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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-

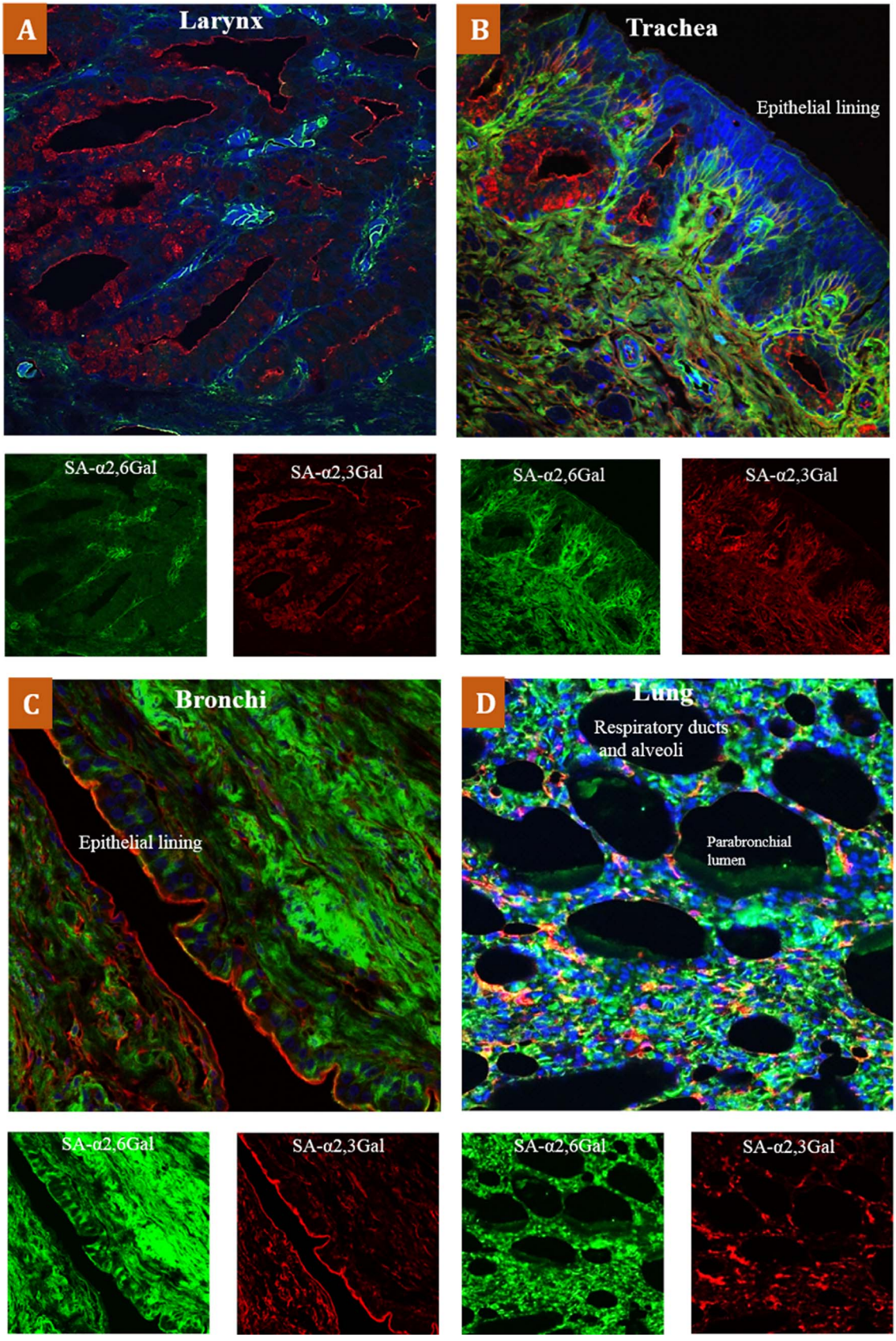

Fig. 1. Co-expression of SAα2,6-Gal and SAα2,3-Gal receptors in emu respiratory tract: Composite confocal images show abundant co-expression of SAα2,6-Gal receptors (green) and SAα2,3-Gal receptors (red) throughout emu respiratory tract. Comparable expression of SAα2,3Gal and SAα2,6Gal was observed in the ciliated epithelial cells, goblet cells and non-ciliated epithelial cells of (A) larynx, (B) trachea, (C) bronchi and (D) alveoli of lungs. Tissue sections were stained with biotinylated MAAII (red-specific for SAα2,3-Gal) and FITC labelled SNA (green-specific for SAα2,6-Gal) lectins, and nuclear staining with DAPI (blue).

