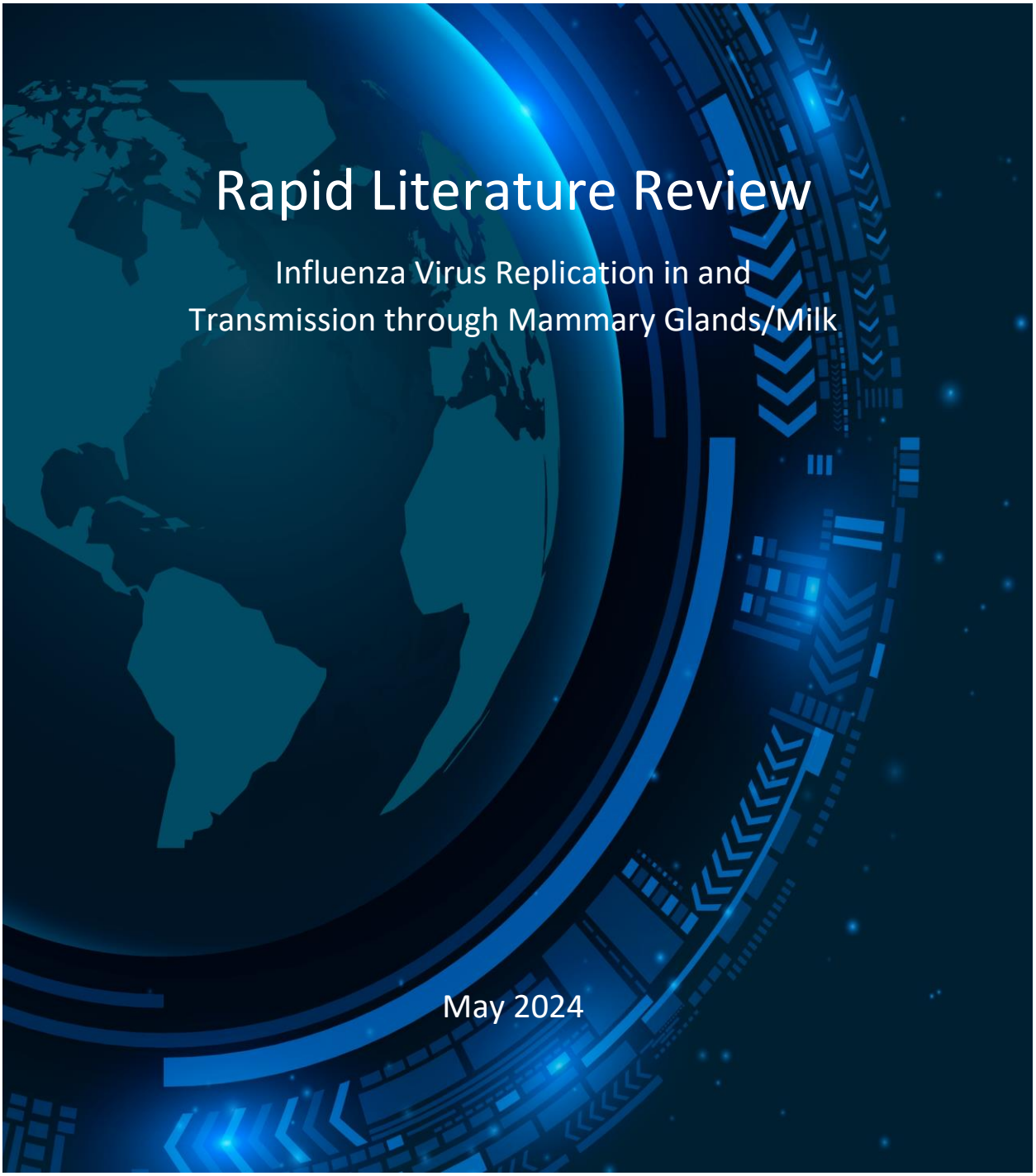

Rapid Literature Review

Influenza Virus Replication in and Transmission through Mammary Glands/Milk

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RAPID LITERATURE REVIEW - INFLUENZA VIRUS REPLICATION IN AND TRANSMISSION THROUGH MAMMARY GLANDS/MILK

INTRODUCTION

There is currently limited information available on influenza A virus[1] replication in and transmission through mammary glands and milk. Specifically, there are no published studies on this topic regarding the circulating H5N1 Eurasian lineage clade 2.3.4.4.b panzootic strain. The summarized scientific literature in this review are experimental or field studies in cattle, swine, ferrets and human breast cells, with limited information available from recent years. Table 1 provides a summary of the studies discussed below.

