Short Communication

Influenza A Virus Receptor Identification in the Respiratory Tract of Quail, Pig, Cow and Swamp Buffalo

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Abstract

Virus infection requires the interaction of a virus protein with the host-cell receptor. The Influenza A virus receptor consists of sialic acid linked to the galactose unit in a α 2,3 or α 2,6 conformation. Various types of these receptors are expressed differently on the epithelial lining of various animal species. Here we characterized the types of receptors that are expressed in the upper and lower respiratory tracts of cow, buffalo, pig and quail. Our findings demonstrate that SA α 2,6-gal linked receptors for human influenza viruses are present in the lower respiratory tract of cow and buffalo, while the SA α 2,3-gal linked receptors for avian influenza viruses are prominent in the upper respiratory tract of buffalo. Both types of influenza virus receptors are expressed in the respiratory tract of quail and pig.

Keywords: buffalo, cow, influenza A virus, pig, quail, receptor

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บทคัดย่อ

การปรากฏและการกระจายตัวของตัวรับที่จำเพาะต่อเชื้อไข้หวัดใหญ่สายพันธุ์เอในระบบทางเดินหายใจของนกกระทา สุกร โค และกระบือ

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การติดเชื้อไวรัสเกิดจากการมีปฏิสัมพันธ์ระหว่างโปรตีนที่จำเพาะของไวรัสกับตัวรับบนผิวเซลล์ของผู้ติดเชื้อ ตัวรับที่จำเพาะต่อเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์เอประกอบด้วยกรดไซอาลิกที่เชื่อมต่อกับ กาแล็กโทสยูนิต ที่ตำแหน่งแอลฟา 2, 3 หรือ แอลฟา 2, 6 การปรากฏและการกระจายตัวของตัวรับทั้งสองชนิดจะมีความแตกต่างกันบนเยื่อบุผิวเซลล์ของสัตว์แต่ละชนิด รายงานนี้ได้จำแนกลักษณะชนิดและการกระจายตัวของตัวรับทั้งสองในระบบทางเดินหายใจส่วนต้นและปลายของโค กระบือ สุกร และนกกระทา รายงานฉบับนี้แสดงให้เห็นว่า ตัวรับที่เชื่อมต่อกับกาแล็กโทสยูนิตที่ตำแหน่งแอลฟา 2, 6 ซึ่งจำเพาะต่อไข้หวัดใหญ่แสดงออกที่ระบบทางเดินหายใจส่วนปลายของโคและกระบือ ในขณะที่ตัวรับที่เชื่อมต่อกับกาแล็กโทสยูนิตที่ตำแหน่งแอลฟา 2, 3 ซึ่งจำเพาะต่อไข้หวัดนกแสดงออกที่ระบบทางเดินหายใจส่วนต้นของกระบือ และพบการแสดงออกของตัวรับทั้ง 2 ชนิดที่ระบบทางเดินหายใจของนกกระทาและสุกร

คำสำคัญ: กระบือ โค เชื้อไวรัสไข้หวัดใหญ่สายพันธุ์เอ สุกร นกกระทา ตัวรับ

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Introduction

Influenza A viruses can infect avian species, land based birds such as chickens and ducks and also humans and other mammalian species such as horses, pigs, minks, seals, and whales (Krug, 1989). Evidence places the origin of Influenza A viruses to waterfowl as all 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes persist in these avian reservoirs (Fouchier et al., 2005). Although the virus can be isolated from a wide range of hosts, it is clear that influenza A viruses do not replicate indiscriminately across these animal species, but rather show a host-range restriction pattern. For example, experimental infection studies demonstrated that human influenza virus could replicate and cause clinical signs in non-human primate species, while avian influenza viruses replicated poorly and did not induce clinical signs in those species (Beare and Webster, 1991). The same is true for studies on avian species such as Japanese quail that showed no clinical symptoms and shed low amounts of virus when experimentally infected with human and swine influenza viruses (Makarova et al., 2003). However, pigs, humans and birds demonstrate partial host restriction facilitating occasional virus-transmission from one species to another. For example human-to-pig virus transmission and vice versa, has been frequently documented (Kundin, 1970; Karasin et al., 2000; Myers et al., 2007; Yu et al., 2007). Cross-species transmission of influenza A viruses can lead to an event known as 'genetic reassortment' since the virus genome comprises 8 RNA segments. This happens when a host cell is infected simultaneously with two different viruses. During viral replication, the exchange of RNA segments from the different parental strains can result in a novel virus with a new combination of genes. If this virus harbors an HA and/or NA protein that is new to the host, it can evade the host immune response and cause an outbreak in that population and other species as well.

