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Co-expression of sialic acid receptors compatible with avian and human influenza virus binding in emus (*Dromaius novaehollandiae*)

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ABSTRACT

Influenza A viruses (IAVs) continue to threaten animal and human health with constant emergence of novel variants. While aquatic birds are a major reservoir of most IAVs, the role of other terrestrial birds in the evolution of IAVs is becoming increasingly evident. Since 2006, several reports of IAV isolations from emus have surfaced and avian influenza infection of emus can lead to the selection of mammalian like PB2-E627K and PB2-D701N mutants. However, the potential of emus to be co-infected with avian and mammalian IAVs is not yet understood. As a first step, we investigated sialic acid (SA) receptor distribution across major organs and body systems of emu and found a widespread co-expression of both SAα2,3Gal and SAα2,6Gal receptors in various tissues that are compatible with avian and human IAV binding. Our results suggest that emus could allow genetic recombination and hence play an important role in the evolution of IAVs.

1. Introduction

Influenza viruses (IAVs) continue to threaten animal and human health globally. In particular, highly pathogenic avian influenza viruses (HPAIVs) have been a major concern to the poultry industry and some of these strains also have a significant impact on public health. Influenza viruses are enveloped, contain 8 segments of single stranded, negative sense RNA genomes and belong to the family *Orthomyxoviridae*. IAVs have a wide host range with clinical outcomes ranging from mild inapparent infections to severe fatal disease depending on the host and the virus strain involved. IAVs undergo constant genetic changes resulting in the occasional emergence of novel variants that can cross species barrier to infect other species.

Though wild aquatic birds are considered as the natural reservoirs for influenza viruses (Alexander, 2000), several terrestrial birds such as chickens, turkeys and quails can act as intermediate hosts and can transmit IAVs to other species (Guo et al., 2007; Wan and Perez, 2006). Ratites (ostrich, emu and rheas) that are either in wild or farmed in open areas have a high chance of getting exposed to avian influenza viruses (AIVs) from wild birds. Emus (*Dromaius novaehollandiae),* second-largest living birds in the world by height after Ostriches, were once commonly found on the east coast of Australia. In the last decade, emu farming has become a popular and lucrative business and continues to grow especially in developing countries such as India and China. There are several reports of isolation of low pathogenic avian influenza (LPAIVs) virus subtypes namely H9N2, H5N2, H10N7 and H7N1 as well as highly pathogenic avian influenza (HPAIVs) H5N1 virus subtypes from emus from different parts of the world (Amnon et al., 2011; Clavijo et al., 2001; Kang et al., 2006; Panigrahy et al., 1995; Shinde et al., 2012; Woolcock et al., 2000).

Emus are susceptible to AIVs of chicken and turkey origin and effectively seroconvert by 7 days (Heckert et al., 1999; Zhou et al., 1998). Experimentally infected emus shed virus for 10 days post infection and show mild or inapparent clinical signs depending on the influenza virus strain similar to the infection in wild birds (Heckert et al., 1999; Kang et al., 2006; Perkins and Swayne, 2002). However, infection with highly pathogenic HongKong-origin H5N1 virus caused neurological symptoms with pancreatitis, meningoencephalitis and mild myocarditis (Perkins and Swayne, 2002). While LPAIVs isolated from emus (H5N2, H7N1, H10N7) were not pathogenic to chickens and turkeys (Panigrahy et al., 1995; Woolcock et al., 2000) except when passaged *in vivo* (Swayne et al., 1996), HPAI H5N1 virus isolated from

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emu was highly virulent when infected in SPF chickens (Amnon et al., 2011). Infection of ostriches with a HPAI virus isolated from emu did not cause significant disease or mortality but the virus was found to replicate extensively (Clavijo et al., 2001). Further, it is known that influenza virus infection of ratites including emus can lead to the selection of mammalian type mutants PB2-E627K and PB2-D701N (Yiu Lai et al., 2013). The sum of this evidence suggest that emus could be important source of HPAIVs to domestic poultry such as chicken and turkeys and also play a key role in the generation of AIVs with increased mammalian pathogenicity. Thus investigating the 'emu-influenza virus interaction' is of great importance considering their exposure to wild birds, close proximity to other terrestrial birds and farm workers.

Influenza virus entry into the host is mediated through the binding of viral hemagglutinin (HA) to the host cell sialic acid (SA) receptors. The receptor binding specificity is influenced by the amino acid sequence of HA protein. Several studies showed that the type of SA receptors is an important determinant of host susceptibility, tissue tropism, pathogenesis and transmission of IAVs (Ito et al., 1997; Kida et al., 1994; Kuchipudi et al., 2009; Murcia et al., 2012; Shinya et al., 2006). SA receptors carry nine carbon monosaccharides on the terminal position of glycan chains and are linked to glycoproteins and glycolipids on cell surfaces (Varki and Varki, 2007). The most common sialic acids, N-acetylneuraminic acid is bound to galactose with either an α2,3 or an α2,6 linkage and their distribution and expression are cell specific. Avian influenza viruses preferentially bind to SAα2,3-Gal receptors (avian like receptors), whereas the classical swine and human IAVS show preferential binding to SAα2,6-Gal receptors (human like receptors) (Matrosovich et al., 1997; Rogers et al., 1983). It was shown that several hosts such as pigs, ducks, pheasants and quails that co-express both types of SA receptors are susceptible to infection with both avian and human IAVs (Ito et al., 1997; Kida et al., 1994; Murcia et al., 2012; Yamada et al., 2012; Yu et al., 2011). It's widely argued that co-infection with avian and human influenza viruses in the species that express both SA receptors, can lead to genetic reassortment between the viruses (Schafer et al., 1993) and possibly result in emergence of strains with pandemic potential (de Graaf and Fouchier, 2014).

