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Repeatability and reproducibility of hunter-harvest sampling for avian influenza virus surveillance in Great Britain

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Abstract

Emerging pathogens can threaten human and animal health, necessitating reliable surveillance schemes to enable preparedness. We evaluated the repeatability and reproducibility of a method developed previously during a single year at one study site. Hunter-harvested ducks and geese were sampled for avian influenza virus at three discrete locations in the UK. H5N1 highly pathogenic avian influenza (HPAIV) was detected in four species (mallard [*Anas platyrhynchos*], Eurasian teal [*Anas crecca*], Eurasian wigeon [*Mareca penelope*] and pink-footed goose [*Anser brachyrhynchus*]) across all three locations and two non-HPAIV H5N1, influenza A positive detections were made from a mallard and Eurasian

wigeon at two locations. Virus was detected within 1-to-4 days of sampling at every location. Application of rapid diagnostic methods to samples collected from hunter-harvested waterfowl offers potential as an early warning system for the surveillance and monitoring of emerging and existing strains of avian influenza A viruses in key avian species.

Key words

Avian influenza, disease surveillance, highly pathogenic, anseriformes

Introduction

The emergence of new multi-host pathogens, including novel strains and variants of pathogens, threatens human health, livestock health and the persistence of some wildlife populations (Cunningham *et al*. 2017). The goose/Guangdong (GsGd) lineage of H5Nx high pathogenicity avian influenza viruses (HPAIVs), emerged over 25 years ago in East Asia as the H5N1 subtype, and these viruses have continued to evolve into distinct clades (Wan 2012; Lee et al. 2017). The ongoing panzootic of the clade 2.3.4.4b of the H5N1 sub-type emerged in 2020, rapidly spread around the world and infected a greater diversity of wild bird species than other clades had during previous outbreaks (Byrne et al., 2023; Caliendo et al. 2022; Lewis et al. 2021; Lo et al. 2022; Alkie et al., 2023). It has also been detected in several wild mammalian species, causing mass mortality among some species (Venkatesan 2023). The apparent jump from birds to mammals causes concern for the virus's ability to infect humans, which it had done in a small number of high-exposure cases by the end of 2022 (EFSA 2022). In contrast, its impact on some seabird communities and on the poultry industry has been severe (Banyard et al., 2022; Falchieri et al. 2022) and it continues to be a major threat to avian health globally.

Novel surveillance schemes are needed to provide early-warning of pathogen emergence to inform decisions on appropriate responses, in addition to providing information on virus evolution in the field (Morner et al. 2002). Such schemes need to be repeatable and reproducible. Surveillance for AIV in wild Anseriformes shot by hunters has been proposed as a method for detection of AIV infection in North America and Europe (Bevins et al 2016; Gobbo et al. 2021; Wade et al. 2022), but its repeatability has yet to be demonstrated. We sought to evaluate whether different strains of AIV could be detected in wild Anseriformes

shot by hunters at three discrete locations in the United Kingdom (UK) during winter 2022-2023. To determine the level of circulating virus in these wild bird populations the presence or absence of viral RNA (vRNA) and/or infectious virus was assessed within swab samples. Since ducks have been observed excreting HPAIV despite no evidence of clinical disease (van den Brand et al. 2018), we hypothesized that HPAIV would be detected in ducks throughout the migration season. Furthermore, since anseriformes are the primary taxon most likely responsible for transporting HPAIV over large distances (Caliendo et al. 2022) we expected coincidence between migration rates of anseriformes and rates of HPAIV outbreaks on poultry holdings.

Methods

Clinical sampling

Sampling and sample analysis methods were undertaken as described in Wade *et al*. (2022) with the approval of the Faculty of Biological Sciences, University of Leeds Ethical Review Panel (reference: BioSci 21-020). Sampling was undertaken at three locations, each of which was on a major estuary: the Humber (northeast England, lat/long: 53.64, 0.02), the Ribble (northwest England lat/long: 52.73, -2.92) and the Solway (southwest Scotland, lat/long: 54.99, -3.58) (Figure 1). Waterfowl hunters took oropharyngeal (OP) and cloacal (C) swabs (Dryswab^{TM} ENT, rayon-bud; MWE Mediwire, Corsham, UK) from Anseriformes that they had shot, and stored them dry in sample tubes. Samples were dispatched on the same day as sampling directly to the Animal and Plant Health Agency (APHA) to enable processing within 48 hours of being collected. Upon receipt swabs were cut into 1 ml of Leibovitz L-15 medium (Slomka et al. 2019) and held at -80℃ prior to testing.