Influenza A virus infections in humans and animals are initiated by interactions between the viral HA and sialic acid (SA)-containing molecules on the target epithelial cells (Krug, 1989). Viruses from different host species have specific binding preference to either the N-acetylneuraminic acid α 2,3-galactose (SA α 2,3-gal) or the N-acetylneuraminic acid α 2,6-galactose (SA α 2,6-gal) linkage (Ito, 2000). Previous research has indicated that the receptor specificity of influenza virus correlates with receptor molecules at the replication site in the hosts' tissue. It has been shown that human influenza viruses (subtypes, H1, H2 and H3) recognize the SA α 2,6-gal linked receptor found predominantly in the humans' upper and lower respiratory tract (Shinya et al., 2006). Avian influenza viruses recognize the SAα2, 3-gal linked

receptor which aligns both the respiratory and digestive tract of bird species. Seal and whale lung epithelial cells express the SA α 2,3-gal linked receptor and influenza A virus isolated from these species recognizes the corresponding linkage type (Ito, 2000). This indicates that avian influenza virus can be directly transmitted to sea mammals. The pig trachea has been shown to express both SA α 2,6- and SA α 2,3-gal linked receptors and thus, both human and avian influenza viruses are able to infect pigs (Ito et al., 1998). Recent data have shown that not all avian species display identical types of influenza virus receptors. The type of linkage expressed on the receptor varies depending on the location of epithelial cells in the host and the species of animal. Therefore, the initial step to identify the hosts for influenza A viruses as well as potential intermediate hosts for re-assorted viruses is to identify the type of receptor expressed in the upper and lower respiratory tract of the different animal species. This is the first study investigating the distribution of the SA α 2,3-gal and SA α 2, 6-gal linked receptors in the upper and lower respiratory tract of cows and water buffalos.

Materials and Methods

Animal and tissue preparation: Trachea and lung samples were collected from the following species (two healthy animals each): five-week-old quails obtained from the Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Thailand; three-week-old pigs purchased from Charoen Pokphand Group (Bangkok, Thailand); cows and water buffalos of unknown age obtained from a slaughterhouse (Bangkok, Thailand).

Animals were humanely sacrificed. Trachea and lung tissues were freshly collected and immersed in phosphate buffered saline (PBS) containing 4% paraformaldehyde for 48 hours. Tissue samples were then dehydrated by successive immersion in various alcohols and embedded in paraffin wax. These paraffin-embedded tissues were cut into 5-μm sections with a microtome, mounted on 3-aminopropyltrethoxy-saline (APS)-coated slides, deparaffinized in xylene and rehydrated by alcohol. At least 4 sections per tissue sample were subjected to immunohistochemistry staining to facilitate detection of each specific type of influenza virus receptor.

Immunohistochemistry: Detection of SA α 2,3-gal and SA α 2,6-gal linked receptors on tissue samples was performed with the digoxigenin (DIG) glycan differentiation kit (Roche, Germany) following the manufacturer's instructions. In brief, the slides were blocked with 1% bovine serum albumin (BSA) in tris-buffered saline (TBS) for 1 hour. To detect the SA α 2,3-gal specific receptor the slides were incubated with digoxigenin (DIG)-labeled Maackia amurensis lectin (MAA; Roche, Germany) 5 μg/ml in buffer I (TBS with Mg^{2+}, Mn^{2+}, Ca^{2+} and 1% BSA) for 1 hour. To detect the SA α 2,6-gal specific receptor the slides were incubated with Sambucus nigra lectin (SNA; Roche, Germany) 5 μg/ml in buffer I for 1 hour. The samples were then washed 3 times with TBS and incubated with anti-DIG-alkaline phosphatase (AP) with 1% BSA for 1 hour. After washing 3 times with TBS the slides were developed in either a NBT-BCIP blue color substrate (for SA α 2,6-gal) or Vector® Red color substrate (Vector Laboratories, USA) (for SA α 2,3-gal). The slides were then mounted and screened for positive signal under a light microscope (Carl Zeiss/Microimaging GMBH, Germany). Quail and pig trachea have been shown to contain both SA α 2,3-gal and SA α 2,6-gal linked receptors and were thus used to serve as positive controls. Slides stained with buffer I containing neither MAA nor SNA served as negative controls.

