Inoculation Of Dairy Bulls With H5N1 B3.13 HPAI Virus Via Natural Routes

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Abstract

Presently, it remains unknown whether dairy bulls are susceptible to H5N1 highly pathogenic avian influenza virus (HPAIV) or whether infection could result in viral shedding via the reproductive tract and semen. To address this knowledge gap, we inoculated sexually mature dairy bulls intranasally, intrapreputially, and repeatedly intranasally or intrapreputially with a high dose of a HPAIV isolated from an infected cow. Rectal and rumen temperatures and various samples including semen and preputial swabs were collected for up to 19 days post inoculation. Five of the bulls were sacrificed, and comprehensive necropsies were conducted collecting more than 50 tissues per animal. No clinical signs were observed for the duration of the study. Viral RNA was detected by rRT-PCR for two to three days only at inoculation sites; no other samples or tissues were positive. Virus isolation performed on swabs, semen, and prepuce samples indicated that neither single nor sequentially inoculated dairy bulls shed infectious virus. All inoculated bulls seroconverted whereas control-inoculated bulls did not express quantifiable antibodies. These data demonstrate that dairy bulls are minimally susceptible without developing a sustained infection despite receiving a high-dose HPAIV challenge and bovine semen is not a potential risk for the spread of H5N1 HPAIV.

INTRODUCTION

Influenza A viruses (IAV) are known to infect multiple avian and mammalian species [1]. Some of these infections, specifically those with highly pathogenic avian influenza virus (HPAIV) strains, often have significant economic, ecological, and social impact [2, 3]. Infections with IAV in bovine species have been reported since the 1940s; however, most cases have been associated with human pandemic viruses of the H1N1 and H3N2 subtypes [4–10]. Experimental studies in the 1950s showed both lactating goats and dairy cows allowed for virus replication when inoculated with human IAV directly in the mammary gland, although clinical disease was not reported [11–13]. An experimental study in 2008 demonstrated that intranasal inoculations of calves with H5N1 HPAIV did not result in established infection and respiratory disease [14]. A detailed review published in 2019 concluded that bovines were largely unaffected by IAV [15]. This dogma dramatically changed in early 2024 when an unprecedented spillover of H5N1 HPAIV into dairy cows was reported in Texas, USA [16–18]. The outbreak, presented in cows with reduced feed intake, reduced rumination, severely decreased milk production, and indications of mastitis, often with thick yellow milk with clots, was caused by a novel reassortant H5N1 2.3.4.4b genotype B3.13 HPAIV [16, 17]. Subsequently, separate wild-bird introductions of genotype D1.1 HPAIV occurred in late 2024 and early 2025 into dairy cattle in Nevada and Arizona, USA, but did not spread further [19].

After the initial introduction into dairy cows, the H5N1 B3.13 HPAIV spread quickly and as of May 21st, 2026, it had affected at least 1,095 dairy farms in 20 U.S. states [19]. While the virus did not affect every cow on infected farms, milk loss was significant, estimated at 900-1,000 [20, 21]. Approximately 4.4 months were needed for a herd to recover to pre-outbreak production metrics [20, 21]. The risk of death of infected cows increased 6-fold, and the probability of premature removal from the herd increased 3.6

times compared to non-clinical cows [20]. The losses due to decreased milk production, mortality, disease management, and early removal from the herd were estimated at ~$160,000-$184,000 per every 1,000 cows [20, 21]. The impact of H5N1 HPAIV on dairy cows' health, productivity, and farm economics was both immediate and lasting, challenging the sustainability of affected dairy farms.

The dairy industry heavily relies on advanced reproductive technologies, particularly, artificial insemination (AI), using fresh or frozen thawed semen. This is the preferred breeding method as it offers a practical, cost-effective, safer alternative to natural service, and provides improved genetics and sex selection in offspring [22, 23]. While global adoption of AI varies significantly—with rates as high as 80–90% in intensive European/North American farms and lower rates in developing nations—AI with frozen thawed semen is the dominant breeding method for dairy cattle worldwide [22, 24, 25]. Natural service bulls are still widely used in some regions as well. After the occurrence of natural infection of dairy cows with H5N1 HPAIV, concerns were raised about the susceptibility of dairy bulls and the potential viral transmission via bovine semen (fresh and frozen) and through natural service. Bovine semen has been shown to carry various viruses, such as bovine viral diarrhea virus, bovine herpesvirus type 1, foot-and-mouth disease virus, lumpy skin disease virus, and bovine leukemia virus [26–28]. Evidence that human semen can harbor 27 different viruses, including influenza, has been documented [29]. A recent study reported the presence of low levels of H5N1 HPAIV RNA in semen from healthy bulls in California, USA, in 2024 during the period of outbreaks in the state. However, the data is from uncontrolled collection on an actively infected dairy farm where environmental contamination was likely and no direct evidence of live virus being detected in the bulls was provided [30]. The available data does not answer the question of whether H5N1 HPAIV can infect dairy bulls and then also have shedding of the virus in semen such that dairy cows could be infected through artificial or natural insemination.

Influenza A viruses initiate infection through hemagglutinin binding to host cell surface sialic acid residues with avian-adapted strains preferentially binding α2,3-linked sialic acids and human-adapted strains binding α2,6-linked residues. We previously performed a comprehensive spatial mapping of α2,3- and α2,6-linked sialic acid receptors across the bovine male reproductive tissues [31]. This study identified regions of the male reproductive tract, including the penis, vas deferens, seminal vesicles, epididymis, and testes, that harbor IAV receptors for both human- and avian-adapted viruses, which raises the possibility of virus attachment to these bovine male reproductive tissues. While attachment is the necessary first step in viral entry, virus replication requires the expression of many different host proteins to allow for replication, virus assembly, and potential transmission. The current study aimed to investigate whether dairy bulls and their reproductive tract are susceptible to challenge with H5N1 HPAIV and whether bovine semen poses a risk of virus transmission. To this end, we performed intranasal and intrapreputial inoculations of dairy bulls with high dose/s of H5N1 2.3.4.4b genotype B3.13 HPAIV and tested various samples collected over a 19-day period.