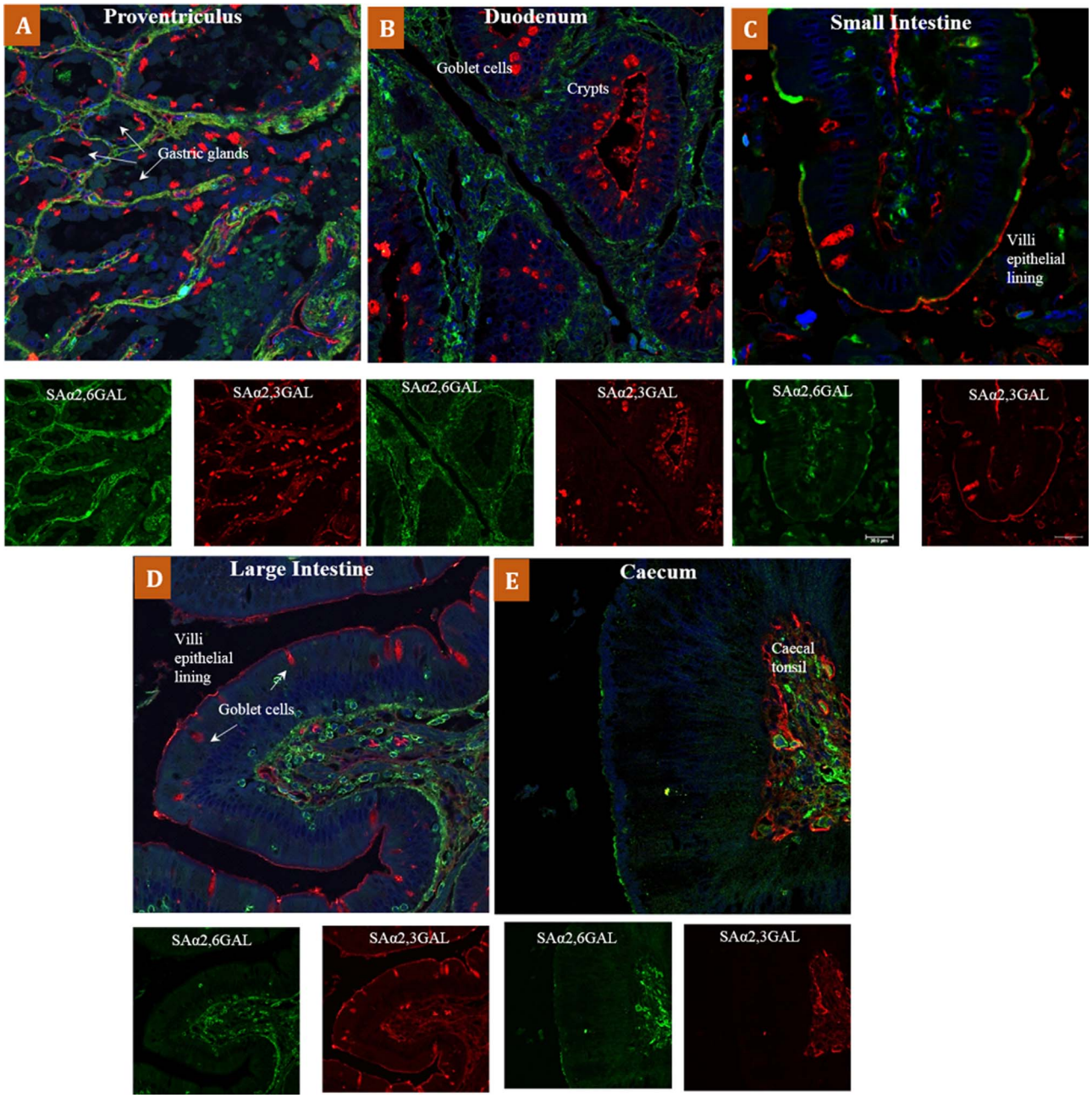

Fig. 2. Co-expression of SAα2,6-Gal and SAα2,3-Gal receptors in emu digestive tract: Composite confocal images show abundant co-expression of SAα2,6-Gal receptors (green) and SAα2,3-Gal receptors (red) in (A) proventriculus, (B) duodenum, (C) small intestine, (D) large intestine and (E) caecum of emu. Expression of both receptors was observed throughout the digestive tract with a gradual increase in the expression of α2,3-SA receptor from duodenum to colon. Tissue sections were stained with biotinylated MAAII (red-specific for SAα2,3-Gal) and FITC labelled SNA (green-specific for SAα2,6-Gal) lectins, and nuclear staining with DAPI (blue).

tors were observed throughout the respiratory mucosa of emu (larynx, trachea, bronchi and alveoli in lungs) (Fig. 1). While the expression of SAα2,3Gal and SAα2,6Gal was comparable in ciliated epithelial cells, goblet cells and non-ciliated epithelial cells, a higher SAα2,6Gal expression was observed in the submucosa of the respiratory tract. Notably, comparable levels of SAα2,3Gal and SAα2,6Gal expression throughout the respiratory tract was also observed in many other avian species such as pheasants, quails, partridges, turkeys, guinea fowls and mallards (Costa et al., 2012; Kimble et al., 2010; Yu et al., 2011), and in the lower respiratory tract of duck (Kuchipudi et al., 2009). This is in contrast to the previously reported predominant expression of SAα2,6Gal receptors in chicken and human trachea and predominant SAα2,3Gal expression in the trachea of duck and all species of Passeriformes (Franca et al., 2013; Kuchipudi et al., 2009; Shinya et al., 2006).

In most avian species, both avian and human type influenza receptors are present at least in one segment of the respiratory tract but not always co-expressed throughout as was observed in emu which is a unique feature of emus among other avian hosts studied. The presence of both the receptors in all of respiratory tract of emu indicates that emu could support co-infection of avian and mammalian influenza viruses similar to what is proposed to occur in pigs, chickens, quails and turkeys (Costa et al., 2012; Pillai and Lee, 2010).

3.2. SA receptor distribution in emu digestive tract

A distinct pattern of SA receptors distribution in emu digestive tract was observed (Fig. 2). In the proventriculus, both SA receptors were

