Canadian Journal of Microbiology

OPEN ACCESS | Note

Longitudinal screening of retail milk from Canadian provinces reveals no detections of influenza A virus RNA (April–July 2024): leveraging a newly established pan-Canadian network for responding to emerging viruses

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Abstract

Highly pathogenic avian influenza (HPAI) H5N1 has caused the deaths of more than 100 million birds since 2021, and human cases since 1997 have been associated with significant morbidity and mortality. Given recent detections of HPAI H5N1 in dairy cattle and H5N1 RNA detections in pasteurized retail milk in the United States, we established the pan-Canadian Milk Network in April 2024. Through our network of collaborators from across Canada, retail milk was procured longitudinally, approximately every 2 weeks, and sent to a central laboratory to test for the presence of influenza A virus RNA. Between 29 April and 17 July 2024, we tested 109 retail milk samples from all 10 Canadian provinces (NL, NS, PEI, NB, QC, ON, MB, SK, AB, and BC). All samples tested negative for influenza A virus RNA. This nationwide initiative was established for rapid retail milk screening as per the earliest reports of similar undertakings in the United States. Our independent testing results have aligned with reporting from federal retail milk testing initiatives. Despite no known HPAI infections of dairy cattle in Canada to date, H5N1 poses a significant threat to the health of both humans and other animals. By performing routine surveillance of retail milk on a national scale, we have shown that academic networks and initiatives can rapidly establish nationwide emerging infectious disease surveillance that is cost-effective, standardized, scalable, and easily accessible. Our network can serve as an early detection system to help inform containment and mitigation activities if positive samples are identified and can be readily reactivated should HPAI H5N1 or other emerging zoonotic viruses be identified in agricultural or livestock settings, including Canadian dairy cattle.

Key words: avian influenza, influenza virus, H5N1, milk, surveillance

Introduction

Influenza A viruses (IAVs) are in the family *Orthomyxoviridae*, which can infect a wide variety of avian and mammalian species, including humans, and cause a wide spectrum of illness, from asymptomatic to severe, including multi-organ failure resulting in death (Canadian Food Inspection Agency 2024*a*; Centers for Disease Control and Prevention 2024*a*). Highly pathogenic avian influenza (HPAI) is an emerging global health threat, and the involved H5Nx viruses have properties that make them of serious concern for potential involvement in a new pandemic. Most recently arriving in North America in late 2021 (Caliendo et al. 2022), HPAI H5N1 clade 2.3.4.4b (A/goose/Guangdong/1996 (H5N1)) has infected a broad range of wild and domesticated bird species, resulting in the death of more than 100 million birds (Youk et al. 2023). This virus and derived reassortants have also infected diverse species of marine and terrestrial mammals (Alkie et al. 2023; Jakobek et al. 2023; European Food Safety Authority et al. 2024; Lair et al. 2024), notably including dairy cattle in the United States (Hu et al. 2024; Nguyen et al. 2024; USDA APHIS 2024*a*). Since 1997, spillovers of HPAI H5N1 from birds to humans have also been documented. Of the nearly 900 known human H5N1 infections, most have exhibited severe disease symptoms, with a case fatality rate of approximately 50% (World Health Organization 2024). However, despite the fact that the currently circulating H5N1 clade 2.3.4.4b virus and derived reassortants have caused extensive infections and deaths of birds and mammals, relatively few human cases have been reported, with only 20 cases globally associated with this clade since January 2022 (data as of 24 July 2024) (Kniss et al. 2023; World Health Organization 2024; Centers for Disease Control and Prevention 2024*f*). Notably, none of these cases has been fatal, with the majority being reported as exhibiting mild disease symptoms with frequent reports of conjunctivitis. Four of the 20 human cases were reported in the spring/summer of 2024 and were associated with close interactions with infected dairy cattle. Another seven human cases of H5N1 clade 2.3.4.4b were reported in early July 2024 in the US following exposure to poultry (Centers for Disease Control and Prevention 2024*f*). The four cases associated with infected dairy cattle all had relatively mild symptoms (conjunctivitis), with one also reporting mild respiratory symptoms, and all individuals recovered (Uyeki et al. 2024; Centers for Disease Control and Prevention 2024*b*, 2024*c*, 2024*e*, 2024*f*). In response to the first human case associated with dairy cattle in Texas, the CDC experimentally infected ferrets with the human isolate, A/Texas/37/2024(H5N1). The isolate from the individual who was infected in Texas was able to infect ferrets efficiently and caused 100% lethal disease. Interestingly, unlike transmission of seasonal human IAVs in ferrets, the Texas isolate was only able to spread ferret-to-ferret through direct contact between ferrets but not through respiratory droplets (Centers for Disease Control and Prevention 2024*d*).

