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TITLE: Spatial Localization of Avian and Human Influenza A Virus Receptors in Male and Female Bovine Reproductive Tissues

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ABSTRACT:

Highly pathogenic avian influenza virus (HPAIV) of the H5N1 type has recently emerged as a major concern in livestock, with widespread outbreaks now confirmed in U.S. dairy cattle. This raises critical questions about the susceptibility of bovine reproductive tissues to viral entry, replication, and potential transmission. Influenza A viruses (IAV) initiate infection through hemagglutinin (HA) binding to host cell surface sialic acid residues, avian-adapted strains preferentially bind α2,3-linked sialic acids, while human-adapted strains bind α2,6-linked residues. This study aimed to characterize the spatial distribution of α2,6-linked sialic acids (human-like receptors), α2,3-Galβ1-4, and α2,3-Galβ1-3 (avian-like receptors) in male and female bovine reproductive tissues using lectin-based histochemistry. Post-mortem reproductive tissues were collected from bulls (n = 4) and multiparous cows (n = 3) and stained with biotinylated lectins. Human-like receptors were detected in the luminal epithelium of the penile urethra, vas deferens, epididymis, seminiferous tubules, vagina, cervix, uterus, oviduct, and mammary gland. Avian-like receptors were also detected in the penile urethra, epididymis, vagina, cervix, oviduct, and mammary gland, though α2,3-Galβ1-4 and α2,3-Galβ1-3 localization varied by tissue. These findings represent the first comprehensive spatial mapping of IAV receptors in bovine reproductive tissues and highlight potential sites for viral entry or shedding.

Keywords: Avian Influenza, Sialic acid receptors, Bovine, MAA-I, MAA-II, SNA

Introduction:

Influenza A virus (IAV), particularly highly pathogenic avian influenza virus (HPAIV) strains such as H5N1, poses a significant threat to global animal and human health because of its capacity for cross-species transmission [1,2]. Influenza A Virus is an enveloped, segmented RNA virus that expresses surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are critical for viral entry and release [3]. Infection begins when the viral HA proteins bind to sialic acids-containing glycans on the surface of epithelial cells [4]. Successful viral binding will facilitate receptor-mediated endocytosis, allowing the virus to then enter the cell [4]. Within the acidic environment of the endosome, HA will undergo a conformational change that will mediate fusion of the viral envelope with the endosomal membrane, resulting in the release of viral RNA into the cytoplasm, which is then transported to the nucleus for replication [5,6]. The newly synthesized viral components are then assembled at the host cell membrane, and the NA protein cleaves the terminal sialic acid residues to facilitate the release of progeny virions from the cell surface [7,8].

Recent outbreaks of highly pathogenic avian influenza (HPAI) have been escalating across multiple regions of the United States and globally, raising urgent questions about how the virus adapts to new hosts, which species are vulnerable, and which tissues are most susceptible to infection. Until recently, cattle were considered unlikely hosts for IAV infections because natural cases in bovine were extremely rare and only involved influenza viruses of low pathogenicity, especially compared to the frequent outbreaks seen in other domesticated and wild animals at the human-animal interface [9]. This resistance was thought to be due to specific bovine host factors, such as certain serum components and secretory proteins, which may have anti-influenza properties and hindered the adaptation and evolution of IAV in cattle. Additionally, the types and distribution of sialic acid receptors in bovine respiratory tracts, key for IAV entry, were believed to be less compatible with most IAV strains, further reducing susceptibility [9]. However, as of August 27th, 2025 more than 1,070 confirmed H5N1-positive dairy cattle herds have been reported across 17 states in the United States, with widespread detection of the virus in milk and mammary gland tissue [10].

The Eurasian strain of H5N1 (clade 2.3.4.4b) was first detected in late 2021 in North America and has since spilled over into several domestic species, including dairy cattle [11-13]. Two major reassortant H5N1 genotypes in the United States have been identified in cattle; B3.13, which has become widespread in dairy herds, and D1.1, a less prevalent but genetically distinct genotype also found in wild birds, humans, and cattle [14,15]. Viruses of genotype B3.13 have been most commonly associated with dairy cattle where they induce clinical necrotizing mastitis, severe histopathologic lesions in the mammary gland, and drastic declines in milk production [16]. Meanwhile, the D1.1 genotype has been detected in bovine and human respiratory specimens, raising concerns about its potential for zoonotic transmission through respiratory or mucosal routes [15,17]. Cross-species transmission of IAV hinges on the HA glycoproteins binding specificity to terminal specific sialic acid residues on host epithelial cells [2]. Avian adapted strains preferentially bind to α2,3-linked sialic acids while human adapted strains bind to α2,6-linked sialic acids [18,19]. Lectins such as *Sambucus nigra* agglutinin (SNA, binds α2,6-linked sialic acids), *Maackia amurensis* agglutinin I (MAA-I, binds α2,3-Galβ1-4), and *Maackia amurensis* agglutinin II (MAA-II, binds α2,3-Galβ1-3) have been widely used to map the distribution of these glycan receptor types in various host tissues [17,20].