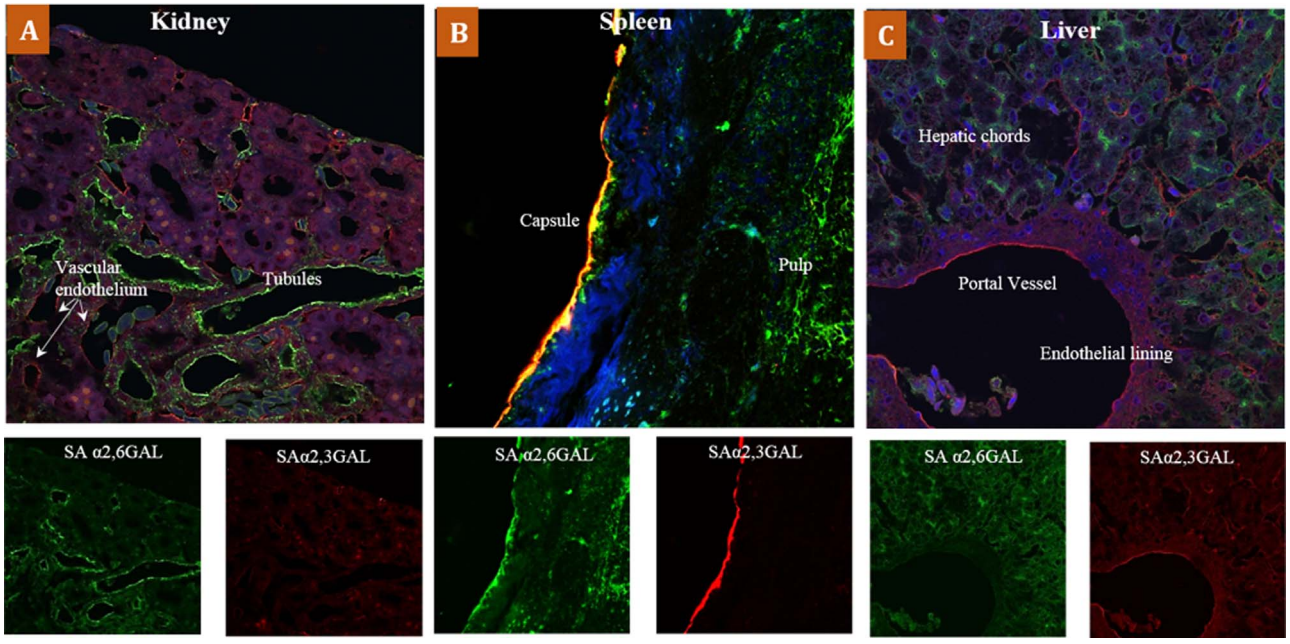

Fig. 3. Abundant co-expression of SAα2,6-Gal and SAα2,3-Gal receptors in internal organs of emu. Abundant expression of SAα2,3Gal and SAα2,6Gal receptors were found in (A) kidney, (B) spleen, and (C) liver. The relative levels of avian and human like receptors was different among the tissues. SAα2,6Gal was the predominant receptor type in the epithelial cells lining kidney tubules, whereas comparable expression of both receptors was found in spleen and liver. Tissue sections were stained with biotinylated MAAII (red-specific for SAα2,3-Gal) and FITC labelled SNA (green-specific for SAα2,6-Gal) lectins, and nuclear staining with DAPI (blue).

uniformly distributed. Unlike chicken digestive tract where SAα2,3Gal expression is seen exclusively, duodenum and the lower part of small intestine of emu express both SA receptors. However, the expression of avian type (SAα2,3Gal) receptors were comparatively less and mostly confined to some parts of epithelial lining and mainly in goblet cells while the human type (SAα2,6Gal) receptors were dominant throughout the epithelial lining, a pattern that is very similar to small intestine of pig (Nelli et al., 2010). Analogous to chickens and other avian species, the epithelial lining of the villi and goblet cells of large intestine, predominantly expressed avian type receptors, while the human type receptors were weak and mostly concentrated towards the luminal region of the villi (Kimble et al., 2010).

The increase in the expression of avian type receptors from duodenum to colon resembles that of pigs but is different from chickens and other avian species where predominantly avian type receptors are present in both small and large intestine (Franca et al., 2013; Ito, 2000; Ito et al., 1998; Kuchipudi et al., 2009; Pillai and Lee, 2010). Abundant expression of both the receptors was observed in caecal tonsil, lymphoid organ which could be important in IAV replication. Abundant SA receptor expression in emu digestive tract corresponds with the observations of significant cloacal shedding of virus in IAV infected emus (Heckert et al., 1999; Kang et al., 2006).