The United States Department of Agriculture (USDA) reported in late March 2024 that dairy cattle on a farm in Texas exhibited lethargy, dehydration, mild respiratory signs, decreased feed intake, decreased milk production, and/or milk with abnormal colour/texture, and further investigation revealed lesions similar to those seen with mastitis (Burrough et al. 2024; Caserta et al. 2024). A pre-print was posted on 13 July 2024 recapitulated this disease state under experimental conditions when dairy cattle were infected through an intramammary route (Baker et al. 2024). Interestingly, on farms with confirmed cases of infected cattle, there were reports of barn cats found dead on the property that were confirmed to have succumbed to infection with H5N1 (Burrough et al. 2024). Phylogenetic analyses subsequently showed that the initial infection of dairy cattle in Texas was the result of a single spillover event of HPAI H5N1 from wild birds in late 2023 (Worobey et al. 2024). This was followed by subsequent cattle-to-cattle transmission possibly mediated by exposure to contaminated milking equipment, or another currently unknown route (Le Sage et al. 2024; Nguyen et al. 2024). Since then, the outbreak has spread to 192 herds in the US as the transport of infected dairy cattle occurred before the extent of the outbreak was known (USDA APHIS 2024*d*). H5N1 clade 2.3.4.4b-infected dairy cattle have been reported in 13 states as of 24 July 2024 (USDA APHIS 2024*d*) and in May 2024 H5N1 clade 2.3.4.4b RNA was detected in wastewater from nine cities across Texas (Tisza et al. 2024), highlighting the expansive geographic spread of this virus.

Soon after the initial dairy infections were reported, the USDA and the US Food and Drug Administration (FDA) began conducting H5N1 surveillance of pasteurized milk and other dairy products, and among those collected from 38 states, reported that up to 20% of samples were positive for H5N1 clade 2.3.4.4b RNA. Importantly, no viable virus was detected in the retail pasteurized dairy samples (Spackman et al. 2024*b*). The USDA tested raw milk ($n = 275$) from states known to have H5N1-infected herds, and found that 158 (58%) of the samples were positive for viral RNA with 39 (25%) of the RNA positive samples also containing infectious virus. Despite the high proportion of positive samples, the group also reported that continuous flow pasteurization (commonly used and FDA approved) of the milk completely inactivated any infectious virus, indicating that the milk supply is safe despite the detection of viral RNA (Spackman et al. 2024*a*). Testing of tissue from dairy cattle and retail beef has also been performed by the USDA. To date, one muscle sample from a dairy cow known to be infected with H5N1 clade 2.3.4.4b has been positive for viral RNA and no meat from any dairy cattle has entered the US food supply (USDA APHIS 2024*c*).

As of 24 July 2024, no H5N1 (clade 2.3.4.4b or otherwise) infections of dairy cattle have been reported in Canada. Due to current mandatory testing by the USDA before transporting cattle across state borders to limit inter-state spread (USDA APHIS 2024*b*), infected cattle are unlikely to enter Canada and threaten Canadian cattle operations. Additionally, since 29 April 2024, the Canadian Food Inspection Agency (CFIA) has implemented a requirement for proof of negative testing on USDA export certificates for all lactating dairy cattle

(Canadian Food Inspection Agency 2024*b*), further protecting Canadian cattle. However, as this virus still circulates extensively in wild birds, there is the potential for the virus to independently spill over from wild birds into Canadian cattle, as occurred on the index farm in Texas. Therefore, monitoring of cattle in Canada is of vital importance to detect if a potential spillover has occurred as quickly as possible.

