MAFFT algorithm (Katoh et al., 2009) within Geneious 7 (Biomatters, New Zealand). Phylogenetic models were determined in MEGA 6 (Tamura et al., 2011), and Maximum Likelihood Trees were built using PhyML (Guindon and Gascuel, 2003) implemented in SeaView (Gouy et al., 2010) and bootstrapped 10,000 times. Reference sequences for phylogenetic analysis comprised of the top BLAST hits for each sequence generated in this study, as well as similar sequences from Sweden. Outgroup sequences were added to root all trees. All sequences generated in this study have been deposited in GenBank under the accession numbers KY320400-39.