Brief report

Reverse spillover of avian viral vaccine strains from domesticated poultry to wild birds

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ABSTRACT

Transmission of viruses from the commercial poultry to wild birds is an emerging paradigm of livestock–wildlife interface. Here, we report the identification and isolation of vaccine strains of avian paramyxovirus serotype 1 (APMV1) and avian coronaviruses (ACoV) from different wild bird species across eight Egyptian governorates between January 2014 and December 2015. Surveillance of avian respiratory viruses in free-ranging wild birds (n = 297) identified three species that harboured or excreted APMV1 and ACoVs. Genetic characterization and phylogenetic analysis of recovered viruses revealed a close association with the most widely utilized vaccine strains in the country. These results highlight the potential spillover of vaccine-viruses probably due to extensive use of live-attenuated vaccines in the commercial poultry, and close interaction between domesticated and wild bird populations. Further exploring the full spectrum of vaccine-derived viral vaccine strains in wild birds might help to assess the emergence of future wild-birds origin viruses.

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1. Introduction

Continuous evolution and emergence of new antigenic and genetic variants of avian respiratory viruses represents the main risk to poultry populations and disease occurrence worldwide. Despite mass vaccination, avian influenza (AIV), avian paramyxovirus serotype 1 (APMV1) (i.e. Newcastle disease viruses, NDV) and avian coronaviruses (ACoV) (i.e. infectious bronchitis virus, IBV) are regarded as the most important avian viruses that are causing serious economic losses, trade restrictions, and food security issues in the poultry industries. Vaccinations are being applied in domestic poultry to achieve high standards of poultry production, control viral infections and to reduce economic losses [1]. The impacts of these vaccines on the interface between domestic and wild bird population are incompletely studied especially when intensive poultry farming are being practiced under poor biosecurity settings.

Wild birds have been proposed to play crucial roles in the spillover and in potentiating the virulence of avian viruses [2]. However, intensive vaccines application, contamination of the

environment with infectious material through disposal or re-use of poultry litter and due to close contacts between wild birds and commercial poultry may lead to reversal of spillover (reverse spillover) from farmed poultry to wild birds [3]. Likewise, the transmission of virulent (e.g. escape mutant) viruses that have evolved either in response to vaccine pressure or through undetected viral contaminants within commercial vaccines are being transmitted to wild birds [2]. In high-tech commercial poultry farming, the possibility of spillover scenario into wild birds can be mitigated by the strict biosecurity measures. However, backyard poultry rearing meliorates these contacts especially in agriculture-enriched countries [4].

It is critically important to improve approaches for viral surveillance and epidemiology that can assist understanding the in-depth evolutionary aspects of viruses in wild birds. In the present study, considering all preliminary evidences on the presence of live vaccines in wild birds [5,6], we examined the hypothesis that APMV1 and ACoV vaccines may spill into wild birds. To this end, we performed virus surveillance in the Egyptian wild birds, and assessed the magnitude of ACoV and APMV1 prevalence in the country. The sequenced data from identified strains of APMV1 and ACoV from Egyptian wild birds were assessed along with the previously reported data in public domains on the vaccine-derived APMV1 and ACoV strains. Cumulative data indicates that ACoV and APMV1

vaccine strains can be identified in wild birds that are housed in the vicinity of commercial poultry farms. These viral vaccine-strains isolated from wild birds have shown significant genetic similarities with the vaccine strains being applied in the commercial poultry farms suggesting the possible reverse spillover of these viruses to nearby wild life. These findings highlight the need to fully investigate the dynamics and spectrum of vaccine-derived viral strains in wild birds and that such virus detection would severely jeopardise the welfare of the wild birds and may in future leads to virus evolution with increased virulence, as has been proposed for avian influenza viruses [1,2].