Although it is known that cattle can be infected by IAVs (Mitchell et al. 1953; Campbell et al. 1977; Brown et al. 1998), including H5N1 (A/cat/Germany/R606/2006(H5N1)) (Kalthoff et al. 2008), no viral genomes had been sequenced from cattle, prior to early 2024. Very little is known about the patterns of disease or the tissue types that allow viral replication, although decreased milk production had been previously associated with influenza antibodies in dairy cattle (Crawshaw et al. 2008). Bordes et al. recently found that H5N1 viruses from the same clade as the dairy cattle viruses (2.3.4.4b) that were originally isolated from a chicken and a fox in the Netherlands were able to efficiently replicate in well-differentiated bovine airway epithelial cells cultured at an air-liquid interface (Bordes et al. 2024). Further, scientists in Germany recently reported that an H5N1 virus isolated from a wild bird was able to infect the udder of experimentally infected cattle (Friedrich-Loeffler-Institut 2024). These findings suggest that other H5N1 viruses may also have the potential to infect cattle, highlighting the need for ongoing surveillance.

Recently, the CFIA, as well as academic colleagues in Ontario, tested retail milk and found that all samples tested negative for IAV RNA. However, these efforts represent longitudinal snapshots in time with the strengths of surveillance related directly to the durability of testing (Blais-Savoie et al. 2024; Canadian Food Inspection Agency 2024*c*). In April 2024, we established the pan-Canadian Milk (PCM) Network, bringing together colleagues from across the country to procure retail milk every 2 weeks and send samples to a central laboratory for testing. Testing was ongoing through this network since its inception in April 2024 until mid-July 2024.

Methods

Milk sampling

Pasteurized whole (3.25%) milk was obtained from local retailers in all Canadian provinces. While additional detailed data was collected about each retail milk sample in case any positives were detected, including brand, expiry date, lot number, dairy cooperative, date of purchase, and city and province of purchase, this is not reported here for the privacy of the dairy cooperatives. Whole milk was chosen as a number of previous studies have found that the thermostability of a several RNA viruses is higher in milk with a higher fat content (Tomasula et al. 2007; Norouzbeigi et al. 2021; Palme et al. 2024). Samples were collected every 2 weeks to ensure different and non-consecutive manufacturing lots of milk were obtained and spaced out in time, beginning 29 April 2024. Milk cartons were externally disinfected, opened in a biosafety cabinet, and a sample was aseptically collected in sterile conical tubes at the originating laboratories. Samples from Saskatchewan were stored at −80 °C and shipped on dry ice. Samples from the other nine provinces were kept at 4 °C and shipped on ice. All samples were shipped to the University of Manitoba for processing and subsequently stored at −80 °C.

RNA isolation

RNA isolation was performed from 140 μL of milk using the Qiagen Viral RNA Mini Kit (Qiagen, Product # 52906) as per the manufacturer's instructions. Isolated RNA was stored at −80 °C.

Screening for influenza A virus

Real-time RT-PCR was used to screen for the presence of IAV matrix gene RNA as per Wight et al. (2024) using a QuantStudio 6 Flex instrument (Applied Biosystems). If any sample had tested positive for the matrix gene, the sample would have been subsequently screened for the H5 subtype of the haemagglutinin gene as per Wight et al. (2024). Had any positive samples been identified, the sample and data, including the associated information relating to each sample, would have been immediately shared with federal agencies through existing collaborations to allow for tracing of the source of viral RNA. These federal collaborators at the CFIA would have performed genome sequencing and attempted virus isolation for any positive sample.

Results

Between 29 April and 17 July 2024, 109 retail milk samples were obtained from all Canadian provinces (NL, NS, PEI, NB, QC, ON, MB, SK, AB, and BC). All 109 samples tested negative for IAV matrix gene by real-time RT-PCR (Fig. 1; Table 1).

Discussion

Screening of retail milk from across Canada between 29 April and 17 July 2024 yielded no detections of IAV RNA. This is in agreement with what has been reported by both the CFIA and academic colleagues in Ontario (Blais-Savoie et al. 2024; Canadian Food Inspection Agency 2024*c*).

