Exploring influenza A virus receptor distribution in the lactating mammary gland of domesticated livestock and in human breast tissue.

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ABSTRACT

The spread of the highly pathogenic avian influenza (HPAI) H5N1 virus among dairy cattle illustrates the adaptability of influenza A viruses (IAV) to infect non-traditional species. While IAV-specific sialic acid (SA) receptors have been identified in the mammary glands of dairy cattle, their presence in pigs, sheep, goats, and alpacas has not been studied until now. The zoonotic transmission of HPAI H5N1 to dairy and poultry farm workers during outbreaks raises public health concerns. This study employed lectin histochemistry to examine the mammary glands of livestock and humans. We found that these tissues were rich in SA α2,6-Gal receptors, followed by SA α2,3-Gal receptors, essential for IAV binding. Notably, the A(H5N1) clade 2.3.4.4b virus could bind to mammary tissue from both cattle and pigs. These findings highlight the potential for HPAI H5N1 to infect and spread within the mammary glands of production animals and humans.

Keywords: Sialic acid, α2,3-Gal, α2,6-Gal, H5N1, mammary gland, cattle, alpaca, sheep, goat, pig, human.

INTRODUCTION

Since the first human case of highly pathogenic avian Influenza (HPAI) H5N1 was reported in 1997, there has been limited evidence to suggest human-to-human transmission. With spillover from birds to humans, there have been years in which the peak annual human cases worldwide reached <150 [1–4]. The case fatality ratios were close to 50%, raising serious public health concerns, albeit with low overall reported case numbers [2,4,5]. Interestingly, the current outbreak of HPAI H5N1 in dairy cattle and poultry in the U.S. has resulted in numerous spillovers into farm workers handling HPAI H5N1-affected dairy herds and poultry flocks. As of March 7, 2025, there have been 70 CDC-confirmed cases, with one reported death associated with HPAI H5N1 [6,7]. Influenza A viruses are known to replicate well in the respiratory and intestinal tract of animals and humans [8,9]. However, the continued HPAI H5N1 viral replication in the mammary gland of novel species like dairy cattle [10,11] with a combination of continued spillover into numerous naive mammalian species [12], raises the concern of HPAI H5N1 virus adaption to mammalian species with a possibility of continued mammal-to-mammal and even human-to-human spread.

To assess the current feasibility of HPAI H5N1 viral replication within the mammary glands of other exposed animals, this study aimed to delineate the spatial distribution of influenza A virus (IAV) specific sialic acids (SA) receptors in the mammary glands of various production animals: pigs, sheep, goats, cattle, and alpacas in comparison to human breast tissue. Due to recent spillovers from production animals into people, this study also examined human breast tissue.

The mammary gland is a remarkable structure responsible for producing milk, a

nutrient-rich fluid that nourishes. The importance of the mammary gland cannot be

overstated, as it ensures species continuity and offspring health and development. It

also contains antibodies that help to protect the young from infections [13,14]. Most

humans depend on milk, or its byproducts, for this highly nutritious fluid that contains

essential proteins, carbohydrates, fats, vitamins, and minerals [15]. The mammary

gland's structure is complex and consists of a network of alveolar lobules

interconnected by ducts and supported by connective, adipose, and smooth muscle

tissues (Figure 1). The lobules are responsible for milk production, while the ducts

transport the milk to the cisterns for suckling. The mammary gland undergoes

significant hormonal and metabolic changes during pregnancy and lactation, adapting

to the demands of milk production and the growth of the fetus [16]. The milk

composition can vary among different species, reflecting the specific needs of their

offspring [17].

However, the mammary gland is not immune to infections. Pathogens, such as

bacteria, viruses, and fungi, can invade the mammary gland and cause mastitis, a

painful and debilitating condition. Interestingly, retrograde infection is the most

common route or method of infection, where microorganisms enter through the teat

opening and into the canal and cistern [18]. In dairy cows, mastitis in animals can lead to

decreased milk production, reduced quality, and even death in severe cases [19]. The

infection can spread to other animals through contaminated equipment or by direct

contact, posing a significant threat to the health of an entire herd [20]. The consequences

of mastitis are far-reaching. In addition to causing pain and suffering for the affected

animal, mastitis can have a negative impact on the economic viability of livestock

production. This includes reduced milk production, increased veterinary costs, and the

potential for culling infected animals, which can result in significant financial losses

for farmers. Moreover, mastitis can lead to transmitting pathogens to humans through

contaminated unpasteurized milk, posing a public health risk.

The recent discovery of HPAI H5N1 clade 2.3.4.4b virus in the mammary gland and

milk of dairy cattle sets a new scientific precedent, emphasizing the adaptability of

H5N1 HPAI viruses to non-avian species and its potential for cross-species

transmission, including to humans. A key factor in the susceptibility to HPAI H5N1

infection is the presence of specific host cell receptors that the virus uses for entry [21].

Influenza A viruses utilize host SA for initial attachment and entry into cells [22]. Sialic

acids are also potential receptors or co-receptors for other viral families such as

Coronaviridae [23], *Paramyxoviridae, Flaviviridae* [24], *Picornaviridae* [25], *Reoviridae* [26],

Parvoviridae [27], *Adenoviridae* [28], *Papillomaviridae* [29], *Polyomaviridae* [30], and

Caliciviridae [31]. In addition, many bacteria, fungi, and parasites exploit sialic acids as

growth substrates for immune evasion, host cell entry, and enhanced virulence,

including those that cause mastitis, such as *E. coli*, *Streptococcus sps.*, *Pasteurella*

sps., *Candida sps.*, *Aspergillus fumigatus*, *Leishmania* and *Trypanosomes* [32–36].