MATERIALS AND METHODS

Animals

All animal studies were carried out in the Texas A&M University's (TAMU) Global Health Research Complex large animal Biosafety Level-3 (BSL-3) facility. Eight sexually mature Holstein-Friesian bulls (630.5 ± 132.5 kg) from 3 to 6 years in age, free of apparent health or fertility issues, were sourced from STgenetics, TX, USA. The bulls were housed in individual pens and allowed to acclimate for at least seven days prior to inoculation. Baseline blood and nasal swabs were collected and analyzed for active or recent IAV infections. Water was provided *ad libitum* and a total mixed ration maintenance diet was offered twice daily. Semen was collected via electroejaculation using a Pulsator V (Lane Manufacturing, USA) five days prior to viral challenge for assessment of sperm motility and morphology. An aliquot of semen was preserved at -80°C for subsequent testing. All bulls had normal semen production and sperm characteristics prior to start of the study. All animal experiments were approved and performed under the regulations of the TAMU's Institutional Animal Care and Use Committee (Protocol #2024 – 0297) and the Federal Select Agent Program.

Virus

The virus used in the study was isolated from a milk sample from a naturally infected cow in Texas in December 2024. The virus isolation was performed on Madin-Darby canine kidney cell line (MDCK) (BEI resources, NR-2628, derived from ATCC CCL-34, Lot 494646-2) following standard procedures [32], with minor modifications described below. Briefly, the milk sample was diluted 1:1,000 (v/v) in minimum essential medium (MEM) supplemented with bovine serum albumin (BSA) and flasks with washed MDCK cells were overlayed with 1mL of the inoculum. The flasks were incubated at 37°C for one hour with manually rocking every 10 minutes. The inoculum was then removed, and cells were washed once with phosphate buffered saline (PBS). The inoculated washed cells were covered with MEM-BSA and incubated at 37°C in a humidified tissue culture incubator with an atmosphere of 5% CO_2 and checked daily for cytopathic effect (CPE). When ≥ 90% of cells detached from the flask, the culture medium was transferred into a conical tube and centrifuged at 3,000 × g and 4°C for 5 minutes to pellet the cellular debris. The virus was expanded on MDCK cells for one more passage. The tissue culture infectious dose 50% ($TCID_{50}$) of the isolated virus was titrated using 10-fold serial dilutions in MEM-BSA in microtubes. Clear flat-bottom 96-well plates were seeded with MDCK cells at 1.5 x 10^4 and at 80–90% confluency the cells were washed twice with MEM-BSA. The virus dilutions were transferred to the plates and were incubated under conditions described above for 2–5 days (until at least 90% of the cells in the positive control wells detached) and CPE was evaluated. $TCID_{50}$ was calculated using the method of Reed and Muench [33]. The virus was stored frozen at -80°C until further use. Full genome deep sequencing utilizing long-read technique was performed on both the original milk sample and the isolated virus. Reverse transcription and amplification were performed with SuperScript™ III One-Step RT-PCR System with Platinum *Taq*™ High Fidelity DNA Polymerase (Invitrogen, USA) using the MBTuni universal primers[34] with the following thermal profile: reverse transcription 42°C for 60 min, 94°C for 2 min, then 5-cycles of amplification at 94°C for 30 sec, 45°C for 30 sec and 68°C for 3 min, an additional 40-cycles of amplification at 94°C for 30 sec, 57°C for 30 sec and 68°C for 3 min, an extension at 68°C for 7 min

and an infinite hold at 4°C. Library preparation was performed with the Oxford Nanopore Technologies (ONT, Oxford, UK), Native Barcoding Kit 96 V14 following the manufacturers' guidelines for amplicon sequencing. Sequencing of the final library product was completed on the ONT MinION Mk1B with the R10 flow cell. Upon completion of sequencing, basecalling in MinKNOW (v25.03.9) was completed using high-accuracy settings. Sequences were imported into Geneious Prime (2025.1.2, GraphPad Software LLC, USA) and reads were mapped to a HPAI H5N1 2.3.4.4b reference genome (GenBank accession numbers PQ833130-PQ833137) with Minimap2 using the Oxford Nanopore data type parameter. A pairwise comparison of each segment was then generated using MAFFT[35] as implemented in Geneious to compare the genome of the original milk sample to the one from the propagated virus.

Animal study design

Serum, nasal swabs, ocular swabs, preputial swabs, whole blood, feces, and semen samples were collected before inoculation. Two bulls (animals #1 and #2) were inoculated intranasally with 10^6 $TCID_{50}$ diluted in 3 mL of MEM. The virus was delivered in the right nostril using a 5 mL syringe with an atomization device (MAD Nasal atomization device, Teleflex, USA) to generate a mist of particles 30–100 µm in size. Two bulls (animals #3 and #4) were inoculated intrapreputially with the same dose. The virus was delivered via a 5 mL syringe, and an insemination pipette inserted at least 4 to 6 inches in the preputial cavity of each bull while applying circular pressure around the preputial orifice to avoid spillage of inoculum and ensure full dose delivery. One bull (#5) was inoculated intranasally for three consecutive days, and one bull (#6) was inoculated intrapreputially for three consecutive days using the respective procedures described above. Two more bulls served as sham-inoculated negative controls and were given 3 mL of the vehicle medium intranasally (#7) and intrapreputially (#8). Animals were monitored daily and were equipped with Farmfit boluses (STgenetics, USA) for monitoring rumen temperature and rumination patterns via a cloud-based system. Rectal temperatures were measured on each sampling day. Control versus inoculated animals were separated by pen and control bulls were worked on prior to sampling any inoculated bulls. Serum, nasal swabs, ocular swabs, preputial swabs, whole blood, feces, and saliva samples (except day 1 post inoculation for this sample type) were collected from the single inoculated and negative control bulls on days 1, 2, 3, 4, 7, 9, 11, and 15 post inoculation (dpi). Semen and urine (when available) samples from the same bulls were collected on 2, 4, 7, 11, and 15 dpi. For the two animals that received three inoculations, the same set of samples, with the addition of a preputial wash, were collected on 1, 2, 3, 4, 7, 9, 12, 14, and 19 dpi with semen and urine collected on 2, 4, 7, 9, 12, 14, and 19 dpi. Semen was collected through electroejaculation as described above. Swabs were collected in 2 mL brain heart infusion (BHI) broth. Animals #1, #3, and #7 at 15 dpi and animals #5 and #6 at 19 dpi were humanely euthanized for pathology and tissues investigation; bulls #2, #4, and #8 were further used in another study. All euthanized animals were necropsied, and gross lesions were recorded. During necropsy, more than 50 tissues were collected from each animal (see Suppl. Table 1 for full list of tissues). All collected tissues were split in two – one section was placed in a Whirl-Pak® Pocket bag (Filtration Group, USA) and stored at -80°C and one section was placed in 10% neutral buffered formalin (NBF). The NBF was replaced with fresh NBF twice every 24 hours before tissues were further