RNA Extraction and AIV Reverse Transcription Real-Time PCR (RRT-PCR)

RNA was extracted using the MagMAX CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific) and the KingFisher Flex system (Life Technologies) according to the manufacturer's instructions. Extracted RNA was tested for the presence or absence of vRNA using the matrix (M)-gene specific RRT-PCR (Nagy et al. 2021). All samples positive for vRNA were further tested by H5 HPAIV (H5-HP) RRT-PCR, for the specific detection of HPAIV H5 as described by James et al. (2022). M gene and H5-HP RRT-PCR Ct values ≤36.00 were considered AIV and HPAIV H5 positive, respectively with higher (weaker) values being interpreted as negative. Samples positive for M gene RRT-PCR, but negative for H5-HP RRT-PCR were further tested for potential low pathogenicity H5 RNA using the H5-HA2 RRT-PCR assay (Slomka et al., 2007) and other influenza A subtypes by H6-HA2 (manuscript in preparation), H7-HA2 (Slomka et al. 2009) and H9-HA2 RRT-PCRs (Monne et al. 2008 and Slomka et al, 2013) assays. The H9 RRT-PCR was undertaken using primers and probes with the thermocycling conditions as described by Monne et al. (2008) but the chemistry was as for the H5, and H7 RRT-PCR assays described by Slomka et al. (2013). Samples collected from unidentified birds were tested by APHA's in-house DNA barcoding method (details can be supplied upon request) for the species identification.

Virus isolation and propagation

Following AIV RRT-PCR testing, where sufficient volumes of Leibovitz L-15 medium remained, 14 swab samples were selected for virus isolation (VI). These swabs were selected because their Ct values were ≤36.00; a threshold above which VI is unlikely (Slomka et al 2012, Mahmood et al, manuscript in preparation). Each sample (100 µL) was diluted 1:1 in antibiotic solution containing gentamycin, 50 $mg.L^{-1}$; penicillin G, 1 million $units.L^{-1}$; streptomycin sulphate, 10 $g.L^{-1}$; and nystatin, 5 million $units.L^{-1}$ (Sigma). After an hour's

incubation at ambient temperature 200µL was inoculated into the allantoic cavity of specific pathogen-free, 9-day-old embryonated fowls' eggs (EFEs). Post infection, allantoic fluid was periodically harvested and tested for the presence of a haemagglutinating agent using the haemagglutinin assay (HA) (WOAH 2015). HA activity of ≥1/16 was considered positive for virus isolation. Negative HA activity corresponded to a score <1/16 and indicated that no virus was isolated. VI was attempted through two successive rounds of passage in EFEs.

Incursions of HPAIV into the UK

The temporal pattern of new HPAIV incidents on poultry holdings during the year 2022 was summarised for England from reports produced by the UK Government's Department for Environment Food and Rural Affairs (https://www.gov.uk/animal-disease-cases-england and https://webarchive.nationalarchives.gov.uk/ukgwa/20221018152851/https://www.gov.uk/guidance/avian-influenza-bird-flu-cases-and-disease-control-zones-in-england#disease-control-zones-no-longer-in-force, accessed 27 July 2023). These include the approximate location and date on which HPAI was confirmed in birds kept at premises, including poultry producers, backyard poultry flocks, zoological collections, and wild animal rehabilitation centres.