Also sialic acids are also one of the major components of milk and play an important

role in human nutrition, particularly in brain development [37,38]. Cattle and goat milk

predominantly contain sialic acid forms N-glycolylneuraminic acid (Neu5Gc) versus

N-acetylneuraminic acid (Neu5Ac), and their content decreases with the progression

of the lactation stage [39,40]. Also, sialic acid levels vary depending on the diet of the

animal and have been shown to regulate the microbiota-gut-mammary axis during

mastitis of Holstein cows [41]. In addition, Neu5Ac was also one of the key metabolites

that was found to be elevated in metastatic breast tumors in humans [42]. Thus,

suggesting that SA levels may vary depending on the lactational stage, diet, and

underlying disease conditions.

Therefore, understanding the sialic acid interactions in a complex organ like a

mammary gland with frequent interactions with microbial and hormonal changes

during the lactational stage is important.

MATERIALS AND METHODS

Sample collection

This study was approved by the Institutional Animal Care and Use Committee

(IACUC) of the College of Veterinary Medicine, Iowa State University, Ames, Iowa

(IACUC-24-090). In the case of the human mammary gland, consultation and

approvals from ISU and GMU [Institutional Review Board approval (GMU IRB#

478170 and 477703)] were obtained before using the archived tissue blocks. Three

animals of each species studied were procured from the Iowa State University

Research farms (sheep, goats, beef cattle) from approved vendors (alpacas), while

archived tissues for the rest (Table 1). Following euthanasia, formalin-fixed and

paraffin-embedded sections of the mammary gland from the area of the teat cistern,

gland cistern, interlobular (collecting) duct, and secretory alveoli (glandular

epithelium) were collected from these animals (Figure 2).

Lectin histochemistry

Mammary tissues were characterized for sialic acids using lectin histochemistry assay

as previously described [43], for chromogenic and fluorescent staining. Briefly,

formalin-fixed, paraffin-embedded tissue sections of the mammary gland were

sectioned at 4 µm with placement on Superfrost® Plus slides (VWR International).

Slides were dried at 60°C for 20 minutes preceding deparaffinization and staining on

Roche Diagnostics' Ventana DISCOVERY ULTRA research platform. Heat retrieval

was accomplished via Cell Conditioning (CC1) solution at 100°C for 24 minutes

(Roche Diagnostics Corporation). Endogenous peroxidase was quenched for

chromogenic staining using Discovery Inhibitor (Roche Diagnostics Corporation).

Slides were then blocked with 1X Carbo-Free blocking solution for 32 minutes

(Vector Laboratories Inc.), followed by a Streptavidin/Biotin Blocking Kit (Vector

Laboratories Inc.) with a separate application each of 12 minutes. The sections were

incubated for 4 hours (fluorescent)/ 1 hour (chromogenic) at room temperature (RT)

with one of the three lectins (SNA, MAL-I, MAL-II) from Vector Laboratories at the

listed concentration in Table 2. For SA α2,6-Gal/GalNAc, slides were incubated with

biotinylated SNA for both fluorescent. A cocktail of fluorescein-labeled MAL-I

(specific for N-linked or O-linked glycans with SA α2,3-Galβ (1-4) GlcNAc) and

biotinylated MAL-II (specific for O-linked glycans with SA α2,3-Galβ (1-3) GalNAc)

were used for fluorescent staining. Biotinylated MAL-I was used separately for

chromogenic staining. Following lectin incubation, streptavidin conjugated with

Alexa Fluor 594 (ThermoFisher Scientific) was applied and incubated for two hours

at RT. In the case of chromogenic staining, streptavidin-HRP RTU (Vector

Laboratories Inc.) was incubated for 40 minutes on all three biotinylated lectins. The

chromogenic signal was further amplified with DISCOVERY Amplification anti-HQ

Multimer (Roche Diagnostics Corporation). Fluorescent counterstaining was

performed with QD DAPI RUO (Roche Diagnostics Corporation) and mounted

manually with Prolong Gold antifade mountant without DAPI (ThermoFisher

Scientific). Chromogenic counterstaining was performed with hematoxylin and bluing

(Roche Diagnostics Corporation), coverslipped with Sakura Tissue-Tek Film.

Negative assay controls consisted of primary lectin or antibody omission. Positive

lectin assay controls were performed on porcine tissues as lectin labeling has been

previously established [44]. Slides were examined and imaged using a BX-53 Olympus

trinocular microscope equipped with an Olympus DP23 camera, Excelitas X-Cite

mini+ compact illumination system, and CellSyns Dimension software.

Virus preparation, inactivation, and labeling for virus histochemistry

A reverse engineered A/bald eagle/Florida/W22-134-OP/2022 (H5N1; rgA/bald

eagle/FL/22) that contained an engineered low pathogenicity 2.3.4.4b H5 by removal

of the polybasic cleavage site, wild type N1, and 6 internal segments from A/Puerto

Rico/8/1943 (H1N1) was kindly provided by Richard Webby from St. Jude Children's

Research Hospital. Confluent layers of Madin-Darby canine kidney (MDCK) cells

were inoculated with LPAIV H5N1 2.3.4.4b rgA/bald eagle/FL/22. After 48 hours,

cell culture flasks went through two rounds of freeze-thaw cycles at -80°C. After the

second cycle, the supernatant was harvested and cleared by low-speed centrifugation.