While prior studies have confirmed the presence of α2,3- and α2,6-linked sialic acid receptors in the bovine mammary gland, a comprehensive spatial mapping of these receptors across the broader bovine male and female reproductive tract has not been conducted. Given the mucosal nature of these tissues and their relevance to potential virus shedding, vertical transmission, and fertility, we hypothesized that both α2,3 and α2,6 sialic acid receptors are expressed throughout the male and female reproductive tracts. Sialic acid receptors have been previously documented to have critical roles in reproductive physiology, influencing sperm transport, selection, trophectoderm-endometrial interactions, and embryo implantation [21-23]. The objective of this study was to characterize the spatial distribution of α2,3 and α2,6-linked sialic acids in both male and female bovine reproductive tissues. To investigate receptor distribution in bovine reproductive tissues, we utilized a validated lectin based histochemistry approach in which biotinylated lectins (SNA, MAA-I, MAA-II) were applied to paraffin-embedded tissue sections. Receptor binding was then detected using a streptavidin-alkaline phosphatase system followed by colorimetric substrate development. This technique enabled spatial visualization of α2,6- and α2,3-linked sialic acid residues, providing insight into potential sites of viral attachment and entry for both avian and human-adapted strains of IAV. This work provides foundational data on receptor localization that may inform future investigations into reproductive tract susceptibility to IAV and contribute to our understanding of viral pathogenesis and zoonotic risk in cattle.

RESULTS:

Three lectins were used to map the distribution of sialic acid receptors: SNA, which is specific to the human influenza receptor type and binds to SAα2,6-Gal, MAA-I which is specific to the chicken influenza receptor type and binds to α2,3-Galβ1-4, and MAA-II, which is specific to the duck influenza receptor type and binds to α2,3-Galβ1-3. Receptor expression varied across male and female (**Table 1**) bovine reproductive tissues, with distinct patterns observed for each lectin.

Penis

The SNA and MAA-II lectins were detected in the urethral epithelium, while MAA-I was not detected in epithelia. Within the penile urethra, MAA-II staining was pronounced in the majority of the basal layers of cells of the stratified epithelium, and sporadic detection of MAA-II was also observed in the most superficial layer of epithelial cells within the urethra (**Figure 1 A2**). SNA staining was intense within the majority of the most superficial cells but was not observed in any other epithelial cells of the stratified epithelium (**Figure 1 C2**). All three lectins, SNA, MAA-I, and MAA-II were detected in the endothelial cells lining the blood vessels and blood sinuses within the corpus spongiosum (**Figure 1 A1, B1, C1**). In addition, MAA-II showed intense staining, SNA exhibited moderate detection, and MAA-I was not present within the connective tissue of the corpus spongiosum (**Figure 1 A1, B1, C1**). MAA-II binding was observed within the connective tissue of the trabeculae of the corpus cavernosum, but this was not the case for SNA or MAA-I, and no appreciable lectin binding was observed in the endothelium of the blood sinuses of the corpus cavernosum (**Figure 1 A3, C3**).

Seminal Vesicles

Similar spatial detection of SNA and MAA-I was observed throughout the seminal vesicles with stronger detection in the epithelium and endothelium than in other cell types and staining more intense for MAA-I than SNA (**Figure 2 B1, C1**). MAA-II lectin was not observed in the seminal vesicles (**Figure 2 A1**).

Vas Deferens

The SNA lectin was strongly detected in all cell types within the pseudostratified epithelium lining the lumen of the vas deferens (**Figure 2 C2, C3**). In contrast, MAA-I and MAA-II were not detected by the epithelial cells of the vas deferens but were present in the connective tissue of the lamina propria with MAA-II showing more extensive detection than MAA-I (**Figure 2 A3, B3**). Detection of MAA-I and SNA was evident in the smooth muscle cells of the muscularis externa, although MAA-I detection was restricted to the connective tissue within this layer (**Figure 2 A2, B2, C2**). None of the lectins demonstrated consistent staining of endothelial cells within the vas deferens.

Epididymis

Both SNA and MAA-I were detected in the epididymis, while MAA-II was largely absent aside from isolated regions within the connective tissue (**Figure 3 A1, B1, C1**). SNA was strongly detected across all epithelial cell types of the pseudostratified epithelium that lines the epididymal lumen, whereas MAA-I staining was limited to the basal layer of the epithelium, near the basement membrane, and to the most apical surface of the epithelium which supports stereocilia (**Figure 3 B2, C2)**. It is likely that the stereocilia bind both SNA and MAA-I. Within the lumen, distinct staining of the mixture of spermatozoa and stereocilia that have broken off of the apical surfaces of epithelial cells during tissue processing was observed (**Figure 3 B1, C1**). Negative controls confirmed that this luminal signal was not due to background staining, in contrast to non-specific mucus staining seen in other tissues (e.g., **Figure 4**., cervix and vagina). Low levels of staining for MAA-I and SNA were also present in the connective tissue of the epididymis (**Figure 3 B2, C2).**

Testis

MAA-I and MAA-II showed low levels of staining in the interstitial space of the testis, while SNA demonstrated strong interstitial staining (**Figure 3 A2, B3, C3**). Within seminiferous tubules, SNA detection increased toward the lumen, especially in regions rich in round and elongating spermatids, indicating receptor presence in late stage, post-meiotic germ cells (**Figure 3 C4, C5**).

Spermatozoa

MAA-I and MAA-II staining was absent in spermatozoa isolated from ejaculated semen. In contrast, SNA was strongly detected in the acrosome and midpiece of sperm cells **(Figure 3 A3, B4, C7)**. SNA staining was also observed along the sperm tail, although the intensity was variable across individual tails, and generally less consistent than in the acrosome and midpiece.