Migration patterns of species in which AIV was detected were plotted using The British Trust for Ornithology's BirdTrack reporting rate data for England during the year 2022 (https://www.bto.org/our-science/projects/birdtrack/maps-reports, accessed 27 July 2023). These report the percentage of complete bird lists submitted by observers, which include a given species during each week of the year. Maximum weekly percentages were calculated for each species and correlated (Spearman's rank) with the weekly number of new HPAI incidents on poultry holdings using IBM SPSS Statistics Release 26.0.0.0.

Results

Between 26th October 2022 and 31st January 2023, hunters submitted swab samples from 404 shot Anseriformes of seven species (Table 1). Samples were collected from 246 birds by three hunters between 26th October 2022 and 30th January 2023 on the Humber, from 149 birds by four hunters between 27th October 2022 and 29th November 2022 on the Ribble and from 9 birds by one hunter on 25th January 2023 on the Solway.

HPAIV was detected in 18 birds across all three locations and a non-H5, non-H7 influenza A virus was detected in three birds at two locations (Ribble and Humber) (Table 2). These latter two samples were positive for the M gene but negative on the H5-HA2, H6-HA2, H7-HA2 and H9-HA2 assays for LPAIVs. HPAIV H5N1 was first detected on the Humber in a Eurasian teal (*Anas crecca*) shot on 27th October 2022 and non-H5, non-H7 influenza A was first detected on the Ribble in a mallard (*Anas platyrhynchos*) shot on 31st October 2022 (Table 2). HPAIV was detected at each location throughout the period over which samples were collected. On the Humber, where sampling continued for the longest, HPAIV was detected in ducks shot during every month of sampling. All positive detections were close to the threshold of detection for the assays used (Table 2). Virus isolation was unsuccessful and genomic analysis could not be undertaken on samples that were so weakly positive for vRNA. Attempts to gain access to carcasses from positive birds, with the aim of gaining material for isolation or genomic purposes were unsuccessful.

HPAIV H5N1 infection was confirmed on 206 premises in England during 2022, with new cases arising throughout the spring and summer (Figure 2). The number of incidents was

lowest in May, with two in Nottinghamshire. During October, 82 incidents were detected nationwide, including locations closely associated with the three sampling regions.

Temporal patterns of migration were highly correlated between pink-footed goose, Eurasian wigeon and Eurasian teal (Figure 2; r_s>0.71, P<0.01, n=53 in all cases), but correlations between these species and mallard were weak at best (wigeon: r_s=0.30, P=0.027; teal: r_s=0.28, P=0.040; pink footed goose: r_s=0.217, P=0.119). The number of HPAIV H5N1 incidents per month was also highly correlated with reporting rates of pink-footed goose, Eurasian wigeon and Eurasian teal (r_s>0.59, P<0.01, n=12 in all cases), but not mallard (P=0.406).

Discussion

The current study supports the utility of hunter harvested Anseriformes for AIV surveillance (Wade *et al*. 2022). We have demonstrated that AIV can consistently be detected in wild Anseriformes shot by waterfowl hunters on the Humber and replicated these results at two other estuaries: the Ribble and Solway. The over-summering of HPAIV H5N1 in some bird species and concomitant outbreaks on poultry holdings during the summer of 2022 prevented assessment of whether strains isolated from shot Anseriformes were novel reassortants that had arrived with migratory species or whether these viruses had been cycling in local populations, potentially in the absence of clinical disease. Enhanced tolerance to HPAIV is likely to occur in some species (van den Brand 2018) and is considered to be the mechanism behind movement of the virus over broad geographical ranges (Caliendo et al. 2022). However, existing passive surveillance initiatives are unable to detect these putatively mild infections of different species as they rely upon investigation of birds

found dead (Bianco et al. 2020). Our detection of HPAIV in ducks shot throughout the migration season, with no discernible temporal pattern, is consistent with the absence of clinical disease despite virus excretion, at least in Eurasian teal.

Multiple attempts to recover live virus isolates from positive samples were unsuccessful and weak PCR results on swab samples precluded genomic assessment and hence phylodynamic modelling to reveal transmission pathways. Nevertheless, the increase in the number of new HPAIV incidents on poultry holdings from August was coincident with the increase in reporting rate of Eurasian wigeon and teal, and the detection of H5N1 in these species was coincident with their peak migration into Great Britain. These observations are consistent with at least some of the autumn poultry cases being due to immigrant strains, although circulation of strains in species with enhanced tolerance of infection or the absence of clinical disease cannot be ruled out.