The cleared supernatant was then centrifuged at 28,000 rpm in an SW32i rotor at 4°C

for 2 hours on a layer of 10% sucrose. Pellets were resuspended using PBS buffer and

measured for hemagglutination activity (HA) by 2-fold serial dilution with turkey red

blood cells. The virus was then incubated at room temperature with an equal volume

of 10% neutral buffered formalin for 1 hour. The inactivated virus was then dialyzed

against PBS. Inactivation was confirmed on MDCK cells, and an HA was performed

to determine viral titer. The inactivated virus was labeled by mixing with an equal

volume of 0.1mg/ml fluorescein isothiocyanate (FITC) (Sigma-Aldrich) in 0.5 M

bicarbonate buffer (pH 9.5) for 1 hour 4°C with constant agitation. The labeled virus

was then dialyzed against PBS, and a final HA titer was determined.

Viral histochemistry

Virus histochemical staining was performed on sections of lactating Holstein dairy

cattle and lactating cross-bred sows. Tissues were deparaffinized with xylene and

rehydrated through a series of graded alcohol solutions. A 3% hydrogen peroxide

solution (Fischer Bioreagents) was used to quench endogenous peroxidases. Slides

were then washed in TNT buffer (Tris (ThermoFisher Scientific), sodium chloride

solution (Sigma-Aldrich), and Tween20® (Sigma-Aldrich)). Slides were then

immersed in a blocking solution for 1 hour at room temperature (TNT buffer plus

blocking reagent, Akoya Biosciences). Slides were incubated overnight at 4°C in a

humidified chamber with FITC-labeled rgA/bald eagle/FL/22 (200 HA units per 50

µl). Tissues were then incubated in peroxidase-labeled rabbit anti-FITC antibody

(Agilent Technologies) followed by signal amplification through a biotin tyramide

amplification system (Akoya Biosciences). Finally, peroxidase was revealed with 3-

amino-9-ethyle-carbazole (AEC, ThermoFisher Scientific), and counterstaining was

performed using hematoxylin stain solution (Epredia) and ammonia hydroxide

solution (Sigma-Aldrich). A sialidase control was run on sow tissue sections. Sections

were pre-treated with 0.4 U/ml sialidase-A (Agilent Technologies) in 50 mM sodium

phosphate buffer (pH 6.0) overnight at 37°C and subjected to the protocol as outlined

above. Intestinal tissues from a chicken with and without the omission of the FITC-

labeled virus were used as positive and negative controls, respectively. Slides were

examined and imaged using a BX43 Olympus microscope equipped with an Olympus

DP28 camera. Photomicrographs were captured using cellSens Standard.

RESULTS

The results were broadly classified into teat and gland cistern and interlobular duct

and secretory alveoli regions and summarized in Table 3.

Lectin labeling in dairy, beef, non-lactating cattle, sheep and goat

In the ruminants (cattle, sheep, and goats), the epithelial lining of the teat (Figure 3,

Panel Ai-Av) and gland cistern (Figure 3, Panel Bi-Bv) showed multifocal to diffuse,

moderate positive labeling of SA α2-6 receptors (SNA) (Figure 3, Panel Ai-Av, Bi-

Bv). This labeling continued into the interlobular duct (Figure 3, Ci-Cv) and secretory

alveoli (Figure 3, Di-Dv) with mild to moderate intensity. There was a multifocal

labeling of MAL II on the epithelial lining of the teat (Figure 3, Ei-Ev) and gland

cistern (Figure 3, Fi-Fv). In the interlobular duct, MAL II labeling was rare,

multifocal, and minimal to mild in intensity (Figure 3, Gi-Gv), while on the surface of

secretory alveoli, the labeling was diffuse and moderate to marked in intensity (Figure

3, Hi-Hv). Interestingly, in nonlactating cattle, the intensity of MAL II labeling was

comparatively low from the gland cistern (Figure 3, Fiii), and it continued to decrease

in the interlobular duct and secretory alveoli (Figure 3, Giii and Hiii). Overall, the

apical labeling intensity was lower for MAL II than for SNA. The spatial distribution

of these receptors was apical on the epithelial cell surface and uniform across all

animals examined of each species.

Lectin labeling in alpacas, pigs, and humans

Overall, the alpaca showed a similar SNA/MAL II distribution pattern to ruminants,

but the labeling intensity along the epithelial lining of teat cistern, gland cistern,

interlobular ducts, and secretory alveoli (Figure 4, Ai-Di and Ei-Hi), was

comparatively more. This labeling on the epithelial lining is multifocal (teat cistern)

to diffuse (all other tissues), intense, and apical from teat cistern to secretory alveoli.

Both SNA and MAL II labeling was observed in all anatomic regions examined in the

closely related monogastric species pigs and humans. In pigs, the epithelial lining of

the teat cistern, gland cistern, interlobular ducts, and secretory alveoli displayed

diffuse apical labeling of moderate to marked intensity for both SNA and MAL II

(Figure 4, Aii – Hii). In humans, teat and gland cistern are absent hence, no images

were captured (Figure 4, Aiii, Biii, Eiii, and Fiii). However, there was multifocal to

diffuse apical epithelial labeling in the interlobular duct and secretory alveoli of

moderate intensity for both SNA and MAL II (Figure 4, Ciii and Diii, Giii, and Hiii)

in four human specimens tested.