Vagina

MAA-I was detected throughout the stratified squamous epithelium that lines the surface of the vagina, whereas SNA presented intermittent detection within the epithelium (**Figure 4 B1, C1**). MAA-II was not detected in vaginal tissues (**Figure 4 A1**). Staining within the vaginal mucus was determined to be non-specific based on negative control slides.

Cervix

Both the SNA and MAA-I lectins were detected in the simple columnar epithelium lining the lumen of the cervix, in the connective tissue and in the endothelium. Staining patterns were very similar between the SNA and MAA-I lectins in terms of localization and intensity (**Figure 4 B3, C2**). MAA-II was not detected in cervical tissues (**Figure 4 A2**). Staining within the cervical mucus was determined to be non-specific based on negative control slides.

Uterus

High levels of SNA and MAA-I lectins were detected in the endometrial vasculature of cycling cows. Much of this staining appeared within the intravascular contents; however, additional linear labeling was also present along the vessel walls. SNA and MAA-I lectins were also present in both the stratum spongiosum and stratum compactum stroma with stromal detection for SNA lectin higher than for MAA-I lectin (**Figure 5 B1, C1**). The SNA and MAA-I lectins were not detected in either the luminal epithelium or glandular epithelium (**Figure 5 A1, B1, C1**), and MAA-II lectin was not detected in any cell types within the endometrium of cyclic cows (**Figure 5 A1**).

Similar to cyclic cows, SNA and MAA-I lectins were highly detected in the endometrial vasculature of pregnant cows, and stromal staining was evident for both SNA and MAA-I lectins with stromal detection just above detectable levels for MAA-I lectin but stromal staining much higher for SNA, particularly in the shallow stratum compactum stroma nearest the luminal epithelium (**Figure 5 B2, B3, B5 C2, C5).** It is noteworthy that in contrast to the endometria of cycling cows, MAA-I and SNA lectins were present within both the luminal epithelium and glandular epithelium of pregnant cows (**Figure 5 B2, B5 C2, C5).** Both SNA and MAA-I lectins showed intermittent detection at the apical surface of the endometrial luminal epithelium which faces the uterine lumen and directly contacts the conceptus (embryo and associated placental membranes) trophectoderm during implantation (**Figure 5 B2, B5, C2, C5).** The SNA and MAA-I lectins were also detected at the apical surfaces of both the superficial and deep glands (**Figure 5 B2, B3, C2, C4**). Interestingly, the SNA staining appeared also included diffuse distribution throughout the cells of the superficial glandular epithelia but was limited to apical localized in the deep glands near the myometrium (**Figure C4, C5**). The SNA lectin was also observed in the myometrium of the uteri of pregnant cows (**Figure 5 C4**). Staining for MAA II was negative throughout the entire endometrium of pregnant cows (**Figure 5 A2**).

Significantly, lectin staining was observed in bovine conceptus. The SNA lectin was detected in the extraembryonic endoderm (**Figure 5 C2, C3**), and the MAA-I lectin was detected at the apical surface of the trophectoderm cells which directly contact the endometrial luminal epithelium during implantation (**Figure 5 B2, B4).**

Oviduct

SNA, MAA-I and MAA-II lectins were highly detected in the epithelial cells lining the oviductal lumen. In all cases, staining was more intense near the apical domain of epithelial cells. Staining for MAA-I and MAA-II lectins was limited to the apical domain of the epithelial cells, while SNA lectin staining was observed throughout the entirety of the epithelial cells (**Figure 6 A2, B2, C2**). The endothelial cells of the vasculature stained positively for SNA and MAA-I but not for the MAA-II (**Figure 6 A2, B2, C2**). The connective tissue underlying the epithelium was positive for SNA but negligible for the MAA-I and the MAA-II lectins (**Figure 6 A2, B2, C2**).

Ovary

SNA and MAA-I were both detected within the ovary, while MAA-II was not (**Figure 6 A3, B3, C3**). MAA-I showed strong staining in the granulosa cells of antral follicles and intense signal within the antral fluid. SNA staining was restricted to the zona pellucida of the ovum of antral follicles but was also observed in primordial follicles (**Figure 6 C3, C4**). The endothelial cells of the ovarian vasculature were detected for both SNA and MAA-I (**Figure 6 B3, C5**).

Mammary Gland

Distinct staining patterns were observed within the mammary glands for all three lectins (**Figure 7 A1, B1, C1**). SNA was strongly detected in the alveolar epithelium and surrounding connective tissue of both lactating and non-lactating mammary tissue (**Figure 7 C1, C2**). In lactating cows, SNA staining was observed in the milk within the alveolar and ductal lumens, while no staining was observed within the alveolar and ductal lumens in non-lactating animals (**Figure 7 C1, C2**). MAA-I was present in the connective tissue but absent in the alveolar epithelium and lumens (**Figure 7 B1, B2**). Any staining observed within the ductal lumen was interpreted as non-specific background staining as it was inconsistent across the gland. MAA-II was strongly detected in the milk within the lumens of alveoli of lactating tissue and was sporadically observed in the alveolar epithelium of both lactating and non-lactating cows (**Figure 7 A1, A2**). Notably, MAA-II was not detected in the connective tissue and any faint coloration in these regions was considered non-specific (**Figure 7 A1, A2**).