2. Materials and methods

2.1. Samples collection and virus isolation

A total of two hundred and nighty seven (n = 297) oral and cloacal swabs were collected from randomly selected and apparently healthy wild birds from eight Egyptian governorates between January 2014 and December 2015 for active and passive surveillance of avian respiratory viruses including AIV, ACoV and APMV1 (Table 1). Wild birds were captured using a combination of hand-nets, drop-nets, mist-nets, and ground traps. Authorized veterinarians of the Central Laboratory for Evaluation of Veterinary Biologics, Egypt obtained swabs samples from live birds. Samples were propagated for three blind passages in the allantoic cavity of 9-days-old specific pathogen free (SPF) embryonated chicken eggs following the OIE standard procedures [7,8]. Pathotyping of APMV isolates were carried out by mean death time (MDT) and intracerebral pathogenicity index (ICPI).

2.2. RNA extraction, PCR amplification and nucleotide sequencing

All swabs were screened for ACoV and APMV1-specific real time and conventional RT-PCR before isolation and genomic characterization [9,10,11,12]. RNA extractions were performed using TRIzol LS (Life Technologies, Carlsbad, CA, USA), as per manufacturer instructions. One-step RT-PCR was performed to amplify the fusion (F) and spike (S1) genes of APMV1 and ACoV using the SuperScript1 III One-Step RT-PCR System with Platinum I *Taq* DNA Polymerase (Life Technologies, Carlsbad, CA, USA) and previously described primers [9,10]. Appropriate positive and negative controls were included, and positive samples were back-screened to exclude the possibility of laboratory- and/or cross-contamination. Amplicons were separated on a 1.2% agarose gel, and desired bands were excised and purified using the QuickClean II Gel Extraction Kit (Gen-Script, Piscataway, NJ, USA). Nucleotide sequencing and assembly were performed as described previously [13]. DNA sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) in ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA USA). Obtained sequences were submitted to GenBank using BankIt tool, and are available under accession numbers: KU251490.1, KU251491.1 and KY549653.

2.3. Evolution and phylogeny

To explore an overall differences in selection pressure on the F and S1 genes, especially on epitopes that defines the cross-neutralization and escape mutant, the occurrences of synonymous (dS) and non-synonymous (dN) substitutions were determined using SNAP web tool (available at https://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html) [14], which plots the cumulative and per-codon occurrence of each substitutions. Phylogenetic trees were constructed utilizing the obtained nucleotide sequences for F and S1 genes of APMV1 and ACoV, respectively based on Bayesian Inference within the program MrBayes version 3.1.2 [15]. Two independent Markov chain Monte Carlo were executed and sampled every 1000 generations using the default parameters of the priors' panel. The analysis was based on the GTR + I + G model, which allow significantly changed posterior probability estimates. To confirm the Bayesian tree topologies, phylogenetic relationship was also established with the MEGA version 6.0 software program using the maximum likelihood method with the Kimura two-parameter model [16]. The evolutionary distances were inferred using the pairwise distance method and expressed as the number of nucleotide substitutions per site giving a statistical significance of the tree topology by 1000 bootstrap resampling of the data [16].

3. Results

3.1. Prevalence of APMV1 and ACoV in wild birds

A total of 297 samples were processed from wild birds-dense, and APMV1 and ACoV-endemic areas from eight governorates of Egypt. We classified all wild bird species that were included in the analysis into three different families, which reflected both their taxonomy and their ecology. These families were *Corvidae* (*C. splendens*, n = 102), *Ardeidae* (*B. ibis*, n = 99) and *Anatidae* (*A. crecca*, n = 96) (Table 1). These samples were individually screened using the RT-PCR targeting the S1 and F genes of the ACoV and APMV1, respectively. This screen yielded a 5.4% (16 out of 297) and 1.7% (5 out of 297) positive samples for ACoV and APMV1, respectively among all tested tracheal and faecal/cloacal samples. Among positive samples for APMV1, a single isolate shown high genetic similarity to a vaccine strain whereas two out of 16 ACoV shown vaccine-alike characteristics. Pathotyping of the APMV1 isolate (NDV/Teal/VRLCU-EG/2015) showed MDT (96 h) and ICPI (0.4375) characteristics for lentogenic strains of NDV.

3.2. Sequencing and phylogeny

All positive samples, detected based upon the RT-PCR for both ACoV and APMV-1, were sequenced and analyzed. A set of sequences, representing previously reported APMV1 and ACoV genotypes in Egyptian poultry sectors were aligned with sequences presented in this study using the ClustalW algorithm in BioEdit [17]. Comparison of nucleotide identity percentages revealed that one APMV1 isolate was genetically identical to the genotype II

Table 1
Proportion and positivity of sampled wild birds across different regions.