MAL I lectin labeling in ruminants and non-ruminants

The labeling of MAL I, which is specific for N-linked or O-linked glycans with SA

α2,3-Galβ (1-4) GlcNAc) varied across tissue regions of mammary glands and species

evaluated. In the teat and gland cistern of dairy and beef cattle, sheep, and goat, the

labeling was multifocal to diffuse and moderately positive compared to occasional

positive cells along the epithelial lining in non-lactating dairy cattle (Figure 5, Ai-Av

and Bi -Bv). The labeling along the epithelial lining of the interlobular duct and

secretory alveoli was weak positive with occasional focal labeling among all

ruminants, again with non-lactating dairy cattle showing poor or no labeling (Figure

5, Ci-Cv and Di -Dv).

In alpaca and pig, the teat and gland cistern MAL I labeling was multifocal (Figure 5,

Ei- Eii and Fi-Fii). Alpacas had multifocal MAL I labeling in interlobular ducts, while

pigs had continuous apical labeling (Figure 5, Gi and Gii). Like ruminants, MAL I

labeling along the epithelial lining of the secretory alveoli was weak or had no

labeling in alpacas and pigs (Figure 5, Hi and Hii). Meanwhile, human samples

showed continuous apical labeling of MAL I in the secretory alveoli (Figure 5, Hiii).

All samples were further tested using chromogenic labeling to confirm the MAL I

labeling. This was done because the labeling for MAL I seemed to be less than for

SNA and MAL II. Therefore, we wanted an additional assay to confirm our MAL I

IFA findings. In lactating dairy cattle, multifocal labeling was seen in the teat cistern

(Figure 6, Ai), with minimal to no labeling in other examined areas (Figure 6, Bi-Di).

In beef cattle, there was multifocal to diffuse labeling in the teat cistern, gland cistern,

and interlobular duct (Figure 6 Aii-Cii). No labeling was seen in the secretory alveoli

(Figure 6m Dii). In non-lactating cattle, minimal to no labeling was noted across all

anatomic locations examined (Figure 6, Aiii-Diii). For sheep and goats, there was

multifocal to diffuse labeling in the teat cistern, gland cistern, and interlobular duct

(Figure 6m Aiv-Civ, Av-Cv), but no labeling in the secretory alveoli in either species

Figure 6 Div and Dv). In alpacas, there was multifocal to diffuse labeling for MAL I

in all examined areas (Figure 6, Ei-Hi). Conversely, pigs only had MAL I labeling in

the teat cistern (Figure 6, Eii). The only examined tissue for humans in this case, the

interlobular duct, had diffuse apical labeling (Figure 6, Giii).

Virus histochemistry – ruminant (dairy cattle) and non-ruminant (pig)

Viral receptors, as detected by virus histochemistry, were identified at the apical

epithelial surface in the interlobular ducts and secretory alveoli of both lactating

Holstein cows and crossbred sows. The distribution of receptors and labeling intensity

in the interlobular ducts of lactating Holstein cows varied within individual animals

(Figures 7A and 7B). Labeled cells of interlobular ducts commonly had a basilar

nucleus and apical cytoplasm. Cells with minimal to no labeling were commonly

attenuated or not organized as a stratified cuboidal epithelial layer (Figure 7A and

7B). Intra-animal variation was also present in secretory alveoli, with some alveoli

having circumferential intense labeling adjacent to secretory alveoli with no to

minimal labeling to more consistent labeling of one or more epithelial cells and

luminal secretions of adjacent alveoli (Figure 7C and 7D). In crossbred sows, viral

receptors were commonly identified on many of the epithelial cells lining interlobular

ducts, one or more epithelial cells lining secretory alveoli, and luminal secretions of

adjacent alveoli (Figure 7E and 7F).

DISCUSSION

As HPAI H5N1 continues to enter farms in the U.S., infecting several animals and

causing human spillover events [7,45], this study aims to assess the risk of HPAI

infecting the mammary glands of other domestic species and humans. There is a

potential health risk for farm workers, and there are concerns that the virus could

appear in other milk-associated products, such as goat milk or cheese, similar to the

presence of HPAI H5N1 viral RNA in dairy products [46]. In addition, many backyard

farms throughout the U.S. practice mixed animal rearing, and a potential virus entry

into these farms may allow for interspecies transmission, as evidenced in Oregon,

USA [47], with the potential to mutate or reassort in these animal species. Influenza A

virus infections are well known to affect the respiratory system, and it is the primary

site of viral replication and subsequent pathology. But in March of 2024, the detection

of HPAI H5N1 in the mammary glands of cows was indeed surprising. Identification

of high levels of virus RNA in the milk and the mammary gland epithelial cells of

infected dairy cattle suggested the mammary gland as a predilection site for HPAI

H5N1 clade 2.3.4.4b replication in this species [43,48]. These observations have further

been confirmed through experimental studies [10,11]. In these challenge studies, cows

inoculated with HPAI H5N1 clade 2.3.4.4b viruses in the udder showed viral mastitis

with epithelial necrosis within the secretory alveoli. Sequelae included exposure of

the basal laminae and myoepithelial cells or replacement of epithelial cells with

fibrous connective tissue [10,11].