Results

SA α 2,6-gal linked receptor detection: The results of the SNA lectin-based staining specific for the SA α 2,6-gal linked receptor are shown in Fig 1 and Table 1. Quail and pig trachea epithelial lining showed a strong reaction with SNA lectin indicating that both species have receptors for human influenza virus in the trachea. Neither cow nor buffalo trachea showed any reaction to SNA indicating absence of receptors for human influenza viruses. Lungs from all animals included in this study showed reaction to SNA lectin specific for SA α 2,6-gal linked receptors indicating expression of the receptor for human influenza viruses (Table 1, picture not shown). Pig lung in particular, demonstrated a strong reaction to SNA lectin on the epithelial lining of the big airways such as the bronchi.

Table 1 Summary of Sialic acid (SA) α 2,3-galactose and SA α 2,6-galactose linked receptor expression in the respiratory tract of quail, pig, buffalo and cow

Animal	Organ	α 2,3-galactose	α 2,6-galactose
Quail	Trachea	+	+
	Lung	+	+
Pig	Trachea	+	+
	Lung	-	+
Buffalo	Trachea	+	-
	Lung	-	+
Cow	Trachea	-	-
	Lung	-	+

SA α 2,3-gal linked receptor detection: The results of the MAA lectin-based staining specific for the SA α 2,3-gal linked receptor are shown in Fig 2 and Table 1. In line with the results of SA α 2,3-gal detection, quail and pig trachea demonstrated intensive reaction to MAA lectin. This indicates that both quail and pig have receptors for avian influenza viruses in their trachea. The epithelial lining of cow trachea showed no reaction to MAA indicating the absence of SA α 2,3-gal linked receptors. In contrast, the epithelial lining of buffalo trachea displayed a strong reaction to MAA. This suggests that buffalo trachea selectively expresses SA α 2,3-gal linked receptors that are specific to avian influenza viruses. The MAA lectin

reacted strongly to quail lung tissue while no reaction was detected in the lungs from pig, cow and buffalo. This suggests that out of the four species studied only quail expresses the receptor for avian influenza virus in the lung.

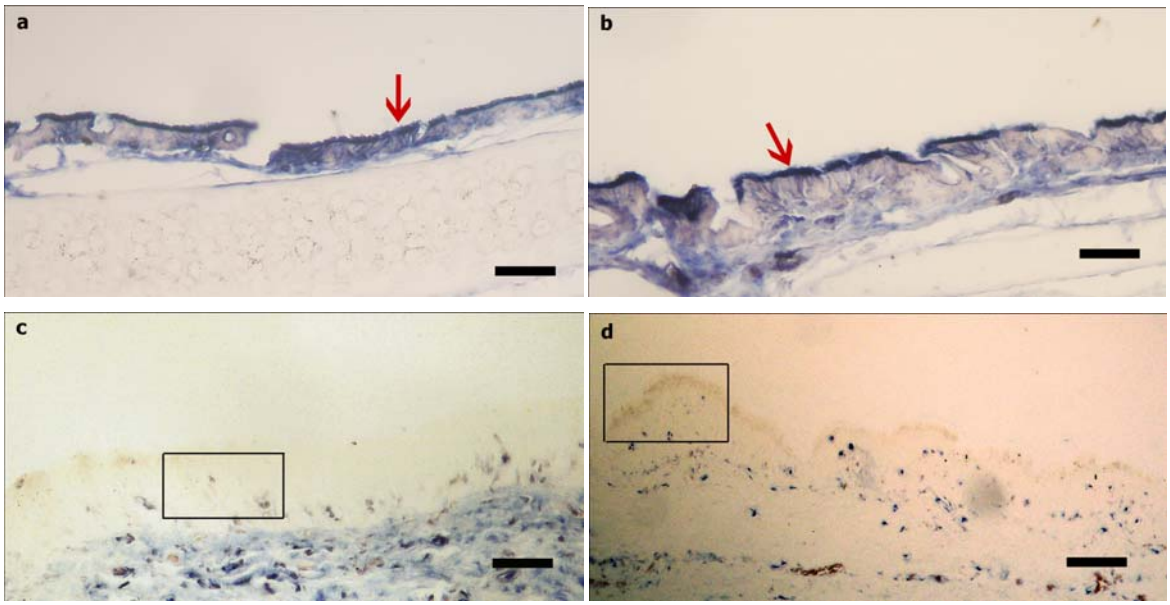

Figure 1 Sialic acid (SA) α 2,6-galactose (gal) linked receptor detection. Trachea sections from (a) Quail, (b) Pig, (c) Buffalo and (d) Cow. Red arrows indicate epithelial cells with positive staining. Dotted box indicate area of epithelial cells with negative staining IHC bar= 50 µm.