3.3. SA receptor distribution in other major organ systems of Emu

Widespread presence of both SAα2,3Gal and SAα2,6Gal receptors in various organs of emu with temporal differences in the relative abundance of the receptors in different tissues was observed. The vascular endothelial wall of kidney expressed both type of receptors (Fig. 3) similar to that of duck (Kuchipudi et al., 2009), whereas the kidney tubules expressed only the human type receptors. The liver showed predominantly avian type receptors especially on endothelium of the portal vein (Fig. 3) similar to other avian species reported previously (Franca et al., 2013). In spleen, the lymphoid organ where replication of influenza virus has been very well documented, uniform distribution of both the receptors in the capsule and weak presence of only the human type receptor in the pulp region was observed (Fig. 3).

Notably avian type receptors were more abundant than the human type receptors in the endothelial lining of veins in the skin (Fig. 4). These are significant observations as endothelium is known to be an important target of IAV in terrestrial poultry and multi organ necrosis and acute inflammation have been reported in association with presence of viral antigen in endothelium (Brown et al., 2008; Perkins and Swayne, 2003; Short et al., 2014). In brain, consistently diffuse but sparse expression of both the receptors was seen and more specifically in the cortex region (Fig. 4). In the skeletal muscle, both the receptors were weakly present around nuclei, while the avian type receptor was weakly present in the basement membrane of muscle fibres (Fig. 4) as observed previously in pigs (Nelli et al., 2010). In heart, presence of both the receptors with comparatively higher expression of the avian type receptor around the areas of blood capillaries was found (Fig. 4).

Widespread distribution of influenza receptors in various organs of emu is consistent with the observation that avian influenza H9N2 virus infected emus showed multi organ involvement including oedema, congestion in lungs, swelling, congestion in liver, and swelling of kidneys, and widespread haemorrhages in the gastrointestinal tract (Kang et al., 2006). Further, in an experimental infection of emus with HongKong-origin H5N1 avian influenza virus, mild myocarditis and meningoencephalitis were identified along with viral isolations from the lung, brain and kidneys (Perkins and Swayne, 2002). Both of the above clinical findings indicated that systemic infection of IAVs which correlates with our results of the widespread presence of both SA receptors in various emu tissues.

3.4. SA receptor in emu were compatible with binding of avian and human IAVs

To evaluate the functional relevance of SA receptors in emu tissues, we performed virus binding assays with an LPAI H5N2 and a human H1N1 influenza viruses. Virus binding assays showed that the SAα2,3Gal and SAα2,6Gal receptors found in emu were compatible with binding of avian and human IAVs respectively (Fig. 5). Extensive binding of LPAI H5N2 virus and the human pandemic H1N1 virus in trachea was found which correlated with the abundant co-expression of avian and human type receptors in the trachea. Notably extensive binding of avian and human influenza viruses was also found in emu