Family	Species (Genus)	Sharqia	Dakahlia	Kafr El Sheikh	Gharbia	Qalubia	Menofia	Giza	Benisuef	Total	Positive%	
											APMV-1 (1.7%)	ACoV (5.4%)
Corvidae	*C. splendens (Corvus)*	10	14	19	10	21	8	9	11	102	0	2
Passeridae	*P. domesticus (Passer)*	11	9	21	14	13	10	12	9	99	1	5
Anatidae	*A. crecca (Anas)*	13	12	18	13	9	10	11	10	96	4	7
Total = 3	**Total = 3**	**34**	**35**	**58**	**37**	**43**	**28**	**32**	**30**	**297**	**5**	**16**

(vaccine strains genotype) with 100% identity to the commercial LaSota vaccine strain (Fig. 1A) whereas the other four isolates showed F protein cleavage site motif (^{112}RRQKRF^{117}) which is characteristic for velogenic strains of NDV. In addition, phylogenetic analysis based on full-length F gene revealed that clustered of these isolates within class II, genotype VII and specifically within subgenotype VIId, which is the most predominant genotype within Egyptian poultry sectors.

However, the two ACoV isolates were closely related to the classic genotype of Massachusetts (MA5) serotype. The ACoV/Crow/Q alubia-Egypt/VRLCU-15/2015 and ACoV/House sparrow/Kafr El Sheikh-Egypt/VRLCU-16/2015 showed 100% and 93% identity to previously reported isolates from commercial poultry (IBV/ck/Egy pt/12vir6109-78/2012 and commercially available MA5 vaccine, respectively (Fig. 1B)). Likewise, the amino acid identity comparison indicated several point mutations, which were prominent within ACoV/House sparrow/Kafr El Sheikh-Egypt/VRLCU-16/2015 isolate compared to MA5 vaccine strain. Briefly, these substitutions include Q242E, N250S, T257K, H264Y, E266V, G268D, N270T, N278T, T281L, Q285H, N308D and I338L. Genetic relatedness analysis for the other 14 ACoV isolates demonstrated the clustering of nine isolates (9/14, 64.3%) within Egy/variant 2 (IS/885 genotype) and five isolates (5/14, 35.7%) within Egy/variant 1 (IS/1494/06 genotype) of GI-23 Middle East lineage.

3.3. Selective pressure

The selection pressure profiling of putative amino acid sequences of all sixteen Egyptian ACoV strains showed two general patterns within the S1 protein sequence. The cumulative difference between the non-synonymous substitution rate (dN) and the synonymous substitution rate (dS) (i.e. dN-dS) revealed a strong positive selection on most of S1 codons for ACoV (Fig. 1C), indicating a potentially ongoing molecular adaptation. However, a strong negative (purifying) selection site was observed in the putative F protein of APMV1 (Fig. 1D).