Although CFIA has not announced any formal plans to continue retail milk testing for H5N1 RNA, they continue to periodically release testing results. Recently, they reported that they have tested a total of 911 milk samples and all were negative for H5 RNA (Canadian Food Inspection Agency 2024*c*), which is in accordance with no outbreaks of disease associated with H5N1 having been reported in dairy cattle in Canada to date. Therefore, it was not unexpected that our samples, collected during nearly the same time as CFIA's, also tested negative for influenza RNA. When considering something as important to human health as the safety of the milk supply, multiple independent testing strategies conducted in parallel can be crucial in providing confidence in the safety of supply, and credibility with the general public is increased when there is transparency relating to testing. Furthermore, parallel testing strategies can be particularly important during emerging outbreaks of novel pathogens when methodologies are being optimized.

Fig. 1. Map of Canada showing provinces where milk was sampled as well as the not sampled territories, with the number of retail milk samples that were tested from each province as of 17 July 2024 indicated.

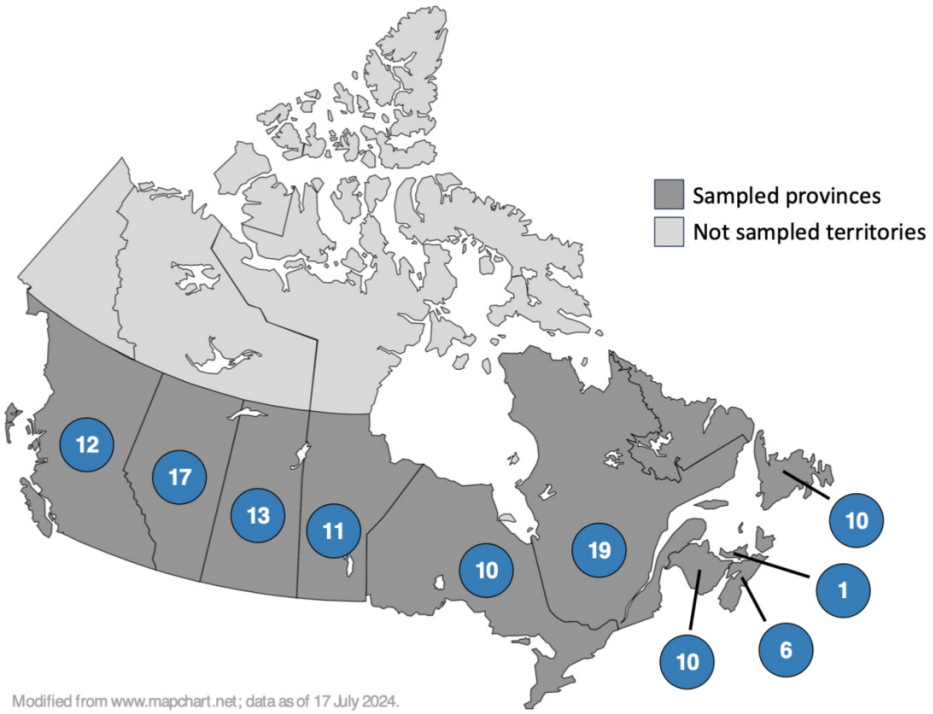

Table 1. Provinces and national totals of retail milk samples tested, the number of influenza A virus (IAV) RNA positive samples, and the percentage of IAV RNA positive samples.

Province	No. of milk samples tested	No. of IAV RNA positive samples	% of IAV RNA positive samples
Newfoundland and Labrador	10	0	0%
Nova Scotia	6	0	0%
Prince Edward Island	1	0	0%
New Brunswick	10	0	0%
Quebec	19	0	0%
Ontario	10	0	0%
Manitoba	11	0	0%
Saskatchewan	13	0	0%
Alberta	17	0	0%
British Columbia	12	0	0%
National Totals	**109**	**0**	**0%**

Note: RNA was isolated from milk samples and screened for IAV matrix gene RNA using real-time RT-PCR.