Contemporary H5N1 is highly-adapted to Anseriformes (James *et al*. 2023) and this order has been considered the natural wildlife host for many years (Verhagen et al. 2021). Nevertheless, such rapid detection of HPAIV and non-H5, non-H7 influenza A following the start of sampling (HPAIV: day 1 on Solway, day 2 on the Humber and day 4 on the Ribble), and despite sampling relatively few birds was surprising. Rapid early detection of AIV is consistent with either a high prevalence of infection or alteration of behaviour of infected birds such that they become easier to shoot (Artois et al. 2009; Gallana et al. 2013). Our detection of AIV throughout the shooting season was not consistent with a disease-induced change in behaviour. Regardless, these characteristics are highly desirable for an efficient surveillance scheme (Artois et al. 2009).

Unforeseen circumstances resulted in sampling starting nearly 2 months after the 1st September start of the waterfowl hunting season. We may have missed the opportunity to detect AIV in some of the first immigrant Anseriformes of the 2022/23 season thus limiting our early-detection capability. Nevertheless, the coincidence between the monthly number of HPAIV outbreaks on poultry holdings and migration rates of pink footed geese, Eurasian wigeon and particularly Eurasian teal, whose migration peaked during the month preceding peak poultry outbreaks, is consistent with the ability to detect HPAIV in Anseriformes in advance of outbreaks on poultry holdings, as identified by Wade et al. (2023). However, unlike years prior to 2021 (Hansen et al. 2018), as HPAIV has remained in circulation among wild and domestic birds over summer in Great Britain, the imperative for early-detection of incursions into the country has diminished. Nevertheless, the reproducibility of the hunter-harvest method for the detection of AIV extends its applicability to disease management by offering the potential to track the emergence of new variants and their movement around the country prior to and during the peak season of incidents among poultry holdings.

Sample sizes and the timing of sampling varied substantially between the three locations. Duration of sampling also varied between hunters, with only two hunters providing samples throughout the season. Five hunters provided samples early in the season but stopped providing samples at least 2 months before the end of the sampling season. Anecdotal evidence implied that the effort required to sample birds and record information was considered excessive by some hunters, particularly on days when large numbers of birds had been shot. Understanding the reasons for cessation of engagement of volunteer sample providers can inform adaptations to sampling methods and study designs in order to improve volunteer retention (Robinson et al. 2021). Moreover, a national surveillance scheme for AIV in wild Anseriformes would benefit from engagement of a larger number of

hunters at each location in order to mitigate the impact of disengagement by some. Sampling at a greater number of more geographically dispersed locations would also be required to reliably track the movement of new strains of AIV around the country. Under such a design and with the alliance of the hunter-harvested sampling with rapid methods for the isolation and typing of AIV (Kwon et al. 2019), such a capability could inform policy or action to mitigate the impact of AIV on poultry before its emergence on holdings. In the absence of active surveillance of healthy birds, the sampling of shot birds, collected through established waterfowl hunting activities, is the only mechanism to generate data on virus circulation although studies are limited to excretion in swabs alone. With the continuation of HPAIV epizootics across the globe, sampling techniques that might enable a greater understanding of virus circulation and impact on different wild bird species is required more than ever to understand factors influencing risk to the poultry industry from the ever-present wild bird risk.