Discussion:

In male tissues, α2,6- (SNA lectin) and α2,3-Galβ1-3 (MAA-II) receptor binding was observed in the urethral stratified luminal epithelium of the penis, with stronger α2,6- receptor expression in the superficial cells and a more intense α2,3-Galβ1-3 receptor expression in the basal epithelial layers. This dual receptor profile indicates that the penis may serve as a mucosal entry point for both human- and avian- IAV. Co-expression of multiple viral receptors within the same tissue, as seen in human respiratory tissues, is a key factor that enables viruses to infect a broader range of cell types (broader tropism) and adapt to new hosts or tissues [24-28]. This phenomenon is not unique to influenza but is observed across various viruses, including coronaviruses and HIV [28-31]. Positive staining in the vasculature of the corpus spongiosum for all three lectins was observed and could therefore indicate potential pathways for systemic viral spread via blood vessels. Systemic viral spread via blood vessels is a crucial step that enables viruses to move beyond their initial site of infection and reach distant organs, often resulting in widespread disease [32,33]. Viruses can enter the bloodstream through several pathways, often involving the lymphatic system, infected immune cells, or direct infection of endothelial cells [33-39]. Additionally, positive staining occurred in the connective tissue of the corpus spongiosum for α2,6- and α2,3-Galβ1-3 receptors allowing for potential IAV access to deep tissue compartments within the penis. This aligns with studies reporting that influenza, Zika, and Ebola can breach mucosal barriers and disseminate through vascular or stromal tissues in other mammals [40-44]. The presence of all three receptor types in connective tissue heightens concern that even if local epithelial infection is controlled, deeper tissue reservoirs may permit virus persistence or delayed transmission. No previous research has characterized IAV receptor presence in penile tissue across any species. However, research in swine has detected IAV RNA in semen, suggesting that reproductive shedding is biologically plausible [45]. There was very similar epithelial and endothelial localization of the α2,6- and α2,3-Galβ1-4 sialic acid receptors throughout the seminal vesicles. Seminal vesicles contribute to a large portion of the seminal plasma within semen, containing proteins and molecules that influence sperm motility, viability, and function [46,47]. Therefore, IAV receptor expression within the seminal vesicles holds important implications for viral transmission and semen quality.

Detection of α2,6- and α2,3-Galβ1-4 sialic acid receptors within the epididymal epithelium, stereocilia and spermatozoa implies that the upper male reproductive tract could potentially harbor the virus following systemic or ascending infection. The presence of IAV receptors within the sperm-rich luminal contents suggests the potential for viral binding to spermatozoa or shedding into seminal fluid, which could facilitate viral transmission through either artificial insemination (AI) or natural breeding [48-53]. Positive staining within the testes was present for all lectins in the interstitial compartment with the SNA lectin staining being the most strongly detected. The α2,6-linked receptor was the only receptor present within the seminiferous tubules with unique staining that intensified in regions that were abundant in round and elongating spermatids. Staining for IAV receptors within testicular compartments is highly relevant because these are the sites of spermatogenesis, and the presence of such receptors could make developing sperm vulnerable to viral contamination, potentially harming sperm development and male fertility [54,55]. Furthermore, disruption or damage to the blood-testis barrier formed by Sertoli cells by viruses could expose germ cells to infection and damage [54].

The vas deferens is a critical organ during ejaculation. Strong staining of the α2,6-linked receptor in the pseudostratified luminal epithelium, alongside the α2,3-linked receptors in the connective tissue surrounding the vas deferens epithelium indicates that this tissue supports multi-receptor binding at both mucosal and stromal interfaces. The connective tissue in the vas deferens plays a significant role in receptor-virus interactions by providing a structural and cellular environment rich in potential viral receptors and mediators of intercellular communication [56-58]. The coexistence of these receptors in the vas deferens may increase the tissue's susceptibility to a wider range of IAV strains, supporting the possibility of cross-species transmission and adaptation [52,59]. Additionally, the expression of the receptors within this organ raises the concern of possible viral contamination within the semen during transport within the vas deferens.

There is currently no documented evidence that IAV or HPAIV H5N1 is found in bovine seminal plasma or spermatozoa. However, our findings extend previous observations by demonstrating dual expression of IAV receptors specific to both human- and avian-adapted strains within multiple regions of the male reproductive tract, including the penis, vas deferens, seminal vesicles, epididymis, and testes. To complement these tissue-level findings, additional lectin histochemistry was conducted on ejaculated semen collected from routine breeding soundness examinations. Consistent with testicular and epididymal localization, sperm cells were negative for avian-adapted receptors but showed positive staining for α2,6-linked receptors within the acrosomal region and mid-piece. The biological significance of this pattern warrants further investigation. The acrosome plays a critical role in fertilization, and localization of sialic acid receptors in this region could theoretically facilitate viral attachment during fertilization or early zygotic development. Previous studies have shown show that extracellular vesicles interact with the acrosome, influencing acrosome integrity and fertilization capacity, suggesting that this region is highly dynamic and susceptible to molecular interactions[60]. Mid-piece localization also raises concern as this region is essential for mitochondrial function and sperm motility, which could be disrupted if viral interactions occur. Disruption of mitochondrial function by other agents (e.g., cannabinoids) has been shown to reduce sperm motility and mitochondrial activity, supporting the plausibility of similar effects from viral interactions[61]. Viruses present in semen can be transmitted to sexual partners during natural mating or AI, leading to infection and, in some cases, persistent or chronic disease [50,62-64]. This risk is well-documented for several viruses, some of which reduce fertility by impairing sperm quality and causing pregnancy complications or fetal infections [64-68]. This is concerning as previous literature has shown that viruses like zika virus are commonly found in semen for weeks to months after infection [69,70]. Most studies report detection of zika virus for up to 3 months, but rare cases show persistence beyond 6 months compromising fertility and transmission risk [71-74]. A recent review reported multiple viruses in semen that can cause subfertility and ultimately negatively impact assisted reproductive technologies [75]. SARS-CoV-2 infection can temporarily reduce sperm concentration, motility, and morphology, likely due to immune-mediated effects and DNA damage [76-78]. However, most sperm parameters return to normal within a few months after recovery. The present findings suggest that the presence of IAV in bovine semen could pose significant risks to fertility and the integrity of breeding programs, given the widespread localization of viral receptors throughout the male reproductive tract. Our study highlights a clear biological potential for both mucosal entry and systemic dissemination of IAV through key male reproductive organs, including the penis, vas deferens, epididymis, seminal vesicles, and testis, depending on the specific viral strain involved. These findings warrant further studies investigating virus binding, replication, and shedding in semen, especially during breeding or semen collection in infected cattle.