One of the factors contributing to virus attachment and replication is SA, which is

abundantly expressed in cow mammary gland epithelium, in particular, O-linked

glycans with SA α2,3-Galβ (1-3) GalNAc, which has more affinity towards IAVs of

avian origin [43]. To the current dairy cattle outbreak, the relative abundance of both the

avian and mammalian type influenza receptors on the surface of secretory epithelial

cells in the mammary glands of dairy cattle most likely allowed for viral binding and

entry of HPAI H5N1 2.3.4.4b into this novel anatomic area [43]. Therefore, comparing

the spatial distribution of these SA receptors in the mammary glands of ruminants and

non-ruminant species, including humans, can help assess viral attachment, entry, and

risk of replication in this tissue. Pathogens usually gain entry to the mammary gland

through the teat canal and spread through the ducts or can be systemic and attach to

the host cells with appropriate receptors. In this study, we looked at the SA along the

epithelial lining of the mammary gland, starting from the teat canal to the alveoli

lobules, where milk secretion occurs (Figure 1).

Although several studies have looked at the distribution of SAs in humans, pigs,

cattle, sheep, goat, and camel respiratory tracts [49,50], to our knowledge, this is the first

study to compare these receptors in the mammary glands across these species. In

alpaca, testis, and placental epithelial cells, both SA α2,3-Gal and SA α2,6-Gal were

present [51]. In addition, sialic acid receptors are associated with triggering early host

immune responses such as cell death signaling (necrosis/apoptosis) [52]. Therefore, it is

of utmost priority to evaluate the predisposing factors responsible for this new affinity

of IAV towards mammary gland epithelium and the consequences it may have for

mammalian species, including humans.

The current study showed that the mammary tissues from ruminants (cattle, sheep,

and goat) and non-ruminants (alpaca, pig, and human) labeled SA α2,3-Gal and SA

α2,6-Gal receptors along the mammary epithelium, suggesting that both mammalian

and avian IAVs have potential to bind. The SA α2,3-Gal spatial distribution identified

using MAL-I specific for N-linked or O-linked glycans with SA α2,3-Galβ (1-4)

GlcNAc and MAL-II specific for O-linked glycans with SA α2,3-Galβ (1-3) GalNAc

lectin labeling, was particularly interesting because of poor MAL I labeling along the

epithelium (teat and gland cistern) of non-lactating cattle (Figure 5, Aiii and Biii)

compared to lactating cattle. A recent study in *ex vivo* explant cultures of bovine

mammary gland showed enhanced HPAI H5N1 (A/dairycattle/Texas/24-008749-

001/2024) viral replication in the teat and gland cistern compared to chicken H5N1

belonging to the same 2.3.4.4b clade [53]. Studies in mice showed selective blocking of

SA α2,3-Gal associated with MAL I showed a reduction in viral replication and

transmission efficiency in contact mice [54]. Therefore, it is possible that N-linked or O-

linked glycans with SA α2,3-Galβ (1-4) GlcNAc (MAL I) may play an important role

in viral attachment and replication. In addition, there is also variability in lectin

binding studies in the mammary gland of cattle both from the studies performed by

our team and others [43,53,55]. Further infectious and glycan biology studies are needed

to confirm the role of MAL I during HPAI H5N1 viral replication in lactating dairy

cattle.

This study also compared the spatial distribution of SAs in human breast tissue and

showed the presence of SA α2,3-Gal and SA α2,6-Gal receptors, which agrees with

recent findings [56]. Their study further demonstrated that the proteins from HPAI

H5N1 (/Texas/37/2024) bound to human mammary gland epithelium but human

H1N1 and H3N2 do not bind to this tissue. The potential for HPAI H5N1 to bind to

this tissue and possible replication could lead to viral mastitis, complications due to

unknown etiology, breastfeeding complications, inflammation, pain, and discomfort,

compromising both maternal and neonatal health. Hence, there is an immediate

urgency to understand the consequences of HPAI H5N1 in human breast tissues.

The LPAIV H5N1 2.3.4.4b rgA/bald eagle/FL/22 was able to bind the apical surface

of a majority of interlobular duct epithelium and a subset of epithelial cells ling

secretory alveoli using virus histochemistry studies. Both structures appeared to have

more consistent viral binding in the sow mammary gland than the cow mammary

gland, suggesting that while not all cells are similarly susceptible to viral attachment,

the sow mammary gland may contain a higher percentage of susceptible cells. The

virus binding pattern partially agrees with multifocal labeling SA α2,3-Gal receptors

(Table 3; Fig 3-6). However, further co-localization studies are required to determine

the specificity. Pigs are susceptible to HPAI H5N1 2.3.4.4b virus strains, but whether

the virus can successfully travel toward the oronasal cavity of the sow or a suckling

piglet to the mammary gland is unknown [57]. Transmission of 2009 H1N1 IAV-

inoculated kits to the dam's mammary gland has occurred [58]. The presence of H5

receptors on the sow mammary gland suggests that attachment and replication may

also be a possibility in the sow mammary gland. Endemic swine-adapted H1 and H3

IAV infection is not uncommon in suckling piglets. Yet, associated mastitis in sows

has not been documented, either due to the inability of H1 or H3 IAV to cause

mastitis or due to a combination of low prevalence, mild disease, and/or difficulty

identifying mastitis in sows as mammary glands are not mechanically milked and not

routinely evaluated without overt clinical illness. Endemic swine H1 and H3 virus

histochemistry on sow mammary tissue is beyond the scope of this study.