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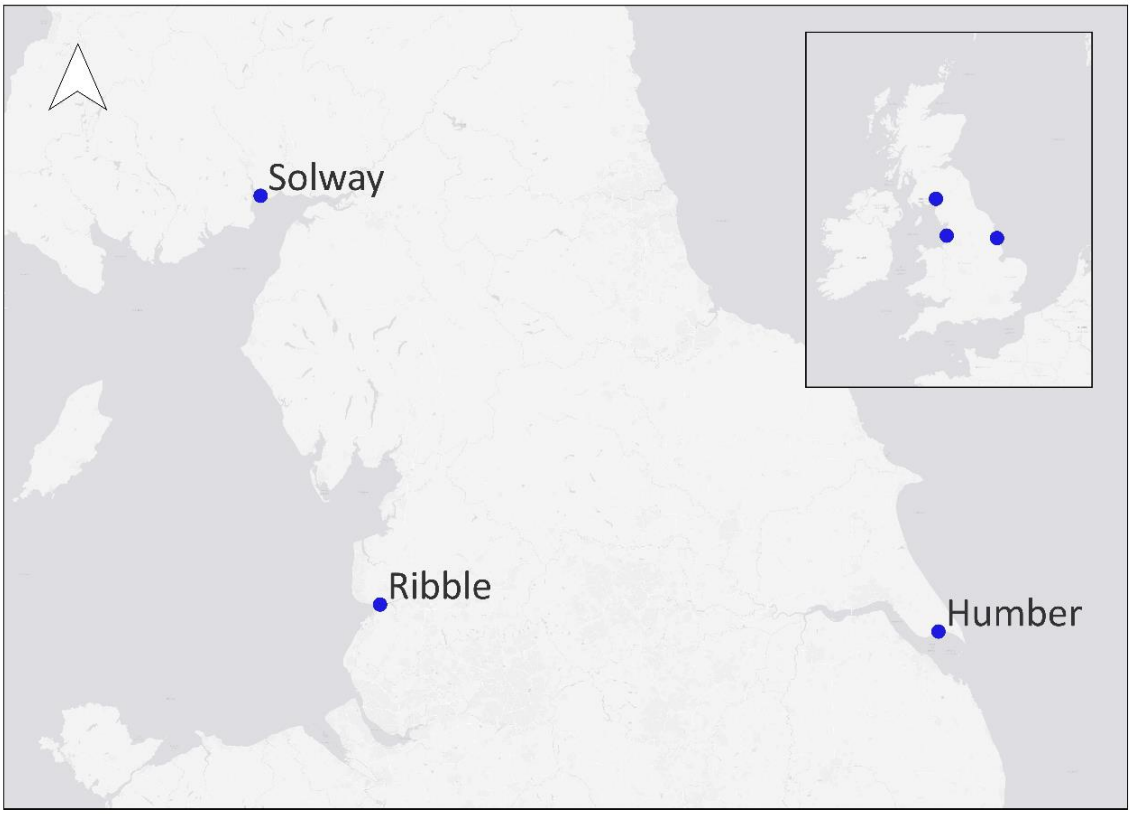

Figure 1. Three UK estuaries at which waterfowl hunters sampled shot wild anseriform birds for AIV.