In the current study, α2,3- and α2,6-linked sialic acid receptors were differentially abundant across the female reproductive tract. Although ipsilateral and contralateral reproductive tissues were not evaluated separately in this study, future work should investigate whether lectin localization differs between sides, particularly during early pregnancy when conceptus-derived signals may drive localized changes in susceptibility to infection. While the vagina, cervix and uterus were all negative for MAA-II, the α2,3-Galβ1-3 receptor, both SNA and MAA-I were present within the luminal epithelium of the vagina and cervix. It has previously been shown that all three regions, especially the transformation zone of the cervix which is a key site for viral entry, can facilitate viral infection [79-81]. The cervical transformation zone is especially vulnerable due to active cell turnover and immune activity, making it central to viral entry and persistence [81-83]. Although direct evidence of IAV contamination within these tissues is currently lacking, our findings demonstrate the presence of IAV-compatible receptors, indicating that both the bovine cervix and vagina may be susceptible to IAV infection. Previous work investigating other viruses found that zika virus, HIV, HPV, and SIV can infect vaginal and cervical mucosa, disrupt the local microbiome, weaken immune defenses, and, if persistent, can lead to precancerous changes or cancer [81,82,84,85]. Viral infection from HIV, HPV or HSV often causes inflammation (cervicitis, vaginitis), which can lead to symptoms like vaginal discharge, pain, and increased risk of secondary infections [86,87]. Furthermore, zika virus has been shown to persist in the vaginal mucosa for days after infection, with prolonged detection of viral RNA in vaginal secretions and its transmission is influenced by hormonal status, such that high progesterone environments increase susceptibility [88-90]. Previous literature has also documented that cervical viral infections, like HPV, during pregnancy are linked to higher risks of preterm birth, miscarriages, premature rupture of membranes, low birth weight, intrauterine growth restriction, and, in rare cases, fetal death [91-96]. However, further research is required to conclude the potential risks that HPAIV poses within the bovine vagina and cervix.

Current research on viral presence in the uterus, particularly regarding IAV, remains limited, as most studies have prioritized respiratory, neurological, or systemic infection over direct uterine involvement [16,97]. Our findings support this trend for our cyclic uterine tissue, as none of the lectins (SNA, MAA-I, or MAA-II) were bound to the luminal or glandular epithelium of the uterus, suggesting a lack of detectable viral receptor expression in these regions. However, in the pregnant uterine tissue, both SNA and MAA-I were detected throughout the uterus, including the vasculature, luminal and glandular epithelium, and within the trophectoderm and endoderm of the conceptus. This raises significant concerns due to the potential for IAV infection at multiple critical sites during early pregnancy. The presence of IAV receptors on both maternal (uterine) and embryonic (trophectoderm, endoderm) tissues means these cells are permissive to IAV entry and therefore could potentially lead to infection of early embryonic cells, resulting in embryonic loss, miscarriage, or developmental defects, as these cells are critical for implantation and organogenesis [98].

In the oviduct, there was high detection of all three receptor types for IAV, particularly at the apical surface of epithelial cells. These data suggests that the oviduct could be a site of viral binding for IAV and possible infection as the virus could bind directly to the oviductal epithelium during gamete or embryo transport. Since the oviduct is the site of fertilization and early embryo development, viral binding here could disrupt these processes and/or potentially lead to early vertical transmission [99,100].

Strong detection of MAA-I in granulosa cells and SNA in the zona pellucida indicate that these ovarian and oocyte structures have receptors that may allow viral binding. This finding suggests a possibility potential for viral entry and persistence during folliculogenesis and early embryo development, raising concerns about vertical transmission through the follicular environment, especially under high viral loads. Viruses such as hepatitis B and C have been detected in ovarian tissue, follicular fluid, and oocytes, supporting the potential for direct infection and vertical transmission to the embryo during fertilization or early development [101,102]. Additionally, the presence of viral receptors in the zona pellucida could facilitate viral binding to the oocyte or early embryo, increasing the risk of infection at these critical stages. Previous studies have shown that viral genetic material (e.g., HIV-1, hepatitis B virus) can be present in gametes and therefore transferred to embryos, where it may integrate and replicate, providing a mechanism for vertical transmission even before implantation occurs [101-103]. Therefore, viral exposure at these early stages of development can negatively affect embryo growth, attachment and survival as previously seen in virally infected animals [104].