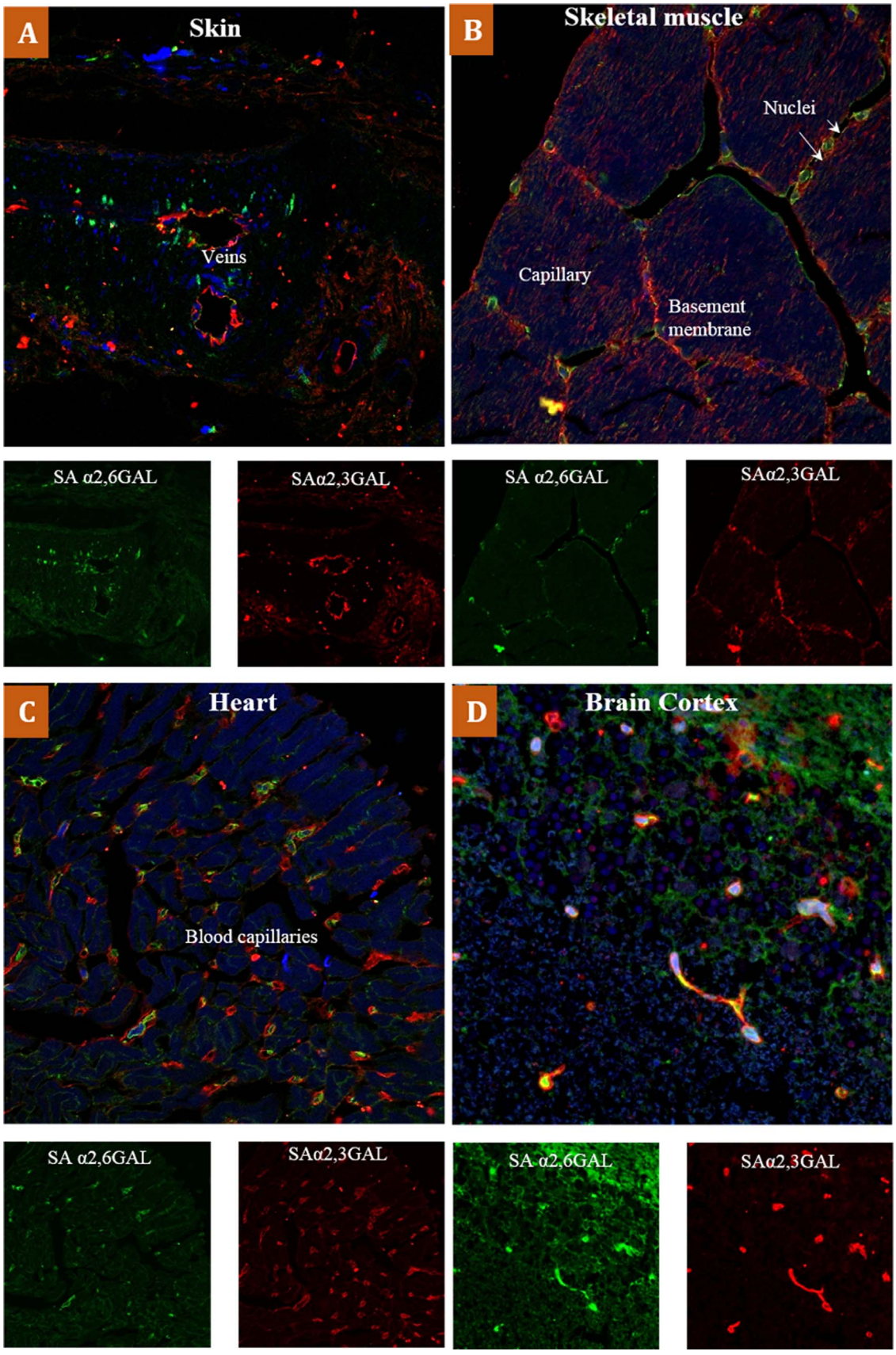

Fig. 4. Diffuse expression of SAα2,6-Gal and SAα2,3-Gal receptors in (A) skin (B) skeletal muscle (C) heart and (D) brain of emu. SAα2,3-Gal was more abundantly expressed in the endothelial lining of veins in the skin and the capillaries of heart. Diffuse expression of both the receptors was seen in the cortex region of the skeletal muscle. Tissue sections were stained with biotinylated MAAII (red-specific for SAα2,3-Gal) and FITC labelled SNA (green-specific for SAα2,6-Gal) lectins, and nuclear staining with DAPI (blue).