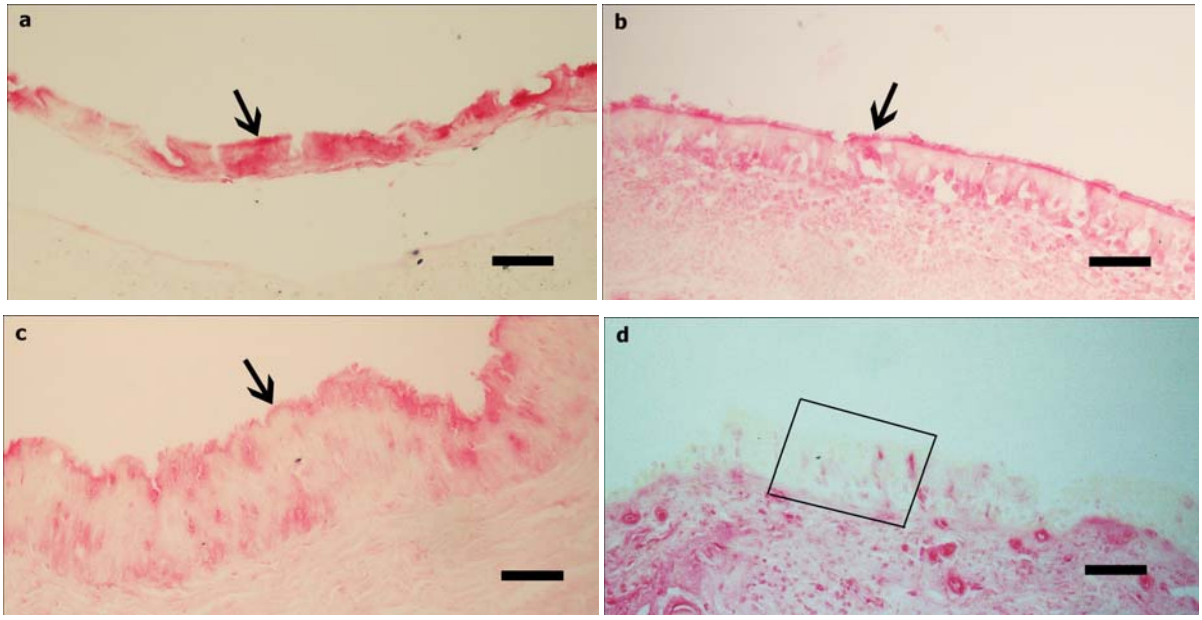

Figure 2 Sialic acid (SA) α 2,3-galactose (gal) linked receptor detection. Trachea sections from (a) Quail, (b) Pig, (c) Buffalo and (d) Cow. Black arrows indicate epithelial cells with positive staining. Dotted box indicate area of epithelial cells with negative staining IHC bar= 50 µm.

Discussion

Many rural populations in Asian countries keep cows and water buffalos as livestock. In Thailand, water buffalos are raised mainly on small farms in rural areas. Water buffaloes are closely associated with rice paddy cultivation and are considered a part of the Thai farmer's life. They are usually tended by the young or elder members of the family and are allowed to graze freely on the rice field and grass land. Dairy and beef production in Thailand have become more industrialized. However, in rural areas cows/cattle farming are still spotted as in the old days where animals are not confined but are allowed to graze freely during the day and are herded back to the village in the evening. Possibly influenza A virus cross-species transmission can happen to both cows and water buffalo. In Thailand both animal species are in daily contact with all the influenza A virus natural hosts and viral reservoirs (wild birds). Thai water buffalos in particular are often seen with starlings and mynahs on their backs in the rice fields and mud puddles. Those two bird species are known to be susceptible to the highly pathogenic avian influenza (HPAI) H5N1 virus (Boon et al., 2007). Moreover, it is known that influenza A virus transmission between humans and other species is common (Van Reeth, 2007). In backyard farms in