METHOD

This scientific literature review includes information related to influenza virus replication in and transmission through mammary glands/milk. The literature search by the Canadian Agriculture Library used Scopus, CAB, and Zoological Abstracts and included the following search strategy: (("influenza a" OR hpai OR "highly pathogenic avian influenza" or influenza) AND (mammal*) AND (replicat* OR transmi*) AND (milk OR (mammary AND gland) OR udder OR mastitis OR breast)) ; ((influenza AND virus) AND (mammals) AND (mammary OR breast* OR milk or mastitis or (mammary w/3 gland))). An additional search was conducted through Google Scholar using a similar combination of key words, including influenza virus replication in milk/mammary gland, influenza virus transmission through milk/mammary gland. Finally, additional relevant articles were also found through an article by Afshar and Bannister (1970) that reviewed reports of infection of the bovine mammary gland with viruses which cause pathological changes and with viruses that propagate in the udder.

STUDIES RELATED TO VIRAL REPLICATION

There are very limited studies available that discuss influenza virus replication in mammary glands or milk. An early study was conducted by Mitchell and colleagues related to the propagation of viruses in ruminant mammary glands. **Mitchell et al. (1953)** inoculated one cow (age 6 years, in lactation for 6 months, producing ca. 20 oz. milk from each quarter) with Newcastle disease virus[2] (NCD) and influenza A (PR8) virus (IAV) on separate sides following removal of milk from the quarters. A tube of lyophilized virus was reconstituted and titred (10^{-7} for NCD and 10^{-5} for IAV) on chick embryos, with 2 cc introduced through the teat canal into the lactiferous sinus (on the right rear quarter for NCD and left front quarter for IAV). Milk samples were collected daily from the injected and control quarters, and titred daily by chick embryo inoculation and by the haemagglutination (HA) test. For IAV, on the second day following inoculation there was an increase in the virus content of the milk which was sustained for four days, dropping for one day to a lower level, but then rose again for a period of five days until a concentration in the milk was greater than in the original inoculum. Overall, the concentration of virus in the milk increased beyond that present on the first day and a sustained titre persisted for several days in spite of daily milking likely indicating propagation of virus within the mammary gland. This was subsequently studied through further investigations on NCD by **Mitchell et al. (1953b)** where the authors conducted experiments that would explain viral propagation had occurred within the gland. Active virus injected into a quarter resulted in

[1] Part of the Orthomyxoviridae family (ICTV, 2011)
[2] Part of the Paramyxoviridae family (ICTV, 2024b)

the virus titre in the milk to increase quickly and persist for several days compared to inactivated virus (using formalin) resulting in no virus being found in the milk in 48h.

Following this, a Master of Science dissertation by **Corner. A. H. (1965)** studied the histological effects of viruses on the lactating bovine mammary gland. Freeze-dried IAV (PR8) was reconstituted and passaged in embryonated eggs. Harvested egg fluids with HA titre of 1:640 were used for intramammary inoculation. Milk sampling was conducted and tissues of the cows were harvested for histological examination. HA titres above 1:10 were not obtained in the whey samples collected from the cows indicating virus propagation had not taken place. Another round of testing was repeated in additional cows, but HA was not demonstrated. Additionally, IAV did not show to cause mastitis as there were no histopathological changes in the mammary tissues. Based on the results, the study discontinued further efforts to propagate IAV in the lactating mammary gland.

A study by **Paquette et al. (2015)** investigated human breast cells to determine their susceptibility to influenza virus infection. Three cell lines of cultured human epithelial breast cells were inoculated with the Cal/07 strain (2009 H1N1) (in the absence of exogenous proteases) to visualize the virus life-cycle in inoculated cells, assess the viral kinetics, and determine cell viability post-inoculation (pi). "Normal" non-tumorigenic (MCF-10A) and adenocarcinoma (MCF-7 and MCDA-MB-231) human epithelial breast cell lines were used to eliminate single cell type biases. To visualize the virus within the cell and determine virus subcellular localization, inoculated cells were stained for IAV. Viral replication was found to occur within the nucleus of all three cell types at 24h pi. vRNA was significantly increased in all cells types between 3 and 24 h pi by ~10 fold. Cell viability analysis showed significant drops in cell viability of MCF-10A and MDA-MB-231 pi reaching ~35% viability at the 72 h time point compared to uninoculated cells at the same incubation. To assess productive infection, live virus was quantified from collected supernatant at each time point. Live viral titers ranged between 3 and 4 $TCID_{50}$/ml (Log_{10}) for all cell types. Baseline control wells had minimal or no detectable live virus. Overall, this experiment showed viral replication of the inoculated virus in human breast cells.