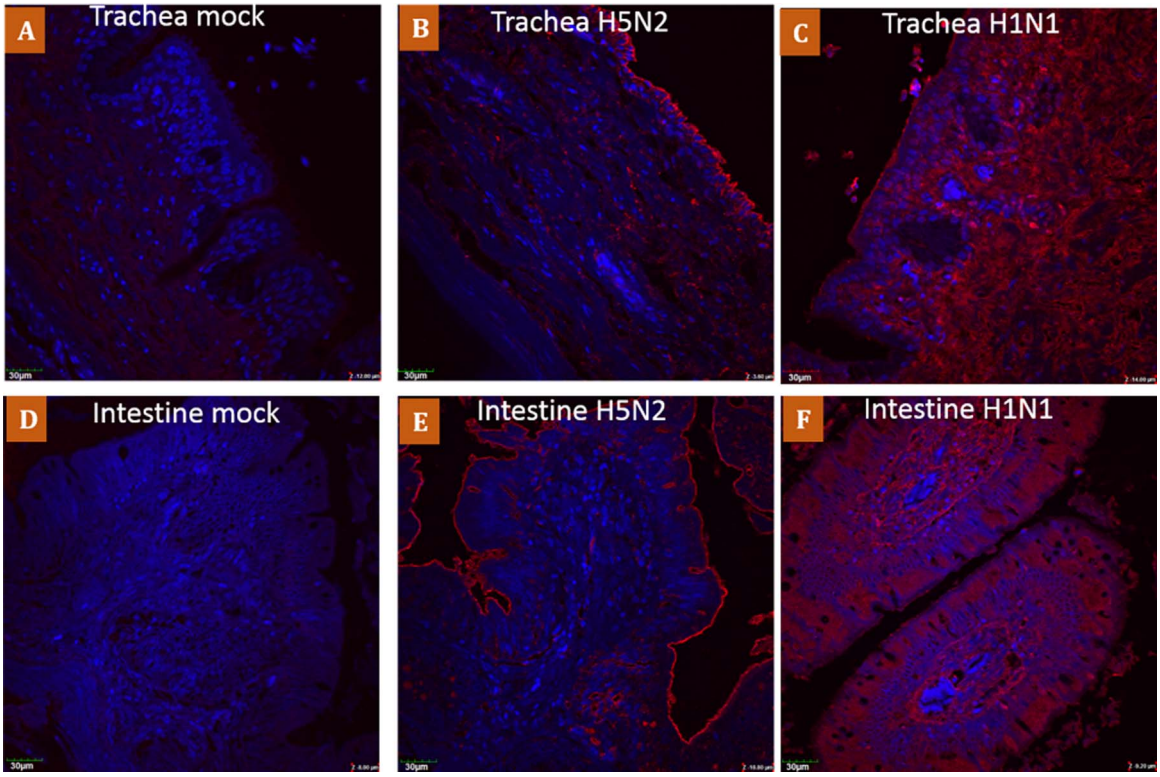

Fig. 5. SAα2,3-Gal and SAα2,6-Gal receptors in emu were compatible with binding of avian and human influenza viruses respectively. Virus binding assays with human H1N1 and avian H5N2 viruses on emu tissues are consistent with virus affinity for particular host receptor type. The presence of both SAα2,6-Gal and SAα2,3-Gal receptors in trachea is mirrored by a similar binding pattern of (B) avian H5N2 virus and (C) human H1N1 virus. Similarly extensive binding of (E) avian H5N2 and (F) human H1N1 viruses to lung was found. Virus binding assays were performed with human pandemic H1N1 virus (A/H1N1/Virginia/2009), or low pathogenic avian influenza H5N2 virus (A/chicken/PA/7659/85). (A & D) Mock controls performed with no virus treatment show absence of fluorescence.

intestine which corroborated the receptor distribution profile in the intestines.

Till date, influenza receptor profile in emus is not known and this is the first comprehensive report of the anatomical distribution of avian and the human type influenza receptors in various tissues of emu. In summary, our results highlight that emus could provide an environment for the reassortment between avian and human influenza viruses in both respiratory and digestive tracts. Further, based on the abundant SAα2,6Gal receptor expression, it is highly likely that emu provides an environment that is conducive for the selection of viruses with increased human receptor binding ability similar to quail and chicken (Kuchipudi et al., 2009; Wan and Perez, 2006). The findings of our study highlight the importance of influenza virus surveillance in ratites especially emus for a better understanding of IAV evolution which is of immense animal and human health significance.

Author contributions

S.V.K conceived the study, S.V.K and M.S supervised the study. S.V.K, M.S, K.K, V.K and B.J designed the experiments and interpreted the data. N.G and M.S performed Lectin histochemistry, S.G, M.S, S.K.C. G.B and R.T performed confocal microscopy. S.K.C, G.B and R.T performed Virus binding assays. S.V.K and M.S prepared the manuscript and all authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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