processed. Due to some microscopic lesions, kidney tissues were also screened for *Leptospira spp.* by real-time PCR [36].

Sample testing

Influenza A Real-time reverse transcriptase polymerase chain reaction (rRT-PCR)

All collected nasal swabs (left and right nostril separately), ocular swabs, preputial swabs and washes, semen, urine, whole blood, feces, and saliva samples were tested for presence of influenza virus by real-time reverse transcriptase polymerase chain reaction (rRT-PCR). Fresh tissue samples were trimmed and 200 milligrams of tissues were placed in pre-filled bead tubes with 2.8 mm ceramic beads and 2 mL of sterile PBS and homogenized on an Omni Bead Ruptor 24 (Revvity, USA) at 5.0 M/s for 1 minute. Tubes were centrifuged at 2,000 × g and 4°C for 2 minutes and the supernatant was used for the next steps. Nucleic acid extraction was performed on the KingFisher Flex Magnetic Particle Processor (ThermoFisher Scientific, USA) with the MagMax CORE Nucleic Acid Purification Kit (Applied Biosystems, USA) following the manufacturer's instructions for sample preparation and extraction with an additional 80% ethanol wash between Wash 1 and Wash 2. Detection of IAV was performed using a matrix-gene rRT-PCR assay [37] and the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, USA). An exogenous internal positive control (XIPC) was added during the nucleic acid extraction procedure to ensure appropriate sample extraction and monitor for PCR reaction inhibitors [38]. The rRT-PCR assay was run on the CFX96 Touch system (BioRad) with the following thermal profile: 45°C for 10 min, 95°C for 10 min, 40 cycles of 94°C for 10 sec, 57°C for 30 sec (data collection step), and 72°C for 10 sec. A positive amplification control (PAC), negative template control (NTC), and negative extraction control (NEC) were also included with each PCR plate. Manual thresholding at 10% dRN of the PAC signal was performed for each rRT-PCR assay run. Cycle threshold (Ct) values < 37 were considered positive.

Serological testing

The collected serum samples were tested using the ID Screen® Influenza A Antibody Competition Multi-species ELISA kit (Innovative Diagnostics, France), targeting anti-nucleoprotein (NP) antibodies, following the manufacturer's instructions for bovine samples. Serum samples were also tested for virus neutralization antibodies using a microneutralization assay, as previously described [39]. Briefly, ten-fold serial serum dilutions in MEM-BSA were mixed with equal volume of virus diluted to 100 to 300 $TCID_{50}$ in 96-well plates. Plates were incubated at 37°C for one hour. A working dilution of 1.5 x 10^5 cells per mL was added to each well and the plates were incubated at 37°C in a humidified tissue culture incubator with an atmosphere of 5% CO_2. Plates were examined for CPE starting at day 2 until > 90% of the cells in the positive control wells detached from the plate. Each sample was tested in duplicate, and the virus neutralization antibody titers were estimated as the highest dilution at which both replicates showed no CPE.

Virus isolation

All nasal swabs, semen, and preputial swabs and wash samples were tested by virus isolation. Fifty microliters of swab media or sample were inoculated in 24-well plates seeded with MDCK cells at 80–90% confluency. Prior to inoculation, cells were washed twice with MEM-BSA and then overlayed with 450 ul of MEM-BSA. Fifty microliters of sample were added to a corresponding well and mixed by gently pipetting. Then 50 ul were transferred to a second designated well of the plate, resulting in 1:10 and 1:100 dilutions for each sample. For semen and prepuce samples, two further transfers to new wells were made resulting in additional 1:1,000 and 1:10,000 dilutions of each of these samples. Plates were then incubated at 37°C in a humidified tissue culture incubator with an atmosphere of 5% CO_2, plates were examined for CPE until > 90% of the cells in the positive control wells detached from the plate. Two passages per sample were performed.

Microscopic evaluation

Collected tissues were prepared for histopathology as previously described [40]. Briefly, the fixed tissues were routinely processed and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin and eosin.

RESULTS

Virus

A HPAIV, designated A/cattle/TX/24/A243460207-59 was recovered from the original milk sample. The complete genome is available in GenBank under accession numbers PQ833130-PQ833137 (designated A/cattle/TX/24-037225-049-original/2024(H5N1)). After isolation and one expansion passage on MDCK cells, the virus titer was established to be $10^{8.5}$ $TCID_{50}$/0.1 mL, corresponding to a rRT-PCR Ct value of 11.6. The complete genome obtained from the original milk sample and the genome obtained after propagation and expansion in MDCK cells were identical, showing that there were no mutations in the virus used for the animal studies. The hemagglutinin protein cleavage sites contained multiple basic amino acid residues (PPREKRRKR↓GLF). Based on criteria utilized by the World Organization of Animal Health (WOAH) to assess pathogenicity of avian influenza virus isolates, such a cleavage site motif is specific for HPAIV viruses (WOAH, 2025) [41]. The virus titers used for animal challenge were confirmed by back titration of the inoculums ($10^{6.25}$ $TCID_{50}$ for animals #1–4 and $10^{5.875}$ $TCID_{50}$ for animals #5–6).