STUDIES RELATED TO VIRAL TRANSMISSION

Studies found that related to transmission of IAV through milk or mammary glands were limited to those conducted in swine and ferrets. **Garrido-Mantilla et al. (2020)** studied the transmission of IAV and porcine reproductive and respiratory syndrome virus[3] (PRRSV) using a novel nurse sow model. For this study sows were used that tested virus-negative by IAV specific real-time reverse transcription polymerase chain reaction (rRT-PCR) assays and antibody-negative by IAV specific ELISA (enzyme-linked immunosorbent assay) serology tests. Sows were housed in two separate BSL-2 rooms. An additional room was used for the nurse sow at the time of new piglet adoption. Pigs were infected with IAV (H1N1 (intranasal inoculation with 10^5 $TCID_{50}$/mL of A/swine/ Iowa/MT_12_07_1920/2012)). They then contaminated the udder skin (confirmed via rRT-PCR) of lactating dams with their nasal and oral secretions while suckling. One day prior to adoption, all of the IAV challenged piglets were IAV positive in their nasal swabs as was the udder skin wipe of the sow, which had a virus titer of 5.6×10^6 $TCID_{50}$/mL. The sow's nasal swab was virus isolation negative. Once the skin was confirmed virus positive for IAV, the sows were moved to separate empty clean rooms to adopt IAV negative suckling piglets. One out of eight (12.5%) piglets tested IAV positive 1-day post-adoption (dpa) and the entire litter (8 out of 8) became positive by 4 dpa. This

[3] Part of the Arteriviridae family (ICTVa)

study showed piglets can transmit viable virus to the udder skin of lactating sows, which can further transmit virus from sow to suckling piglets.

Similar research by **Garrido- Mantilla et al. (2021)** involved a field cohort study in three influenza positive breed-to-wean farms to determine the impact of nurse sows on IAV transmission in pigs. To assess IAV status, udder skin wipes and oral swabs were collected from nurse sows (at enrollment (~5–7 days after farrowing) and weaning) from three IAV positive swine breeding herds in Minnesota and Iowa from October 2018 to February 2019. Similarly, oral swabs of 6 piglets per litter were sampled randomly from nurse and control litters at various time points. IAV status of the samples was determined by rRT-PCR. The proportion of IAV positive oral swabs was significantly higher at weaning (17.9%, 14.9%) than at enrollment (0%, 3.4%) for control and nurse sow groups, respectively. Similarly, the proportion of IAV positive udder wipes was significantly higher at weaning (60.2%) than at enrollment (22.8%) for the control group, however, there was no significant difference in the proportions of IAV positive udder wipes collected at weaning (63.3%) and enrollment (75.8%) for the nurse sow group. Nevertheless, a higher proportion of positive udder skin wipes was obtained at enrollment in nurse sows (75.8%) compared to controls (22.8%). The IAV positivity for control / nurse sow litters resulted in around 12% positivity for both groups at enrollment, 14.9% / 30.2% 2 days post-enrollment (dpe), 22.9% / 37.0% 4dpe, 46.8% / 59.4% at day 14 of lactation, and 64.0% versus 56.4% at weaning. Overall, the nurse sow may be shedding virus from the respiratory tract as well as through secretions left on the udder skin by the recently weaned piglets during suckling, which could become an infection source for the piglets in the newly adopted litters. Litters adopted by the nurse sows had significant higher odds of testing IAV positive. The study results show that nurse sows can contribute to IAV transmission and persistence of infections in pigs prior to weaning.

Another study **(Paquette et al., 2015)**, mentioned in the previous section, investigated 2009 H1N1 influenza virus infection and transmission in breastfeeding mothers and infants utilizing an infant-mother ferret influenza model. Ferrets were shown to be seronegative by haemagglutination inhibition assay against currently circulating influenza A and B strains before infection. For each infection experiment three litters at 4-weeks postpartum were randomly chosen for each experimental group (inoculation or mock control). To determine the transmission of influenza virus to mother mammary glands, infant ferrets were first intranasally inoculated with A/California/07/2009 (Cal/07) at a dose of 10^5 50% egg infectious dosage (EID_{50}) and housed with mother ferrets for a 7 day time course. All mammary glands were collected from mothers on day 4 and 7 post-infant-inoculation (pii) (3 per time point). Live viral load was quantified via MDCK titration assay that revealed live virus in 7 of the glands, with some viral loads reaching 6 $TCID_{50}$/g (Log_{10}) or higher. All mothers assessed had at least one gland positive for live IAV. Nipples of positive mammary glands also had significant amounts of live influenza virus on day 4 pii (3–7 $TCID_{50}$/g (Log_{10})). Live virus assessment in milk collected between day 3 and 7 pii revealed virus presence where some samples contained high virus levels between 6–7 $TCID_{50}$ (Log_{10}). Viral RNA (vRNA) analysis through quantitative real-time PCT (qRT-PCR) from milk collected on separate occasions showed vRNA was present in some milk samples as high as ~10,000 copies/5 ng totRNA. Most samples had between 10 and 1000 copies. vRNA and live virus were not detected in milk collected at baseline. Histopathological assessment was conducted following harvest of mammary gland tissues to determine IAV localization and gland pathology following infection. Virus staining was not detected in control mammary glands, but observed day 4 pii and increasingly by day 7 pii with loss of significant tissue structure. Viral staining was more pronounced in epithelial cells. The results summarized above suggest influenza-infected infant ferrets

transmitted the virus to the mother mammary glands, since they contained IAV and viral shedding occurred through milking.