While virus histochemistry may provide a more accurate evaluation of the presence

and distribution of viral receptors than lectins or recombinant HA proteins, it has

limitations. The duration of tissue fixation, tissue autolysis, and the type of fixative

may alter results. In this study, livestock tissues were collected shortly after

euthanasia and formalin-fixed for less than 48 hours. Additionally, the LPAIV H5N1

2.3.4.4b rgA/bald eagle/FL/22 virus was cultivated in MDCK cells. While the impact

of variable virion shapes and sizes on AIV binding to receptors is not yet completely

known, a virus originating from cell culture rather than a clinical specimen could

impact virus binding and interpretation [59]. In addition, the current study also showed

intra-animal variability in the distribution of viral receptors based on virus

histochemistry in lactating Holstein dairy cattle, suggesting that not all cells are

equally susceptible to viral attachment and, therefore, infection. This variable in

susceptibility is also supported by the finding that not all alveoli in inoculated animals

are infected or affected by IAV and, at some level, may also contribute to the

variability in the clinical disease reported from animals in the field and lesion severity

of inoculated animals [10].

Manifestation of clinical disease is multifactorial, and while this study evaluated more

Holstein dairy cattle than prior publications, it did not actively evaluate Holstein dairy

cattle at different stages of lactation or known pathologic conditions that may

contribute to differential glycan expression [39,40]. The observation that epithelial cells

of probable different maturation vary in labeling by virus histochemistry supports

investigating differential glycan expression across different pathologic and

physiological states to elucidate host factors that could influence susceptibility.

CONCLUSION

Based on these findings, we suggest IAVs have the potential to bind to the mammary

glands of cattle, sheep, goats, alpacas, pigs, and humans. Further investigation into the

possible sustained IAV replication in the mammary glands of these species, both in

experimental and natural environments, needs to be performed. Meanwhile, routine

milk or milk product testing from these species, together with continued surveillance

of the human agricultural interface, will aid in containing the virus spread,

establishing mitigation strategies, and understanding evolutionary changes in IAV and

their interactions with different species.

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Table 1. Samples collected

	Breed	**Age**
Dairy Cattle	Holstein Friesian	multiparous
Beef Cattle	Simmetal/Angus cross	5-9 years old
Non-lactating heifer calves	Holstein/Angus cross	14-16 weeks
Sheep	Polypay composite	2-6 lactations
Goat	American alpine	2-6 freshenings/lactations
Alpaca	Brown and Light fawn	2-8 cria/lactations
Pig	Large White and Yorkshire cross	parity 2 or greater
Human*	De-identified	De-identified

* one normal control breast tissue slide from a commercial vendor (Mopec, Madison Heights, MI, USA; Cat no. SC936) and three formalin-fixed paraffin-embedded (FFPE) archived breast tissues collected with informed consent from George Mason University provided de-identified.

Table 2. Lectin histochemistry and Viral binding assay reagents.

	Conjugate	Working Concentration	Manufacturer	Product number
Lectin Staining				
Maackia amurensis -I	Fluorescein	20 µg/ml	Vector Laboratories	FL-1311-2
Maackia amurensis -I	Biotinylated	20 µg/ml	Vector Laboratories	B-1315-2
Maackia amurensis -II	Biotinylated	4 µg/ml	Vector Laboratories	B-1265-1
Sambucus nigra	Biotinylated	20 µg/ml	Vector Laboratories	B-1305-2
Streptavidin	Alexa Fluor-594	2 µg/ml	ThermoFisher Scientific	S11227
Streptavidin	Horseradish Peroxidase (HRP) - R.T.U	1mg/ml	Vector Laboratories	SA-5704-100
Virus histochemistry				
Fluorescein Isothiocyanate isomer (FITC)	none	0.1 mg/ml	Sigma-Aldrich	3326-32-7
Polyclonal Rabbit Anti-FITC antibody	HRP	20 µg/ml	Agilent Technologies	P5100
Biotin tyramide Amplification System	Streptavidin conjugated-HRP	10 µg/ml	Akokya Biosciences	NEL700A001KT
3-amino-9-ethyle-carbazol (AEC) Substrate Chromogen	none	Stock	ThermoFisher Scientific	001122

Table 3. Sialic acid distribution in the mammary glands of ruminants, monogastric, and pseudo-ruminant/camelid.