several Southeast Asian countries including Thailand, cows and water buffalos can occasionally intermingle with pigs and quails. The two lateral species have the potential to act as intermediate hosts of influenza A viruses (Ito et al, 1998; Wan and Perez, 2006). Pigs in particular have been marked as a mixing vessel to generate new influenza A viruses with pandemic capability as demonstrated by the 2009 H1N1 pandemic (Neumann et al., 2009). In theory, quail is capable of being a mixing vessel similar to pigs as it holds both human and avian influenza A type receptors. A previous study showed that quail was broadly susceptible to infection with a variety of subtypes of both mammalian and avian influenza viruses (Makarova et al., 2003). The results from this study indicate that cows and water buffalos have potential to be infected with influenza A viruses and may possibly transmit these viruses to other animal species, including humans, highlighting their potential roles in interspecies transmission of influenza A viruses.

Published data on influenza A virus infection in cattle are scarce. Research reported from Japan in 1978 studied sera from 728 cattle during 1975-1977 and detected 1.5% and 1% of hemagglutination-inhibition (HI) antibody response to human H1 and H3 influenza A virus, respectively (Onta et al., 1978). Another article reviewed the reports on serological findings and virus isolation from cattle in many countries (Lopez and Woods, 1984) indicating that 20% of more than 600 cattle sera in the USA had HI antibodies to swine H1N1, equine H7N7 and also human influenza B viruses. In addition, the article provided data on virus isolation showing that H1N1 and H3N2 virus have been isolated from cattle in Hungary and the USSR respectively. A more recent study (Jones-Lang et al., 1998) in Minnesota used an ELISA based test to detect H1 subtype-specific antibodies in 2,345 bovine sera. The group detected 27% positive along with 31% weak-positive samples that appeared to peak during the months of seasonal influenza epidemics found in the swine population. These previous data indicated that bovine species were susceptible to influenza A virus infection, in particular human and swine influenza. The most recent influenza A virus study conducted in bovine species comprised an experimental infection with the HPAI H5N1 virus (Kalthoff et al., 2008). Four Holstein-Friesian calves were experimentally infected with a high dose of HPAI H5N1 virus and were monitored for 7 days post infection (dpi). It was concluded in the study that the infection rate of cattle with HPAI H5N1 in disease-endemic regions should be low since no animals showed any clinical signs. Although seroconversion was detected in all animals at 14 (dpi), very low amounts of virus were shed by two of the animals for only 2 dpi. These findings provide an explanation for the data from previous reports. It is well known that the initial step for virus infection is the successful attachment of the viral protein to the specific receptor on the target cells (Knipe et al., 2001). This study demonstrated that the SA α 2,3-gal linked receptors which were the receptors for avian influenza viruses (including the HPAI H5N1 virus) were absent from the upper and lower respiratory tract of cows. Only the SA α 2,6-gal linked receptors which are the receptors for human and swine influenza viruses are present in the lower respiratory tract. Therefore, human and swine influenza viruses can replicate in cows more efficiently than avian influenza viruses. It should be noted however that influenza virus infection in cows may not be as prevalent as in the swine population because the SA α 2,6-gal linked receptors were detected only in the cow's lung but not the trachea. Therefore, it is possible that a higher viral load is required for both human and swine influenza virus to infect and establish influenza A virus-associated respiratory disease in cows compared to the human and swine population. No studies have been conducted to assess influenza A virus infection in water buffalos. The findings of the study indicate that the types of influenza virus receptor expressed in water buffalos are not consistent in the upper and lower respiratory tract. Water buffalos express SA α 2,3-gal linked receptors which are the receptors for avian influenza viruses in the trachea while SA α 2,6-gal linked receptors which are the receptors for human and swine influenza viruses are expressed in the lungs. Since water buffalo expresses both types of receptors in the respiratory tract, it would be interesting to conduct experimental infection studies to further evaluate the possibilities of buffalo as an influenza virus intermediate host. However, such studies require high level biosecurity laboratories and thus, seroprevalence studies to assess the HI antibody levels against influenza A viruses particularly to HPAI H5N1 may be more practical as the results can also imply virus transmission from birds to buffalo. In conclusion, our study showed that both types of influenza virus receptor were expressed in the respiratory tracts of quails, pigs and buffalos, while the respiratory tract of cows expresses only SA α 2,6-gal linked receptors that are specific for human influenza viruses. This study is the first to underline the potential role of buffalo and cow as being a susceptible host for influenza A virus infection and their roles in influenza interspecies transmission. Future studies such as serological surveillance in these species can provide information on the disease prevalence and reflect their role in the influenza A virus ecology.

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