Clinical signs and observations

No apparent clinical signs were observed in any of the bulls for the duration of the study. Animals remained normal and responsive for the duration of the study. Rumen and rectal temperatures were within the ranges of 36.1–38.7°C and 38.0-39.2, respectively. No deviations from normal feed and water

intake were observed. Rumination time did not differ between inoculated and control animals and remained within normal ranges during the study period.

rRT-PCR, serology and virus isolation

All bulls were negative for influenza A virus via rRT-PCR and were also negative for IAV antibodies via ELISA prior to the start of the study. Nasal swabs from the inoculated right nostrils of the two animals (bulls #1 and #2) that received a single high dose of H5N1 HPAIV intranasally tested positive by rRT-PCR at 1 and 2 dpi. The Ct values ranged between 32.0 and 35.1 (Fig. 1A). The nasal swabs from the uninoculated left nostrils were negative. Preputial swabs collected from the bull that received three consecutive intrapreputial inoculations (bull #6) were positive by rRT-PCR at 1, 2, and 3 dpi. The swabs at 1 and 2 dpi were collected prior to the next inoculation. The Ct values ranged between 36.2 and 36.8 (Fig. 1B). Testing of all other collected samples by rRT-PCR yielded negative results. Virus isolation did not result in a successful propagation of a virus from any of the tested samples. *Leptospira spp.* was confirmed by real-time PCR in the kidney samples from all tested animals.

Anti-NP antibodies were not detected in any of the animals for the first nine days of the study. At 11 dpi, bulls #1 and #2 (single intranasal inoculation) showed seroconversion by ELISA. The presence of antibodies was confirmed at 15 dpi. Bull #5 (triple intranasal inoculation) seroconverted at 14 dpi and bull #6 (triple intrapreputial inoculation) seroconverted at 12 dpi as shown by ELISA results. The presence of antibodies was confirmed at 19 dpi for these two animals. The seroconversion of all four animals was also confirmed by the presence of virus neutralization antibodies. Bulls #1 and #2 (single intranasal challenge) had detectable neutralizing antibodies at 9 dpi with titers of 4 and 8, respectively. The titers at 15 dpi were 16 and 32 (Fig. 1C). Bull #5 (triple intranasal inoculation) and #6 (triple intrapreputial inoculation) had detectable neutralizing antibodies at 14 dpi (titers of 8 and 16, respectively), confirmed at 19 dpi (with titers of 32 and 32, respectively) (Fig. 1D).

Gross and microscopic lesions.

Bulls #1, #3, #6, and #7 had mildly to moderately enlarged retropharyngeal lymph nodes. Bull #6 had moderately enlarged inguinal lymph nodes, while the sub iliac and perirenal lymph nodes in bulls #5 and #6 and the ileocecal lymph nodes in bull #5 were moderately to markedly enlarged with edema and multifocal areas of hemorrhage. The capsular surface of kidneys from bulls #5, #6, and #7 had pinpoint, white-gray areas that extended into the superficial cortex. The omentum of bull #6 had multifocal areas of hemorrhage. Bull #7 had evidence of chronic pleuritis characterized by multiple fibrous adhesions that extended from the lateral surface of the lung to the corresponding costal pleura. Observed changes were not consistent with an active viral infection.

All bulls had some evidence of bacterial pathogenesis in the heart, lung, liver, and spleen with small numbers of lymphocytes, plasma cells, and macrophages and scattered multifocally distributed neutrophils. The interstitial space of the kidneys from bulls #1, #5, #6, and #7 was multifocally expanded by a moderate to large number of lymphocytes, macrophages and plasma cells. The small and large

intestines contained a moderate histiocytic and eosinophilic inflammation with presence of coccidia in bulls #5 and #6. The submucosa of the trachea in bulls #1, #3 and #6 had mild to minimal inflammation containing few lymphocytes, plasma cells and macrophages and scattered neutrophils. A mild to minimal neutrophilic inflammatory infiltrate was observed in the oropharynx of all bulls. The lymph nodes of all bulls had moderate to marked lymphoid hyperplasia with sinus histiocytosis, draining neutrophils, and varying degrees of hemorrhage.

Microscopic lesions in the reproductive organs were few and mild. The interstitial space of the seminal vesicles in bulls #3 and #5, the interstitial space of the ampulla in bull #5 and the interstitial space of the epididymis in bulls #5 and #6 contained rare aggregates of lymphocytes, plasma cells, and macrophages. The interstitium of the testicle in bull #5 had a focal aggregate of lymphocytes, plasma cells, and macrophages accompanied by a focal area of fibrosis and mineralization. The submucosa of the penis of bull #3 was expanded with a moderate number of lymphocytes, plasma cells, macrophages, and lesser neutrophils accompanied by granulation tissue. The preputial submucosa of all bulls had a mild inflammatory infiltrate composed primarily of lymphocytes, plasma cells, and macrophages with few neutrophils. No significant lesions were observed in the bulbourethral gland or prostate glands of any of the bulls.