Mammary gland regions	MAL I (SA α2,3-Galβ (1-4) GlcNAc)	MAL II (SA α2,3-Galβ (1-3) GalNAc)	SNA (SA α2,6-Gal/GalNAc)
Ruminants (Goat/Sheep/Dairy/ Beef cattle)			
Teat Cistern	+ (apical; m/f)	+ (apical; m/f)	+ (apical; d)
Gland Cistern	+ (apical; m/f)	+ (apical; m/f)	+ (apical; d)
Interlobular (Collecting) Duct	+ (apical; * m/f)	+ (apical; * m/f)	+ (apical; d)
Secretory Alveoli (glandular epithelium)	+/– (apical; * m/f)	+ (apical;(m/f) * to d)	+ (apical; d)
Non-ruminant monogastric (Pig/Human)			
Teat Cistern	+ (apical; m/f)/DNE	+ (apical; d)/DNE	+ (apical; d)/DNE
Gland Cistern	+ (apical; m/f)/DNE	+ (apical; d)/DNE	+ (apical; d)/DNE
Interlobular (Collecting) Duct	+ (apical; m/f)/DNE	+ (apical; d)/ + apical; m/f	+ (apical; d)/+apical; m/f
Secretory Alveoli (glandular epithelium)	+/– (apical; m/f)/ + apical; d	+ (apical; d)/+ apical; m/f	+ (apical; d)/+ apical m/f
Pseudo-ruminant/camelid (Alpaca)			
Teat Cistern	+ (apical; m/f to d)	+ (apical; m/f)	+ (apical; d)
Gland Cistern	+ (apical; m/f to d)	+ (apical; d)	+ (apical; d)
Interlobular (Collecting) Duct	+ (apical; m/f to d)	+ (apical; d)	+ (apical; d)
Secretory Alveoli (glandular epithelium)	+ (apical; m/f to d)	+ (apical; d)	+ (apical; d)

Data representative of n = 3 for all animal species; n= 4 for humans. + denotes positive labeling; – no or minimal labeling; apical means labeling

lectins on the epithelial lining; m/f = multifocal labeling; d=diffuse labeling; * denotes non-lactating cattle; DNE = did not examined.

FIGURE LEGENDS

Figure 1: Illustration of sagittal section anatomy of the mammary gland of a cow.

Inset image showing the microscopic anatomy of alveolar lobules with secretory

epithelial cells, myoepithelial cells, and milk secretions.

Figure 2: Cross-sectional gross anatomy of the mammary gland of a cow.

Lines indicate representative areas where samples were collected for histological

studies.

Figure 3: Distribution of sialic acids in the mammary gland of ruminant species.

The mammary glands of dairy cattle, beef cattle, nonlactating cattle, sheep, and goats

showing *Sambucus nigra lectin* (SNA, purple pseudocolor, Alexa Fluor 594) and

Maackia amurensis lectin-II (MAL-II, red pseudocolor, Alexa Fluor 594), using

fluorescent labeling.

The labeling described is in reference to the epithelial surface of each image. In

images Ai-Cv, there is apical, multifocal to diffuse SNA labeling in the teat cistern,

gland cistern, and interlobular duct of ruminants. In Di - Dv, there is positive, diffuse,

apical labeling for SNA of the epithelial surface of secretory alveoli.

In images Ei-Fv, there is multifocal labeling of MAL II on the epithelial lining of the

teat and gland cistern. In Gi-Gv, MAL II labeling was rare, multifocal, and minimal to

mild, with the exception of the nonlactating cow in Giii, which showed minimal

appreciable labeling for MAL II. In Hi-Hv, there was diffuse MAL II labeling of the

epithelial surface of secretory alveoli, again with the exception of the nonlactating

cow in Hiii, which also showed minimal appreciable labeling for MAL II. However,

the apical labeling intensity was less for MAL II than for SNA labeling. Arrows

indicating the labeling on the epithelial lining. Scale bar =50 μm.

Figure 4: Distribution of sialic acids in the mammary gland of non-ruminant species.

The mammary glands of alpaca, pig, and human showing *Sambucus nigra lectin*

(SNA, purple pseudocolor, Alexa Fluor 594) and *Maackia amurensis lectin-II* (MAL-

II, red pseudocolor, Alexa Fluor 594), using fluorescent labeling.

In micrographs, Ai-Di, the alpaca, showed intense diffuse labeling along the epithelial

lining of the teat cistern, gland cistern, interlobular duct, and secretory alveoli for

SNA. Apical, multifocal, intense labeling was seen in the teat cistern for alpacas (Ei),

and labeling for MAL II was diffuse, intense, and apical for MAL II on the gland

cistern, interlobular ducts, and secretory alveoli (Fi, Gi, Hi). In Aii – Hii, images of

pigs showed diffuse, apical labeling of moderate to marked intensity for SNA and

MAL II in all anatomic regions examined. In humans, Ciii, Diii, Giii, and Hiii, a

multifocal to diffuse apical epithelial labeling for both SNA and MAL II was

observed in sections of the interlobular duct and secretory alveoli. Teat and gland

cistern are uncommon in the human breast, hence no images, Aiii, Biii, Eiii and Fiii.

Arrows indicating the labeling on the epithelial lining. Scale bar =50 μm.

Figure 5: Distribution of sialic acids in the mammary gland of ruminant and non-

ruminant species, MAL I specific images using IFA.

The mammary glands of dairy cattle, beef cattle, nonlactating cattle, sheep, goats,

alpacas, pigs, and humans showing *Maackia amurensis lectin-I* (MAL-I, green

pseudocolor, Alexa Fluor 594), using fluorescent labeling.

Micrographs Ai-Cv, showing apical, multifocal MAL I labeling along the epithelial

lining of the teat and gland cistern, as well as the interlobular duct of ruminants. The

labeling in nonlactating cattle teat and gland cistern, Aiii, and Biii, was relatively low

than in other species, including the interlobular duct (Ciii). In the secretory alveoli of

all ruminant species, Di - Dv, there is minimal to no apical labeling for MAL I on the

epithelial surface. Ei-Gii, there is apical, multifocal MAL I labeling in the teat cistern,

gland cistern, and interlobular ducts of alpacas and pigs. In the secretory alveoli of

alpacas and pigs, Hi and Hii, there is apical, multifocal, minimal labeling for MAL I.