The mammary gland was included as a positive control, and the findings in the present work are in agreement with previously published findings demonstrating strong SNA and MAA-II detection in alveolar epithelium and ductal lumens in lactating cows [20,105]. The high levels of H5N1 virus in milk, together with the distribution of specific viral receptors in the mammary gland, provide a explanation for why the mammary gland is a key site for viral attachment, replication, and transmission in dairy cattle. The mammary gland expresses abundant avian influenza virus receptors, allowing H5N1 to efficiently infect and replicate in mammary tissue, leading to high viral loads in milk and enabling milk-borne transmission [105-107]. The H5N1 virus has been documented to undergo robust replication in the mammary gland, particularly in the gland cistern and teat canal, resulting in severe mastitis and high viral titers in milk [12,20,108,109]. This enables direct transmission to suckling offspring (with high mortality in animal models) and poses a risk for cow-to-cow transmission and possible zoonotic transmission through contaminated milk and milking equipment [13,17,110,111]. This result highlights the mammary gland as a critical site for a primary route of transmission and viral persistence among dairy herds.

Collectively, our data support that both α2,3- and α2,6-linked receptors are broadly detected throughout the bovine reproductive tract, with sex- and specific tissue differences. Male reproductive tissues appear to offer more consistent epithelial receptor expression across the tract, suggesting a potentially greater susceptibility to viral entry or shedding through semen. Female reproductive tract expression appears more localized to the vagina, cervix and oviduct, which may still allow for transmission risk during breeding or AI, especially under highpathogen load scenarios. Combined, the findings from the present study suggest that the reproductive tract and associated fluids may be a vector for transmission, carrying potential implications for livestock biosecurity.

In conclusion, our histochemical analysis highlights the presence of IAV-compatible receptors in bovine reproductive tissues. This foundational map provides a framework for assessing the potential for reproductive transmission, viral shedding via semen or birth fluids, and vertical transmission. These insights are especially timely given the expanding host range of HPAIV strains and the emerging evidence of cattle-to-human spillover.

Methods:

Ethics statement

This study did not involve live animal experimentation. Bovine reproductive tissues were collected post-mortem from animals processed at a USDA-inspected commercial abattoir. Animals were not euthanized or sacrificed for the purposes of this research. Therefore, institutional animal care and use committee (IACUC) approval was not required. All procedures complied with applicable federal and institutional guidelines for the use of animal-derived tissues.

Animals and Tissue Collection

Bovine reproductive tissues were collected from mature bulls (n = 4), and non-pregnant cows (n = 3) of mixed *Bos taurus* and *Bos indicus* breeds at a local USDA-inspected abattoir in July 2024 (San Angelo, Texas, USA). Additionally, pregnant cows bred via artificial insemination (n = 2) were euthanized at gestational day 20, and only the pregnant uterine tissue (ipsilateral horn) was collected for analysis. Day 20 was selected to allow collection of an elongated, pre-implantation conceptus while the uterine structure remained intact. All tissues were collected post-mortem and transported to the lab in warmed sterile phosphate buffered saline (PBS), with approximately 15 minutes between slaughter and immersion in warmed PBS. Upon laboratory arrival, tissues were collected from anatomically equivalent regions of interest for all animals by dissecting the anatomical structure from the collective reproductive tract. Female anatomical tissues of interest were vagina, cervix, uterus (non-pregnant and pregnant), oviduct, ovary, and mammary gland tissues (lactating and non-lactating). Male anatomical tissues of interest were the penis/urethra, epididymis, vas deferens, seminal vesicles and testis. Immediately after collection, tissues were trimmed to approximately 2.5 x 2.5 x 1cm (length x width x height) and placed into prelabeled 50 mL conical tubes containing 25 - 30 mL of freshly prepared 4% paraformaldehyde (PFA) in 1X PBS and gently rocked for 48 h at room temperature. After fixation, tissues were transferred to 70% ethanol for an additional 24 hours, followed by a second 24-hour 70 % ethanol wash prior to paraffin embedding. Tissues were sectioned both at 2 and 5 µm thickness and mounted onto Fisherbrand™ Superfrost™ Plus Microscope Slides (Fisher Scientific, Waltham, MA, USA); however, all slides used for imaging in this manuscript were 5 µm thick.".

To assess receptor localization in spermatozoa, semen was collected from beef bulls (n = 2) via electroejaculation during routine breeding soundness examinations (BSE). Semen was diluted in PBS and centrifuged (e.g., 500 × g, 10 min) to remove seminal plasma. The sperm pellet was washed twice in PBS, and an aliquot was smeared onto glass slides, air-dried, and fixed in 4% PFA for 20 min at room temperature. Fixed smears were processed in parallel with tissue sections for lectin histochemistry.