DISCUSSION

In this study, we evaluated the susceptibility of sexually mature Holstein-Friesian bulls to infection with H5N1 HPAIV. This investigation was performed to gain knowledge about the potential role that dairy bulls and semen may play in transmission of HPAIV. Six animals were challenged intranasally or intrapreputially with a high dose of A/cattle/TX/24/A243460207-59 (H5N1 2.3.4.4b genotype B3.13 HPAIV). Over the course of two weeks, the inoculated bulls did not develop any clinical signs. The challenge virus was not identified in any collected samples apart from the inoculation sites within the first few days after inoculation. No live virus was recovered from any tested samples, including semen and preputial swabs or washes. This is the first study of its type and represents a comprehensive investigation of dairy bulls' susceptibility to H5N1 HPAIV, providing valuable information produced from a controlled experiment.

Despite the high challenge H5N1 HPAIV dose, the bulls in this study did not develop clinical disease. This provides evidence to suggest low susceptibility through likely natural routes of exposure. Furthermore, no detection of live virus was found in bovine semen from any inoculated bulls, showing semen is of low risk for spread of HPAIV. Even when a high viral dose was delivered intranasally and intrapreputially, these inoculations did not result in sustained replication of the virus in the animals. While we are unaware of previous data demonstrating potential routes of natural transmission of IAV into bovine species and bulls specifically, the intranasal and intrapreputial routes were examined in this study. The intranasal route was selected due to abundance of reports and existing knowledge that this is one of the most common influenza virus transmission modes in mammals [42, 43]. In many mammalian species, infection with HPAIV is commonly limited and efficient transmission between mammals is not widely established [43, 44]. However, in situations where animals are in close proximity, for example fur farms

or seals and sea lion colonies, mammal-to-mammal spread of the virus through respiratory droplets, aerosols, and contaminated surface appears likely [45, 46]. During the unexpected outbreak that occurred in dairy cows in the USA the spread of virus was initially attributed to mechanical transmission via milking machines; however, aerosols or droplets transmission needs to be further elucidated as the virus was abundantly found in air samples from farms with affected cows [47]. The intrapreputial route was also investigated, as this was considered a potential risk for natural service bulls to become exposed from cows, allowing further transmission of the virus [48–50]. In this study, we did not explore the intraoral inoculation route. Some evidence for intraoral transmission was found in a study where calves were fed large volumes of milk with high viral load, freshly obtained from infected cows, for four consecutive days. That resulted in limited distribution of the virus to the respiratory tract of the calves; however, this mechanism seems unlikely in a bull with a functional rumen [51]. Nevertheless, the main objective through both intranasal and intrapreputial routes of inoculation was to determine if H5N1 HPAIV can efficiently infect dairy bulls and be shed in the semen or reproductive tract of the animals, for which we did not find evidence.

Inoculations with a high dose of HPAIV for three consecutive days did not result in shedding in dairy bulls. Initial studies of intranasal inoculations in lactating cows did not reproduce the disease observed in the field[52, 53] and transmission through milking equipment under controlled conditions has been unsuccessful thus far [53]. The only reliable method of virus infection is by direct inoculation into the mammary gland through the teat [52, 53]. Given the high viral loads found in air samples and environment from affected facilities [47, 54], we speculated that sequential inoculations may result in development of an infection in dairy bulls, similar to farm conditions where animals may be continuously exposed to the virus. While the virus was identified in the inoculation site of one of the bulls, neither intranasal nor intrapreputial consecutive (x3) inoculations resulted in detection of the virus or viral RNA in any other samples. Our previous study identified multiple reproductive tissues with receptors indicating potential binding sites for HPAIV [31]; however, it appears that the virus could not reach these tissues or did not replicate in them. These data provide additional evidence to indicate that dairy bulls are minimally susceptible to challenge with H5N1 HPAIV and clear the virus prior to development of sustained infection.

The virus and/or its antigens were presented to the immune system of the inoculated bulls at a high enough level to elicit immune response. While no productive or clinical infection was observed, detectable antibodies were demonstrated in the inoculated bulls by both ELISA and microneutralization assays. Detectable antibodies in the absence of clinical or productive infection are consistent with early immune recognition and rapid neutralization and elimination of the infectious pathogen before systemic dissemination or disease is established [55, 56]. Interestingly, neutralizing antibodies detected in bulls #5 and #6 were of lower titer compared to those in bulls #1 and #2. This is likely due to the slightly higher challenge titer of the inoculum in the latter; however, both groups responded to the inoculations.

Gross and microscopic lesions were few and non-specific. The observed minor findings during necropsy were not indicative of an active viral infection. The inflammation in the kidneys was consistent with

Leptospira spp. infection [57, 58]. The eosinophilic and histiocytic inflammation in the small and large intestines was attributed to the presence of coccidia [59]. The changes in the lymph nodes are those of reactive hyperplasia. While the cause of nodal hyperplasia was not observed microscopically, a combination of leptospirosis, bacteremia from a leaky intestinal tract and/or prior viral exposure could explain the lesion; however, no viral nucleic acid was detected in the lymph node tissues or any other tissues tested by rRT-PCR. The cells observed in the ampulla, seminal vesicle and testicle were minimal or focal and interpreted as incidental findings. The inflammatory changes observed in the prepuce and penis are attributed to manipulation and irritation during repetitive collection and/or inoculation.

A recently published study reported the detection of low levels of IAV RNA in semen from a single natural service bull from an infected dairy farm [60]. While the investigators identified RNA in one semen sample, no live virus was recovered from the tested specimen, and none of the tested males seroconverted to IAV. The study does not provide evidence that the bull from which the sample was collected was infected and recognizes the possibility of the ejaculate and semen being contaminated during collection as viral environmental contamination on a farm with infected dairy cows is readily detectable. Moreover, semen samples from two more bulls with similar service types and exposures, and collected at the same time from the same farm, were negative. In our study, inoculated bulls, regardless of the exposure route, clearly seroconverted. Therefore, the lack of antibody detection in any of the bulls tested by Lim and co-authors further supports collection contamination, as it has been well documented that very high environmental viral loads are detected on actively infected dairy farms [47, 54, 61].