4. Discussion

RNA viruses such as influenza, coronaviruses and paramyxoviruses are composed of complex and dynamic mixtures of mutant genome variants [18,19]. The continuous fluctuations in mutant viruses caused by host immune system or environmental factors lead to tissue and host switches with altered pathogenicity and virulence. Especially for viruses with broader host range such as influenza and APMV, poor vaccination may help the host survival but these don't prevent the spread of the virus and thus resulting in the evolution of virulent viruses which may predispose
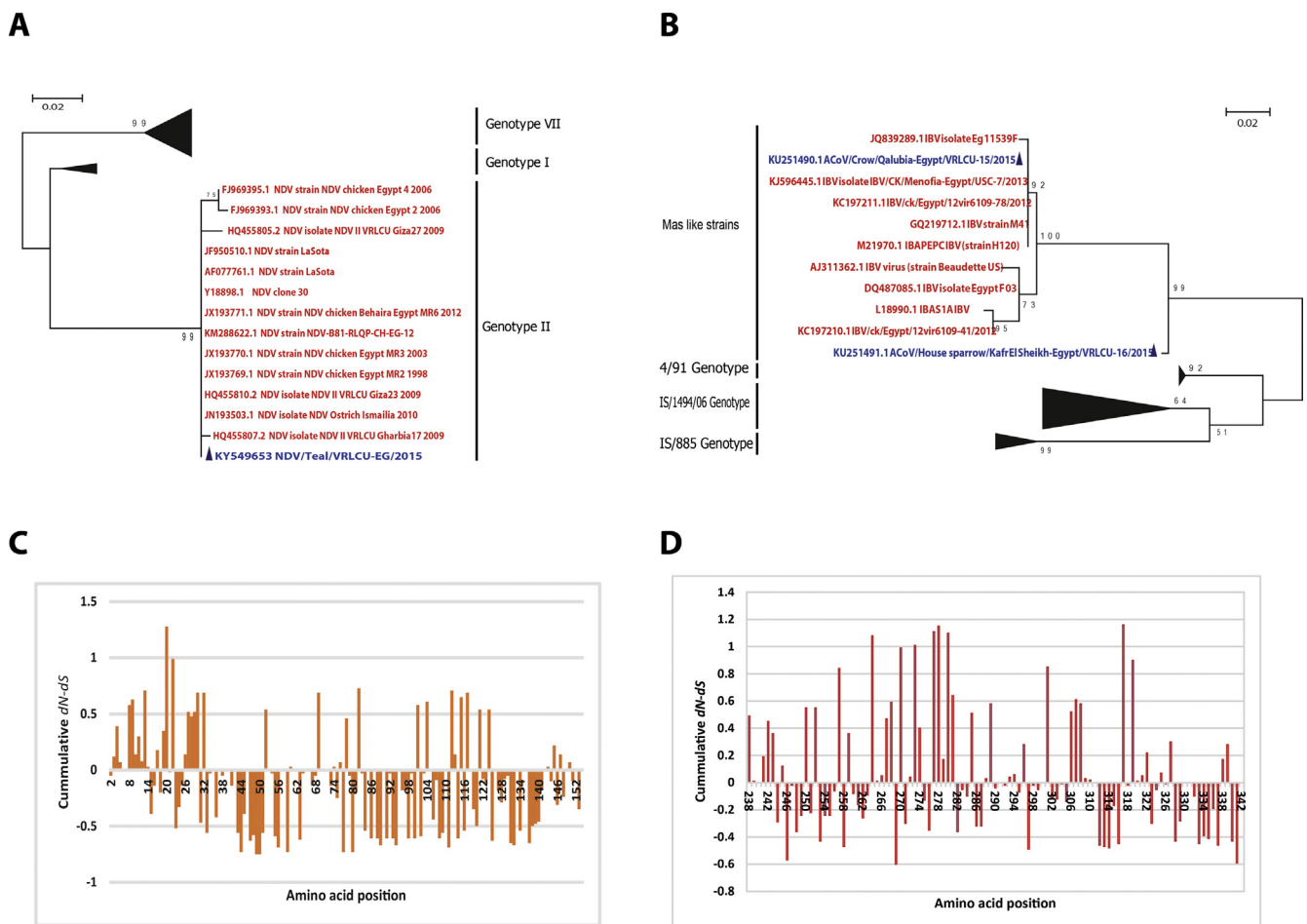

Fig. 1. Phylogenetic and evolutionary analysis of APMV1 and ACoVs in domesticated and wild birds. (A) Phylogenetic tree based on complete sequences of the F gene, showing the relationship between the commercial NDV vaccines (genotype I and II) and the circulating Egyptian genotypes (genotype VII) in commercial poultry sectors in relation to APMV1 isolates from wild birds reported in this study. (B) Phylogenetic tree based on partial sequences of the S1 gene, indicating the relationship between the commercially used vaccines (Mass like strains, MA5) and currently circulating Egyptian genotypes (IS/885 and IS/1494/06) in commercial poultry sectors. The robustness of individual nodes of the tree was assessed using 1000 replications of bootstrap re-sampling of the originally aligned nucleotide sequences. Scale bar represents the number of substitutions per site. The year of isolation and geographical origin of the virus sequences are included in the tree. (C) Distribution of negative and positive selection motifs along the HVR3 region of ACoV strains. Accumulated dN-dS are shown that are spanning amino acid residues from 238 to 342. (D) Distribution of negative and positive selection motifs along the F protein of the NDV strain sequenced in this study. Accumulated dN-dS are shown which are spanning the amino acid residues from 1 to 552.