Lectin Histochemistry

Lectin staining was performed according to manufacturer's instructions (Vector Laboratories, Newark, CA, USA) and as previously described, with minor modifications [18,20]. Briefly, tissues mounted on slides were deparaffinized and subsequently rehydrated. Antigen retrieval was performed using a 10 mM sodium citrate buffer (pH 6.0) heated in a steamer for 40 minutes followed by a 25-minute cooldown. Slides were then washed three times for five minutes in lectin Tris-buffered saline (TBS), composed of Tris hydrochloride, NaCl, $CaCl_2$, and $MgCl_2$, adjusted to a pH 7.6, as previously described [112]. To reduce nonspecific binding, slides were blocked sequentially with 5% bovine serum albumin (BSA) for 30 minutes, followed by avidin (SP-200, Vector Laboratories) for 15 minutes, and biotin (SP-200, Vector Laboratories) for an additional 15 minutes. Washing was performed after each blocking step in lectin TBS as described above. Biotinylated lectins were used to detect specific sialic acid linkages, SNA; α2,6-linked sialic acids (B-1305-2, Vector Laboratories), MAA-I; α2,3-Galβ1-4, and (B-1315-2, Vector Laboratories), MAA-II; α2,3-Galβ1-3 (B-1265-, Vector Laboratories). Biotinylated lectins were diluted as previously described [18], in lectin TBS to final concentrations of 1:5000 (SNA), 1:4000 (MAA-I), and 1:4000 (MAA-II), and incubated overnight at 4°C in a humidified chamber. After washing the sections, slides were incubated with streptavidin-alkaline phosphatase (SA-5100-1, Vector Laboratories), diluted to 1:100 in lectin TBS for 10 minutes. The staining was developed by adding Vector® Blue substrate (SK-5300, Vector Laboratories) according to the manufacturer's instructions, for 20 minutes. Hereafter, slides were washed three times with distilled water for five minutes. Sections were then mounted using glycerol gelatin (GG1-15ML, Millipore Sigma, Burlington, MA USA). Lectin staining was performed on the following tissue sections. For each tissue, 2 sections per animal were analyzed, collected from comparable regions within the tissue, and all sections showed consistent staining patterns for each lectin. Female tissues (non-pregnant cows, n = 3 animals): vagina, cervix, uterus, oviduct, ovary, mammary gland (2 sections per tissue per animal). Pregnant cows (n = 1 animal): ipsilateral uterine horn only (2 sections analyzed). Male tissues (n = 4 animals): penis/urethra, epididymis, vas deferens, seminal vesicles, testis (2 sections per tissue per animal). Both ovaries, where applicable, were assessed and showed consistent staining patterns. In female tissues, biological replicates were consistent across animals, and no side-specific differences were observed except for the ipsilateral horn in the pregnant cow, which was the only tissue collected.

Controls

The bovine mammary gland served as the positive control tissue based on previously published receptor localization results [20]. Two negative controls were utilized for each tissue type, 1) No-lectin control: slides underwent the complete staining protocol with the omission of the biotinylated lectins and 2) Neuraminidase control: this pretreatment cleaves terminal sialic acids, preventing lectin binding and confirming staining specificity. However, due to consistent results between the two negative controls, only negative 1 was included as the control in the results.

Imaging and Analysis of IAV Histochemistry

All slides were imaged using an Axio Imager.M2 microscope (Zeiss, Oberkochen, Germany EU) under brightfield illumination at 10X magnification. Tissues exhibiting clear and specific lectin staining were additionally imaged at 20X magnification to enhance visualization of cellular detail. Images were captured and adjusted using ZEISS ZEN lite 3.11 microscopy software. All slides were thoroughly evaluated for lectin binding intensity and spatial localization across epithelial, endothelial, and stromal compartments. After scanning the entire tissue section, representative regions from each tissue type were selected for qualitative comparison and figure preparation. For Figure 1 (panels A3 and C3) whole-slide images were generated by digitally stitching together multiple overlapping 10X images arranged in a 5×5 grid to create high-resolution panoramic views of the penile cross-section. For Figure 5, whole-slide images were also generated by digitally stitching together multiple overlapping 20X images arranged in a 5×5 grid to create high-resolution panoramic views of the uterine interaction with the conceptus.

Data Availability Statement

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

Sample collection was performed by B.P., D.S., and O.P. Lectin histochemistry was conducted by B.P. Data analysis and interpretation were carried out by B.P., T.M., J.C., and G.J. The manuscript and figures were prepared by B.P. with contributions, edits, and approval from G.J., T.M., D.S., J.C., Z.S., L.L., P.R., K.D., G.C.L., and K.P.

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Competing interests

The authors declare that there are no conflicts of interest related to this article. This work was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.

Figure Legends

Table 1. Summary of the most physiologically relevant expression of influenza A virus (IAV) receptors within bovine male and female reproductive tissues. Symbols indicate tissue-specific status: ‡ = presence/absence of lectin staining in the pregnant uterus, * = presence/absence of lectin staining in the non-pregnant uterus.

Figure 1. SNA, MAA-I and MAA-II lectin histochemistry within the bovine penis. Panels A1-3 represent MAA-II lectin staining which is specific for the α2,3-Galβ1-3 sialic acids which bind the duck adapted strain of IAV. Panel B represents MAA-I lectin staining which is specific for the α2,3-Galβ1-4 sialic acids which bind the chicken adapted strain of IAV. Panels C1-3 represent SNA lectin staining, which is specific for the α2,3-linked sialic acids which bind the human adapted strain of IAV. Panels A3 and C3 represent whole slide images assembled by stitching together multiple overlapping, high magnification images (10X 5x5, 500 µm.). Panel D represents the negative control in which no lectins were added. Arrows in panel A2 indicate the

stratified urethral epithelium. UL, urethral lumen; CS, corpus spongiosum; CC corpus cavernosum. Scale bar at 10X is 100µm and at 20X is 50µm.