In summary, we performed intranasal, intrapreputial, and sequential intranasal and intrapreputial inoculations of dairy bulls with a high dose/s of H5N1 2.3.4.4b genotype B3.13 HPAIV. The results generated from the study did not identify productive infection caused by the inoculations and no virus or viral RNA shedding was detected from the multiple tested sample types. These findings are valuable towards evaluating the existing risks of H5N1 spread among bovine species. These data provide evidence that dairy bulls are not likely to be infected with H5N1 HPAIV and bovine semen and male reproductive tract are not a mechanism for the spread of HPAIV.

Declarations

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References

1. Peacock, T. P. *et al.* The global H5N1 influenza panzootic in mammals. *Nature 2024 637:8045* **637**, 304–313 (2024).
2. Zamani, O., Bittmann, T. & Ortega, D. L. The effect of avian influenza outbreaks on retail price premiums in the United States poultry market. *Poult. Sci.* **103**, 104102 (2024).
3. Nisaa, Z. *et al.* Global economic impacts of Highly Pathogenic Avian Influenza: A systematic review and impact framework. *Prev. Vet. Med.* **251**, 106827 (2026).
4. Tanyi, J., Romváry, J., Aldásy, P. & Mathé, Z. Isolation of influenza a virus strains from cattle. Preliminary report. (1974).
5. Wagner, K., Becker, W. & Brömel, J. Influenza of cattle. Enzootic bronchopneumonia of cattle. *Tierarztl. Prax.* **6**, 51–61 (1978).
6. Brown, I. H., Crawshaw, T. R., Harris, P. A. & Alexander, D. J. Detection of antibodies to influenza virus A in cattle in association with respiratory disease and reduced milk yield. (1998).
7. Graham, D. A., Calvert, V. & McLaren, I. E. Retrospective analysis of serum and nasal mucus from cattle in Northern Ireland for evidence of infection with influenza A virus. *Veterinary Record* **150**, 201–204 (2002).
8. Gunning, R. F., Brown, I. H. & Crawshaw, T. R. Evidence of influenza A virus infection in dairy cows with sporadic milk drop syndrome. *Vet. Rec.* **145**, 556–557 (1999).
9. Crawshaw, T. R., Brown, I. H., Essen, S. C. & Young, S. C. L. Significant rising antibody titres to influenza A are associated with an acute reduction in milk yield in cattle. *Cattle Practice* **15**, 219–223 (2007).
10. Fatkhuddinova, M. F., Kir'ianova, A. I., Isachenko, V. A. & Zakstel'skaia, L. I. Isolation and identification of the A-Hong Kong (H3N2) virus in respiratory diseases of cattle. *Vopr. Virusol.* **18**, 474–478 (1973).
11. Mitchell, Chas. A., Walker, R. V. L. & Bannister, G. L. Persistence Of Neutralizing Antibody In Milk And Blood Of Cows And Goats Following The Instillation Of Virus Into The Mammary Gland. *Can. J. Comp. Med. Vet. Sci.* **18**, 426 (1954).
12. Mitchell, C. A., Walker, R. V. L. & Bannister, G. L. Preliminary Experiments Relating to the Propagation of Viruses in the Bovine Mammary Gland. *Can. J. Comp. Med. Vet. Sci.* **17**, 97 (1953).
13. Mitchell, C. A., Walker, R. & Bannister, G. L. Further Experiments Relating to the Propagation of Virus in the Bovine Mammary Gland. *Can. J. Comp. Med. Vet. Sci.* **17**, 218 (1953).
14. Kalthoff, D., Hoffmann, B., Harder, T., Durban, M. & Beer, M. Experimental Infection of Cattle with Highly Pathogenic Avian Influenza Virus (H5N1). *Emerg. Infect. Dis.* **14**, 1132 (2008).
15. Sreenivasan, C. C., Thomas, M., Kaushik, R. S., Wang, D. & Li, F. Influenza A in Bovine Species: A Narrative Literature Review. *Viruses 2019, Vol. 11, Page 561* **11**, 561 (2019).

16. Nguyen, T. Q. *et al.* Emergence and interstate spread of highly pathogenic avian influenza A(H5N1) in dairy cattle in the United States. *Science (1979).* **388**, (2025).
17. Caserta, L. C. *et al.* Spillover of highly pathogenic avian influenza H5N1 virus to dairy cattle. *Nature 2024 634:8034* **634**, 669–676 (2024).
18. Burrough, E. R. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus Infection in Domestic Dairy Cattle and Cats, United States, 2024. *Emerg. Infect. Dis.* **30**, 1335 (2024).
19. HPAI Confirmed Cases in Livestock | APHIS. https://www.aphis.usda.gov/livestock-poultry-disease/avian/avian-influenza/hpai-detections/hpai-confirmed-cases-livestock.
20. Peña-Mosca, F. *et al.* The impact of highly pathogenic avian influenza H5N1 virus infection on dairy cows. *Nature Communications 2025 16:1* **16**, 6520- (2025).
21. Rodriguez, Z., O'Connor, A., Bradford, B. J. & Picasso-Risso, C. Characterization and health, productivity, and economic effects of highly pathogenic avian influenza H5N1 outbreak in dairy cattle. *J. Dairy Sci.* **108**, 6349–6358 (2025).
22. Vishwanath, R. Artificial insemination: the state of the art. *Theriogenology* **59**, 571–584 (2003).
23. Diskin, M. G. Review: Semen handling, time of insemination and insemination technique in cattle. *Animal* **12**, s75–s84 (2018).
24. Colazo, M. G. & Mapletoft, R. J. A review of current timed-AI (TAI) programs for beef and dairy cattle. *The Canadian Veterinary Journal* **55**, 772 (2014).
25. Survey of present status of the use of artificial insemination in developing countries. https://www.fao.org/4/u9550t/u9550T0d.htm.
26. El-Mohamady, R. S., Behour, T. S. & Rawash, Z. M. Concurrent detection of bovine viral diarrhoea virus and bovine herpesvirus-1 in bulls' semen and their effect on semen quality. *Int. J. Vet. Sci. Med.* **8**, 106–114 (2020).
27. Kahrs, R. F., Gibbs, E. P. J. & Larsen, R. E. The search for viruses in bovine semen, a review. *Theriogenology* **14**, 151–165 (1980).
28. Givens, M. D. Review: Risks of disease transmission through semen in cattle. *animal* **12**, s165–s171 (2018).
29. Salam, A. P. & Horby, P. W. The Breadth of Viruses in Human Semen. *Emerg. Infect. Dis.* **23**, 1922 (2017).
30. Lim, A. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Virus RNA in Bovine Semen, California, USA, 2024 - Volume 32, Number 5—May 2026 - Emerging Infectious Diseases journal - CDC. *Emerg. Infect. Dis.* **32**, (2026).
31. Poliakiwski, B. D. *et al.* Spatial localization of avian and human influenza A virus receptors in male and female bovine reproductive tissues. *Scientific Reports 2026 16:1* **16**, 9974- (2026).
32. Eisfeld, A. J., Neumann, G. & Kawaoka, Y. Influenza A virus isolation, culture and identification. *Nature Protocols 2014 9:11* **9**, 2663–2681 (2014).
33. Reed, L. J. & Muench, H. A simple method of estimating fifty per cent endpoints. (1938).