There is apical, diffuse labeling for MAL I in human secretory alveoli (Hiii). Arrows

indicating the labeling on the epithelial lining. Scale bar =50 μm.

Figure 6: Distribution of sialic acids in the mammary gland of ruminant and non-

ruminant species, MAL I specific images using chromogenic staining.

The mammary glands of lactating dairy cattle, beef cattle, non-lactating cattle, sheep,

and goats with *Maackia amurensis lectin-I* (MAL-I, brown chromogen), using lectin

histochemistry. There is similar multifocal, apical labeling for MAL I, with no

significant apical labeling observed in Aiii, Bi, Biii, Ci, and Ciii. Similarly, to the IFA

technique, there is minimal labeling observed in secretory alveoli, Di-Dv. The most

intense apical labeling was observed in the alpaca mammary gland, with strong apical

labeling in the teat cistern, moderate labeling in the gland cistern, scant labeling in the

interlobular duct, and moderate labeling within the secretory alveoli (Ei, Fi, Gi, and

Hi). Faint apical labeling was observed in the teat cistern of the pig (Eii). No

significant labeling was detected in the gland cistern, interlobular duct, or secretory

alveoli (Fii, Gii, Hii). Strong apical labeling was present within the interlobular duct

of human breast tissue (Giii). Image panel from one representative animal. Scale bar

=200 μm.

Figure 7. Distribution of LPAIV H5N1 2.3.4.4b rgA/bald eagle/FL/22 2.3.4.4b labeling by

virus histochemistry (red) in the mammary gland of lactating Holstein cows and cross-

bred sows.

Interlobular ducts in the same tissue section of a Holstein dairy cow with minimal labeling (A;

arrow) and intense locally extensive labeling (B; brace). Cells with minimal to no labeling were

commonly attenuated (A; arrowhead) or not organized as a stratified cuboidal epithelial layer

(B; arrowhead). C) Nearly circumferential intense labeling of two secretory alveoli (circled)

adjacent to secretory alveoli with no to minimal labeling of the apical epithelial surface in a

Holstein dairy cow (asterisk;). D) More consistent labeling of the apical surface of one or more

epithelial cells (arrows) and luminal secretions of adjacent alveoli in a Holstein dairy cow. E)

Interlobular duct of a crossbred sow with apical labeling of a single epithelial cell (arrow) or row

of epithelial cells (brace). F) Consistent labeling of the apical surface of one or more epithelial

cells (arrows) and luminal secretions of adjacent alveoli in a crossbred sow. Scale bar = 20 μm

(A, B, and E); 50 μm (C, D, and F).

Sample 4
(alveoli)

Sample 3
(interlobar
duct/alveoli)

Sample 2
(gland
cistern)

Sample 1
(teat
cistern)

Column headers: Dairy Cattle, Beef Cattle, Non-lactating Cattle, Sheep, Goat

Sambucus nigra (SNA)

Teat Cistern: Ai, Aii, Aiii, Aiv, Av
Gland Cistern: Bi, Bii, Biii, Biv, Bv
Interlobular duct: Ci, Cii, Ciii, Civ, Cv
Secretory alveoli: Di, Dii, Diii, Div, Dv

Maackia amurensis lectin–II (MAL-II)

Teat Cistern: Ei, Eii, Eiii, Eiv, Ev
Gland Cistern: Fi, Fii, Fiii, Fiv, Fv
Interlobular duct: Gi, Gii, Giii, Giv, Gv
Secretory alveoli: Hi, Hii, Hiii, Hiv, Hv

Column headers (top panel): Dairy Cattle | Beef Cattle | Non-lactating Cattle | Sheep | Goat

Left axis label: *Maackia amurensis lectin–I* (MAL-I)

Row labels (top panel): Teat Cistern | Gland Cistern | Interlobular duct | Secretory alveoli

Panel labels (top): Ai, Aii, Aiii, Aiv, Av | Bi, Bii, Biii, Biv, Bv | Ci, Cii, Ciii, Civ, Cv | Di, Dii, Diii, Div, Dv

Column headers (bottom panel): Alpaca | Pig | Human

Left axis label: *Maackia amurensis lectin–I* (MAL-I)

Row labels (bottom panel): Teat Cistern | Gland Cistern | Interlobular duct | Secretory alveoli

Panel labels (bottom): Ei, Eii, Eiii (Not present) | Fi, Fii, Fiii (Not present) | Gi, Gii, Giii (Not performed) | Hi, Hii, Hiii

Maacki Amurensis lectin-I (MAL-I)

Dairy cattle | Beef cattle | Non-lactating cattle | Sheep | Goat

Teat cistern: Ai, Aii, Aiii, Aiv, Av
Gland cistern: Bi, Bii, Biii, Biv, Bv
Interlobular duct: Ci, Cii, Ciii, Civ, Cv
Secretory alveoli: Di, Dii, Diii, Div, Dv

Alpaca | Pig | Human

Teat cistern: Ei, Eii, Eiii (Not present)
Gland cistern: Fi, Fii, Fiii (Not present)
Interlobular duct: Gi, Gii, Giii
Secretory alveoli: Hi, Hii, Hiii (Not performed)