Due to the reach, resources, and lab capacity of the CFIA, we have decided to suspend the PCM network's efforts at the current time as our network is unable to test retail milk on the same scale as the CFIA. Although suspending testing at the current time, members of this PCM network have agreed to re-instate the network if needed, which could be readily re-established and recommence testing within a matter of days. At present, network members are continuing to monitor ongoing milk testing updates as well as virus circulation updates and remain connected through regular contact. This includes multiple members that are connected through ongoing emerging virus research activities and through national laboratory network initiatives, including the Canadian Consortium of Academic Biosafety Level 3 Laboratories. These ongoing research connections ensure continual connectivity among PCM members that will expedite reconvening testing activities, if needed. While the PCM was created rapidly, there were logistical hurdles that were encountered. These were primarily related to procurement of reagents and coordination of sample shipments between locations. The latter was easily addressed through coordination of shipping and receiving logistics. The former included manufacturing delays for primers and probes as well as acquisition of specialty reagents which added time between network formation and initial testing. The acquisition of these reagents by our central testing laboratory will ensure that re-activation of the

network for renewed testing is not delayed by the need for re-acquiring these reagents. This academic network has created new collaborations between scientists from across the country, enabling the development of scientific relationships, protocols, and capacity that will aid those in the network individually, as well as this network as a whole, should we be called upon to serve, even if in response to a different emerging viral pathogen.

Limitations of this study are primarily related to sample size. With an estimated 1.4 million dairy cows in Canada, the ability to perform large-scale testing from across the country, without direct access to raw milk, is limited. Despite this, we were able to confirm findings by other groups, cementing the role and importance of academic collaborative networks such as ours for responding to infectious diseases.

Although our negative findings are important and confirm other reports from academic and government collaborators, what became clear throughout the course of this study was the importance and ability to rapidly establish a pan-Canadian network of academic infectious disease researchers in the face of new and emerging infectious disease threats. The value of a network such as this should not be overlooked in the necessary and quick responses needed for infectious disease outbreaks. This network, with a rapid, scalable, and comprehensive approach to screening and public sharing of data in real time, is a model for other outbreak responses.

Given the expansive nature of the H5N1 clade 2.3.4.4b outbreak in dairy cattle, spillover to other species, and at least four human infections linked to close contact with dairy cattle in the US, continued longitudinal monitoring of milk samples in Canada may allow early detection of HPAI infection of dairy cattle. These ongoing efforts by government agencies may enable early containment and preventative measures to limit further spread, should H5N1 or other subtypes of IAVs, infect dairy cattle in Canada. The PCM network will remain abreast of the evolving situation with dairy cattle in the US and testing will be re-implemented should the situation change significantly.

Conclusions

We successfully established a pan-Canadian network of academic colleagues who are willing and able to quickly respond to emerging infectious disease threats. Through this work, we further supported findings from government and other academic colleagues that there is no evidence to date of H5N1 clade 2.3.4.4b infection of dairy cattle in Canada, despite expansive outbreaks just south of the Canadian border in the US.

Acknowledgements

We would like to acknowledge all PCM Study collaborators for contributing milk samples from across the country. We would also like to acknowledge the individuals who were involved in shipping milk samples, including Ishraq Rahman (Memorial University), Isabelle Dubuc (Université Laval), Carolina Camargo (University of British Columbia), Kim Pageau (Université Laval), Danielle G. Gordon (University of British Columbia), Ivan Villanueva (University of British Columbia), and Karla Fisher (Toronto General Hospital).

Article information

History dates

Received: 26 July 2024
Revised: 22 August 2024
Accepted: 26 August 2024
Accepted manuscript online: 27 September 2024
Version of record online: 27 November 2024

Copyright

Data availability

All data are available from the authors upon request.

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Andrew S. Lang served as Editor at the time of manuscript review and acceptance; peer review and editorial decisions regarding this manuscript were handled by another editorial board member.

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Competing interests

The authors declare that they have no competing interests.

Funding information

This work was supported by the Canadian Institutes of Health Research (Tier 2 Canada Research Chair, grant number 950-

231498 to JK) and by the Natural Sciences and Engineering Research Council Discovery Grant (RGPIN-2018-06036 to JK).

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