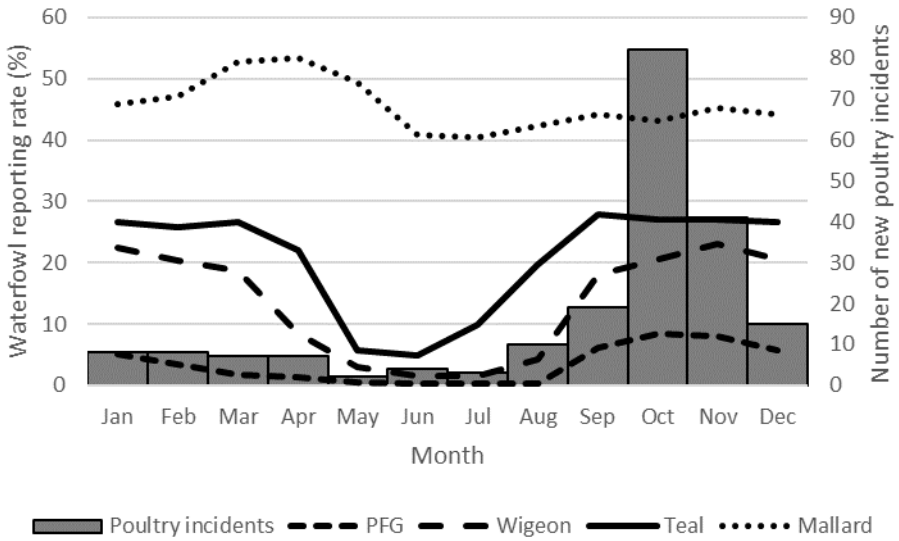

Figure 2. Migration patterns of four species of anseriform birds, as the monthly average percentage of complete lists of bird records on which each species was present, in England during 2022. The waterfowl reporting rates were from birdtrack.net and used with permission from the British Trust for Ornithology. The four species were those in which H5N1 avian influenza virus was detected from samples collected by waterfowl hunters. PFG = pink footed goose. Bars show the number of new incident cases of H5N1 on poultry holdings in England during 2022.

360 **Table 1.** Sample sizes and AIV test results for Anseriformes birds shot at three estuaries.

361 Numbers in parentheses are percentages. HPAIV = high pathogenicity AIV, AIV= non-H5,

362 non-H7 AIV positive.

Species	**Number of birds sampled**	**Number of birds AIV Positive (%)**	**Number of birds H5 HPAIV positive (%)**	**Number of birds non-H5, non-H7 AIV positive (%)**
Greylag goose (*Anser anser*)	4	0	0	0
Mallard (*Anas platyrhynchos*)	72	3 (4.2)	2 (2.8)	1 (1.4)
Pink-footed goose (*Anser brachyrhynchus*)	34	3 (8.8)	3 (8.8)	0
Northern pintail (*Anas acuta*)	2	0	0	0
Northern shoveler (*Anas clypeata*)	7	0	0	0
Eurasian teal (*Anas crecca*)	189	13 (6.9)	11 (5.8)	2 (1.1)
Eurasian wigeon (*Mareca penelope*)	92	2 (2.2)	2 (2.2)	0
Unidentified	4	0	0	0
Total	**404**	**21 (5.2)**	**18 (4.5)**	**3 (0.7)**

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365 **Table 2.** Dates and species of Anseriformes shot at three estuaries, which tested positive for
366 AIV. Ct = RT-PCR cycle threshold value. No Ct = No Ct value for H5 or H7, so considered
367 LPAIV. *Indicate the 14 swabs for which VI was attempted.

Species	**Estuary**	**Collection date**	**Cloacal swab**		**Oropharyngeal swab**	
			M gene Ct	H5HP Ct	M gene Ct	H5HP Ct
Eurasian teal	Humber	27.10.2022	33.23	29.79	*32.56	*28.88
Mallard	Ribble	31.10.2022	*34.5	*No Ct		
Eurasian wigeon	Ribble	04.11.2022	35.22	31.28	*30.25	*29.32
Mallard	Humber	10.11.2022	33.97	34.58		
Eurasian wigeon	Ribble	11.11.2022	39.19	34.95		
Eurasian teal	Humber	18.11.2022	*35.98	*No Ct		
Eurasian teal	Humber	18.11.2022	*26.63	*No Ct		
Eurasian teal	Humber	19.11.2022			36.84	33.96
Eurasian teal	Humber	19.11.2022			*34.53	*32.57
Eurasian teal	Humber	19.11.2022	37.39	34.76		
Eurasian teal	Humber	19.11.2022			*34.85	*32.22
Mallard	Humber	09.12.2022			*34.45	*32.8
Eurasian teal	Humber	09.12.2022	*30.25	*28.75		
Eurasian teal	Humber	16.12.2022	28.08	26.79	*27.04	*25.38
Eurasian teal	Humber	16.12.2022	36.5	35.03		
Pink footed goose	Humber	21.12.2022	*25.88	*23.46	27.81	25.6

Eurasian teal	Humber	30.12.2022	*33.43	*30.89	35.32	32.26
Eurasian teal	Humber	06.01.2023			*31.91	*29.49
Eurasian teal	Humber	20.01.2023			38.14	35.86
Pink-footed goose	Solway	25.01.2023	32.35	30.02	*30.31	*28.92
Pink-footed goose	Solway	25.01.2023	37.96	35.52		

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