the nearby life. Live attenuated vaccines, being used for several avian viruses including APMV1 and ACoV, are stable in poultry litter [20] and thus sub-standard biosecurity measures and practices can facilitate the transmission of these viruses to wild birds that may come in close contact with vaccinated poultry or nearby farms.

In the present study, we demonstrate the isolation of vaccine-derived APMV1 and ACoV from different wild bird species and identified at least three species from three avian orders that harboured and/or excreted vaccine-derived viruses. However, it is likely that APMV1 strains could infect any bird species due to broader host spectrum of these viruses [20,21]. While this and previous studies [2,4,5] have highlighted the reverse spillover, it is yet to be determined if these spilled over viruses retain their immunization potential or in the event of concurrent infection may lead to virus evolution and altered pathogenicity.

The overall percentage of vaccine-acquired virus strains in unvaccinated wild bird has been overlooked. Given the number of APMV1 vaccines (n = 12 in Egypt only such as LaSota, Clone 30 and Avinew) and ACoV vaccines (n = 8 in Egypt only including MA5, H120 and 4/91) being applied in the field, these viruses are yet major threat in the industry. Thus most attentions of diagnostic laboratories have been to identify pathogenic or virulent isolates whereas avirulent strains are potentially ignored. Although our data do not allow for the identification of the direct sources of APMV1 and ACoV infection for the wild birds, it is reasonable to suggest that these wild bird isolates originated from recent spillover of live APMV1 and ACoV vaccines, instead of representing strains that naturally circulate in these birds. Spillover of APMV1 and ACoV vaccines into wild birds reflects the most commonly used live vaccines. In addition, LaSota is the most commonly used APMV1 vaccine [22] and is more likely to be shed in the environment.

Positively selected fragments of genes encoding viral proteins exposed on the surface of the capsid have been documented in other viruses [23,24]. There is an association between positively selected sites along the S1 and F genes for ACoV and APMV1, respectively. It has been reported that mutations in the S1 protein often result in changes in antigenicity [25]. Likewise, parts of the hypervariable region 3 (HVR3) defined in this study were shown to be under strong positive selection in the ACoV strains. Taken together, variable purifying selection pressures on putative S1 and F proteins may be considered an outcome of virus adaptation in un-intended wild bird hosts.

Among factors that are enforcing the close association of wild life to domesticated life are mainly contributed by the human-triggered industrialization and urbanization. Proper mitigation of these factors will not only increase the wild-domesticated interface but will also alleviate the disease and stress burdens on wild-life. In conclusion, further studies are required to evaluate ecological impacts of transmission of vaccines or infectious agents from poultry operations into free-ranging avifauna. Since the identified APMV-1 and ACoV isolates from wild birds were closely related to the currently used commercial vaccines in the Egyptian poultry sectors, the extensive use of vaccines poses possible threat into wildlife. Intensified surveillance of wild birds is an important means of assessing the relative prevalence of avian vaccine strains, and this knowledge would aid risk assessments, and disease management. Moreover, our findings are fundamental to assess the poor biosecurity measures, possible threats for virus evolution in these asymptomatic birds, and the impact of such spillover on the ecology and survival of wild birds and provide insights into the reverse spillover of avian viral vaccine strains in wild birds, which are considered reservoirs for these viruses. These findings highlight the need to fully investigate the dynamics and spectrum of vaccine-derived viral strains in the wild birds. Such investiga-

tions would be helpful to ameliorate the welfare of wild birds and may reduce the possible virus evolution in potentially asymptomatic hosts. Likewise, bio-security measures should be enhanced to reduce the escape of vaccine strains to wild birds and disease transmission from domesticated to wild birds and vice versa.

Conflict of interest

All authors declare that they have no conflict of interest.

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