34. Zhou, B. *et al.* Single-Reaction Genomic Amplification Accelerates Sequencing and Vaccine Production for Classical and Swine Origin Human Influenza A Viruses. *J. Virol.* **83**, 10309–10313 (2009).
35. Katoh, K. & Standley, D. M. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Mol. Biol. Evol.* **30**, 772–780 (2013).
36. Smythe, L. D. *et al.* A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. *BMC Infectious Diseases 2002 2:1* **2**, 13- (2002).
37. Spackman, E. *et al.* Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol.* **40**, 3256–3260 (2002).
38. Schroeder, M. E. *et al.* Development and performance evaluation of calf diarrhea pathogen nucleic acid purification and detection workflow. *Journal of Veterinary Diagnostic Investigation* **24**, 945–953 (2012).
39. Spackman, E. Animal Influenza Virus. **2123**, (2020).
40. Prophet, E. B. *Laboratory Methods in Histotechnology*. (American Registry of Pathology, 1992).
41. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals - 10th edition. *WOAH* 2229 p. (2025) doi:10.20506/WOAH.3729.
42. Killingley, B. & Nguyen-Van-Tam, J. Routes of influenza transmission. *Influenza Other Respir. Viruses* **7**, 42–51 (2013).
43. Larsen, S. V. *et al.* Transmission, Pathological and Clinical Manifestations of Highly Pathogenic Avian Influenza A Virus in Mammals with Emphasis on H5N1 Clade 2.3.4.4b. *Viruses* **17**, 1548 (2025).
44. Bertran, K. & Swayne, D. E. High doses of highly pathogenic avian influenza virus in chicken meat are required to infect ferrets. *Veterinary Research 2014 45:1* **45**, 60- (2014).
45. Uhart, M. M. *et al.* Epidemiological data of an influenza A/H5N1 outbreak in elephant seals in Argentina indicates mammal-to-mammal transmission. *Nature Communications 2024 15:1* **15**, 9516- (2024).
46. Restori, K. H. *et al.* Risk assessment of a highly pathogenic H5N1 influenza virus from mink. *Nature Communications 2024 15:1* **15**, 4112- (2024).
47. Campbell, A. J. *et al.* Surveillance on California dairy farms reveals multiple possible sources of H5N1 influenza virus transmission. *PLoS Biol.* **24**, e3003761 (2026).
48. Dennett, D. P., Barasa, J. O. & Johnson, R. H. Infectious bovine rhinotracheitis virus: studies on the venereal carrier status in range cattle. *Res. Vet. Sci.* **20**, 77–83 (1976).
49. Jones, A. L. Sexually Transmitted Diseases of Bulls. *Vet. Clin. North Am. Food Anim. Pract.* **40**, 111–119 (2023).
50. Macías-Rioseco, M. , A. S. S. , A. J. , C. T. , O. J. , W. D. R. , R.-P. R. A. , M. R. B. , J. W. , E. W. R. , C. B. , U. F. A. Detection of highly pathogenic influenza virus (HPAIV H5N1), clade 2.3.4.4b, genotype B3.13 in placentas from aborted bovine fetuses. *Journal of Veterinary Diagnostic Investigation.* (2026).

51. Davila, K. M. S. *et al.* Transmission of highly pathogenic avian influenza H5N1 to calves fed unpasteurized milk from experimentally infected cows. https://doi.org/10.31220/AGRIRXIV.2025.00303 (2025) doi:10.31220/AGRIRXIV.2025.00303.
52. Halwe, N. J. *et al.* H5N1 clade 2.3.4.4b dynamics in experimentally infected calves and cows. *Nature 2024 637:8047* **637**, 903–912 (2024).
53. Baker, A. L. *et al.* Dairy cows inoculated with highly pathogenic avian influenza virus H5N1. *Nature 2024 637:8047* **637**, 913–920 (2024).
54. Singh, G. *et al.* Detection and characterization of H5N1 HPAIV in environmental samples from a dairy farm. *Virus Genes 2024 60:5* **60**, 517–527 (2024).
55. Eschbaumer, M. *et al.* Systemic immune response and virus persistence after foot-and-mouth disease virus infection of naïve cattle and cattle vaccinated with a homologous adenovirus-vectored vaccine. *BMC Veterinary Research 2016 12:1* **12**, 205- (2016).
56. Lu, L. L., Suscovich, T. J., Fortune, S. M. & Alter, G. Beyond binding: antibody effector functions in infectious diseases. *Nature Reviews Immunology 2017 18:1* **18**, 46–61 (2017).
57. Saglam, Y. S., Yildirim, S., Ozkaraca, M. & Altun, S. Investigation of leptospiral antigen with immunohistochemical and immunofluorescence methods in cattle kidney. *Microb. Pathog.* **164**, 105434 (2022).
58. Yener, Z. & Keles, H. Immunoperoxidase and Histopathological Examinations of Leptospiral Nephritis in Cattle. *Journal of Veterinary Medicine Series A: Physiology Pathology Clinical Medicine* **48**, 441–447 (2001).
59. Friend, S. C. E. & Stockdale, P. H. G. Experimental Eimeria bovis infection in calves: a histopathological study. *Canadian Journal of Comparative Medicine* **44**, 129 (1980).
60. Annibaldis, G. *et al.* Highly Pathogenic Avian Influenza A(H5N1) Virus RNA in Bovine Semen, California, USA, 2024 - Volume 32, Number 5—May 2026 - Emerging Infectious Diseases journal - CDC. *Emerg. Infect. Dis.* **32**, (2026).
61. Stenkamp-Strahm, C. *et al.* Dairy Sites with Milk Exposure Are Most Likely to Have Detection of Influenza A Virus. *Microorganisms* **14**, 584 (2026).