Figure 2. SNA, MAA-I and MAA-II lectin histochemistry within the bovine seminal vesicles and vas deferens. Panels A1-3 represent MAA-II lectin staining which is specific for the α2,3-Galβ1-3 sialic acids which bind the duck adapted strain of IAV. Panels B1-3 represent MAA-I lectin staining which is specific for the α2,3-Galβ1-4 sialic acids which bind the chicken adapted strain of IAV. Panels C1-3 represent SNA lectin staining which is specific for the α2,3-linked sialic acids which bind the human adapted strain of IAV. Panels D1-2 represents the negative control in which no lectins were added. VL, vas deferens lumen; VE, pseudostratified vas deferens epithelium; CC, connective tissue; SM, smooth muscle. Scale bar at 10X is 100µm and at 20X is 50µm.

Figure 3. SNA, MAA-I and MAA-II lectin histochemistry within the bovine epididymis, testis and spermatozoa. Panels A1-3 represent MAA-II lectin staining which is specific for the α2,3-Galβ1-3 sialic acids which bind the duck adapted strain of IAV. Panels B1-4 represent MAA-I lectin staining which is specific for the α2,3-Galβ1-4 sialic acids which bind the chicken adapted strain of IAV. Panels C1-7 represent SNA lectin staining which is specific for the α2,3-linked sialic acids which bind the human adapted strain of IAV. Panels D1-3 represent the negative control in which no lectins were added. In panel C4 the white arrowhead is pointing towards a seminiferous tubule containing developing germ and Sertoli cells whereas the asterisk indicates the interstitium containing Leydig cells, blood vessels, nerves and connective tissue. In panel C5, the black shaded arrowhead is pointing to the elongated spermatids whereas the grey shaded arrowhead is pointing towards the round spermatids. VL, vas deferens lumen; VE, pseudostratified vas deferens epithelium; CC, connective tissue; SM, smooth muscle; EL, epidydimal lumen containing spermatozoa and broken stereocilia; EE, epididymal epithelium. Scale bar at 10X is 100µm and at 20X is 50µm.

Figure 4. SNA, MAA-I and MAA-II lectin histochemistry within the bovine vagina, and cervix. Panels A1-2 represent MAA-II lectin staining which is specific for the α2,3-Galβ1-3 sialic acids which bind the duck adapted strain of IAV. Panels B1-4 represent MAA-I lectin staining, which is specific for the α2,3-Galβ1-4 sialic acids which bind the chicken adapted strain of IAV. Panels C1-2 represent SNA lectin staining, which is specific for the α2,3-linked sialic acids which bind

the human adapted strain of IAV. Panels D1-3 represent the negative controls in which no lectins were added. VE, stratified squamous vaginal epithelium; VL, vaginal lumen; CC, connective tissue; CE, simple columnar cervical epithelium; CL, cervical lumen. Scale bar at 10X is 100 µm and at 20X is 50 µm.

Figure 5. SNA, MAA-I and MAA-II lectin histochemistry within the bovine uterus. Panels A1-2 represent MAA-II lectin staining which is specific for the α2,3-Galβ1-3 sialic acids which bind the duck adapted strain of IAV. Panels B1-4 represent MAA-I lectin staining, which is specific for the α2,3-Galβ1-4 sialic acids which bind he chicken adapted strain of IAV. Panels C1-4 represent SNA lectin staining, which is specific for the α2,3-linked sialic acids which bind the human adapted strain of IAV. Panels D1-2 represent the negative controls in which no lectins were added. Panels A2, B2, C2 and D2 represent whole slide images assembled by stitching together multiple overlapping, high magnification images (20X 3x3, 150µm). Panels B5 and C5 also represent whole slide images assembled by stitching together multiple overlapping, high magnification images (10X 5x5, 500µm.). White arrowhead indicates endometrial glands and black arrowhead indicates vasculature. UL, uterine lumen; ULE, uterine luminal epithelium; UST, uterine stroma containing glands, blood vessels and connective tissue. Scale bar at 10X is 100µm and at 20X is 50µm.

Figure 6. SNA, MAA-I and MAA-II lectin histochemistry within the bovine oviduct and ovary. Panels A1-3 represent MAA-II lectin staining which is specific for the α2,3-Galβ1-3 sialic acids which bind the duck adapted strain of IAV. Panels B1-4 represent MAA-I lectin staining which is specific for the α2,3-Galβ1-4 sialic acids which bind the chicken adapted stain of IAV. Panels C1-5 represent SNA lectin staining which is specific for the α2,3-linked sialic acids which bind the human adapted strain of IAV. Panels A3, B3, C3 and D2 focus on antral follicles. Panel C4 focuses on primordial follicles whereas panel C5 focuses on ovarian vasculature. Panels D1-2 represent the negative controls in which no lectins were added. Black arrowhead indicates vasculature. OL, oviductal lumen; OE, oviductal epithelium; CC, connective tissue. Scale bar at 10X is 100µm and at 20X is 50µm.

Figure 7. SNA, MAA-I and MAA-II lectin histochemistry within the mammary glands of lactating and non-lactating cows. Panels A1-2 represent MAA-II lectin staining which is specific for the α2,3-Galβ1-3 sialic acids which bind the duck adapted strain of IAV. Panels B1-2

represent MAA-I lectin staining which is specific for the α2,3-Galβ1-4 sialic acids which bind the chicken adapted strain of IAV. Panels C1-2 represent SNA lectin staining which is specific for the α2,3-linked sialic acids which bind the human adapted strain of IAV. Panel D represents the negative control in which no lectins were added. AL, alveolar lumen; AE, alveolar epithelium; CC, connective tissue. Scale bar at 10X is 100μm and at 20X is 50μm.