SA receptor profile of several avian species belonging to the following orders has been documented so far: Accipitriformes, Anseriformes, Charadriiformes, Ciconiiformes, Columbiformes, Falconiformes, Galliformes, Gaviiformes, Gruiformes, Passeriformes, Pelecaniformes, Psittaciformes and Struthioniformes (Ellstrom et al., 2009; Guo et al., 2007; Kimble et al., 2010; Kuchipudi et al., 2009; Pillai and Lee, 2010; Wan and Perez, 2006; Yu et al., 2011) which helped to understand the molecular basis of species-related differences in the susceptibility to IAV infection.

A major determinant of susceptibility to avian and/or mammalian IAVs is the relative distribution of appropriate SA receptors. There is a strong correlation between the abundance of SA receptors and susceptibility to IAV infection in birds. For example birds that are highly susceptible to IAV infection such as chickens and Pekin ducks, show abundant expression of avian type (SAα2,3-Gal) receptors (Kuchipudi et al., 2009; Pillai and Lee, 2010; Wan and Perez, 2006), whereas a weak expression of SAα2,3-Gal receptors correlate with resistance of birds such as pigeons to AIV infection (Liu et al., 2009).

The role of emus in the epidemiology of IAVs appear to be significant and raises number of key questions. "What are the mechanisms underlying the selection of mammalian like PB2 mutant IAVs? ", "Why emu origin HPAIVs are asymptomatic in ostriches but are highly virulent in chicken?", "Can emu act as a mixing vessel to generate IAVs with pandemic potential?". To unravel a key piece of this puzzle, we investigated influenza virus receptor distribution across major organs and body systems of emu by lectin histochemistry using linkage specific lectins followed by confocal microscopy. Compatibility of the SA receptors in emu tissues to allow binding of avian and human IAVs was also investigated.

2. Materials and methods

2.1. Birds and tissue samples

Tissue samples from three male emu birds around 2 years of age, were collected under aseptic conditions immediately after they were slaughtered for meat purpose. The tissue samples were collected and transported in buffered neutral formalin. The following tissues were included in the study to investigate influenza virus receptors: larynx, trachea, bronchi and lungs, representing the respiratory tract, proventriculus, duodenum, small intestine, large intestine and caecum representing the digestive tract and brain representing the nervous system. Other major organs such as liver, heart, spleen, kidney, skeletal muscle and skin were also included for studying the SA receptor distribution.

2.2. Lectin histochemistry

Tissue sections of 5 μm thickness were used for lectin histochemistry using *Sambucus nigra* agglutinin (SNA) and *Mackia amurensis* agglutinin II (MAAII) lectins (Vector Laboratories, USA) following the protocol described previously with minor modifications (Kuchipudi et al., 2009). Briefly, sections deparaffinised in xylene and rehydrated by serial alcohol dips were pre-soaked in Tris-buffered saline (TBS) for 10 min and then blocked with 1% BSA in PBS for 3 h at room temperature (RT). Sections were incubated overnight at 4 °C in the dark with FITC labelled SNA and biotinylated MAAII lectins, each at 10 μg/ml concentration. After three washes with TBS, sections were incubated with streptavidin-Alexa Fluor 594 conjugate at RT for 2 h. The sections were then washed and mounted with Prolong gold antifade reagent with DAPI (Invitrogen). After an overnight curing at RT in dark, the sections were imaged with confocal microscope (Leica SP8). Negative controls were performed omitting the primary reagents. Settings for each of the blue, green and red channels were determined using the negative controls to avoid any background fluorescence. Subsequently, all the lectin stained sections were scanned with the same settings. Further, we also ruled out nonspecific binding of the lectins by treating sections with Sialidase A (N-acetylneuraminate glycohydrolase; Prozyme, San Leandro, CA), which cleaves all non-reducing terminal sialic acid residues in the order α(2,6) > α(2,3) > α(2,8) > α(2,9).

2.3. Virus binding assays

Virus binding assays with human pandemic influenza H1N1 virus (A/H1N1/Virginia/2009), and a LPAI H5N2 virus (A/chicken/PA/7659/85) were performed as previously described, with minor modifications (Kuchipudi et al., 2009). Briefly, paraffin embedded 5 μm sections of trachea and intestines were deparaffinised in xylene and rehydrated by serial alcohol dips. Deparaffinised tissue sections were incubated with 250 μl each of avian or human influenza virus at 10^6 $TCID_{50}$/ml in medium containing TPCK trypsin for 2 h at RT. The sections were washed, blocked with goat serum for 30 min, and incubated with a mouse monoclonal antibody specific for influenza H5 (Abcam) or nucleoprotein (Abcam) in 1:1000 dilution, for 2 h in a humidified chamber at RT. A secondary antibody, Cy5-labelled goat anti-mouse IgG (Abcam,), was applied at 1:500 dilution for 1 h at RT. After three further washes with TBS, the sections were mounted with ProLong Gold anti-fade reagent with DAPI and viewed under confocal microscope (Olympus FluoView™ FV1000).

3. Results

3.1. SA receptor distribution in emu respiratory tract

A widespread expression of both SAα2,3Gal and SAα2,6Gal recep-