Figures

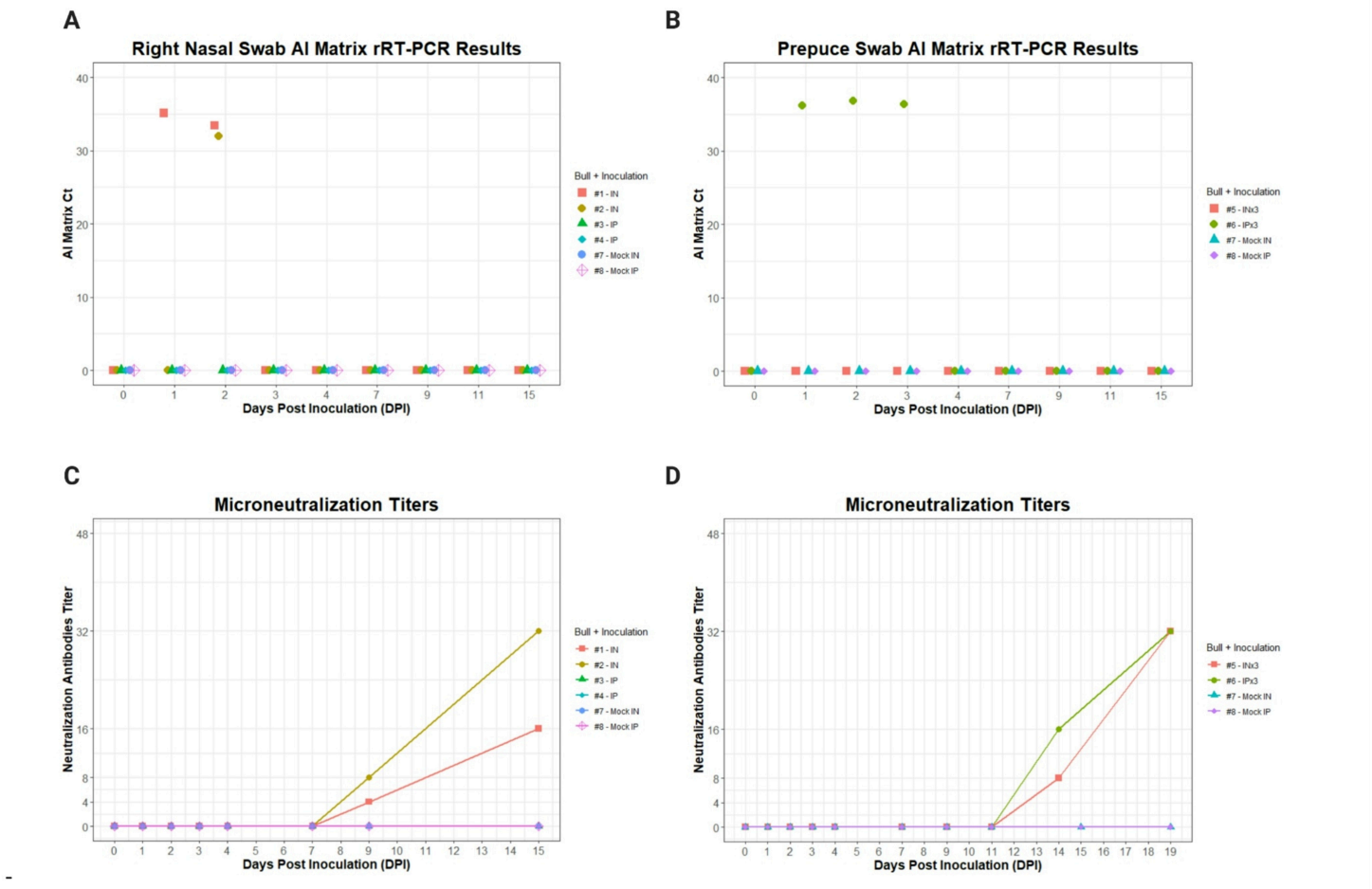

Figure 1

Results from performed real-time reverse transcriptase polymerase chain reaction (rRT-PCR) and microneutralization assay testing of samples from dairy bulls inoculated intranasally and intrapreputially with 10^6/$TCID_{50}$ of H5N1 2.3.4.4b genotype B3.13 highly pathogenic avian influenza virus. (A) rRT-PCR results from inoculation site of intranasally inoculated dairy bulls. (B) rRT-PCR results from inoculation site of triple intrapreputially inoculated dairy bulls. (C) Microneutralizaion assay antibody titers of intranasally inoculated dairy bulls. (D) Microneutralizaion assay antibody titers of triple intranasally and intrapreputially inoculated dairy bulls.

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