To determine the transmission of influenza virus from mammary glands, active A/California/07/2009 (Cal/07) at a dose of 10^5 50% egg infectious dosage (EID_{50}) or PBS was inoculated into active glands of lactating mother ferrets through the lactiferous ducts following expression of milk. Following direct inoculation with the virus, mothers and their infants developed significant disease compared to the control inoculation groups. Infants feeding on virus inoculated mammary glands also had significant weight loss with a 30% survival rate by day 7 post-mammary-inoculation (pmi). Live virus, average 6 $TCID_{50}$/ml (Log_{10}), was detected in the nasal washes (NW) of infants feeding from virus inoculated glands day 4 pmi. Virus was also detected in mother NW after detection in the infants (day 7 pi). Live virus was present in expressed milk, day 2 and 4 pmi, between 3 and 13 $TCID_{50}$/ml (Log_{10}), thereby confirming successful inoculation and infection. The findings suggest respiratory infection in infants resulted from virus that was shed from the inoculated mammary gland.

SUMMARY

Despite the limited studies that looked at influenza virus replication in or transmission through milk or mammary glands, there is evidence of IAVs being capable of infecting cells and replicating in the mammary gland of various animals or in human breast cells. Live virus has been detected in the milk produced from those glands and viral shedding can occur through milking. Research shows the transmission of IAV from young nursing animals to mothers and from mothers to nursing animals via the mammary gland. Nursing animals can transmit viable virus to the udder of lactating animals, with onward transmission to other nursing animals. Additionally, lactating animals can shed the virus through their milk, which can lead to infection in the young nursing animal as well as continued influenza infection in a herd of animals. Further research is needed, specifically in bovines and on the current H5N1 avian influenza strain.

Table 1 – Summary of Influenza A Replication and Transmission Studies

Study	Species	Inoculation/Transmission	Results
VIRAL REPLICATION STUDIES			
Mitchell et al, 1953a	Cattle	Intramammary inoculation (IAV (PR8))	Chick embryo inoculation and haemagglutination (HA) test demonstrated presence of virus in milk.
Corner. A. H., 1965	Cattle	Intramammary inoculation (IAV (PR8))	No viral propagation was obtained (HA titre below 1:10 in whey samples) and no histopathological changes were detected in mammary tissues.
Paquette et al, 2015	Humans (in vitro)	Viral inoculation (Cal/07 strain (2009 H1N1)) of three lines of cultured breast cells	Virus was able to enter human breast cells leading to virus replication at 24h post-inoculation. Live viral titers ranged between 3 and 4 $TCID_{50}$/ml (Log_{10}) for all cell types
VIRAL TRANSMISSION STUDIES			
Garrido-Mantilla et al, 2020	Swine	Transmission of IAV (H1N1 A/swine/Iowa/MT_12_07_1920/ 2012) from piglet to udder skin of sow, with subsequent transmission to suckling piglets	One out of eight (12.5%) piglets tested IAV positive 1-day post-adoption (dpa) and the entire litter (8 out of 8) became positive by 4 dpa.
Garrido-Mantilla et al, 2021	Swine	Transmission of IAV (natural infection) from control and nurse sows to suckling piglets	The odds of IAV positivity were significantly higher ($p<0.05$) for litters from nurse sows 2 days post-enrollment (DPE), 4 DPE, and at day 14 of lactation. There were no differences in the proportion of positive samples at weaning.
Paquette et al, 2015	Ferrets	Transmission of IAV (H1N1 A/California/ 07/2009 (Cal/07)) from infant ferret (intranasal inoculation) to mother ferret mammary glands	All mothers assessed had at least one gland positive for live IAV and nipples of positive mammary glands had significant amounts of live IAV on Day 4 post-infant-inoculation (3–7 $TCID_{50}$/g (Log_{10})). Most milk samples collected had between 10 and 1000 copies per 5 ng totRNA.
Paquette et al, 2015	Ferrets	Transmission of IAV (H1N1 A/California/07/2009 (Cal/07)) from mother ferret (intramammary inoculation) to infant	Live virus, average 6 $TCID_{50}$/ml (Log_{10}), was detected in the nasal washes of infants feeding from virus inoculated glands day 4 post-mammary-inoculation (pmi). Live virus was present in milk day 2 and 4 pmi, between 3 and 13 $TCID_{50}$/ml (Log_{10})

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