Genomic Surveillance of a Canadian Airport Wastewater Samples Allows Early Detection of Emerging SARS-CoV-2 Lineages

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Abstract

The SARS-CoV-2 pandemic has shown that wastewater (WW) surveillance is an effective means of tracking the emergence of viral lineages in communities, arriving by many routes including via transportation hubs. In Ontario, numerous municipal WWTPs participate in WW surveillance of infectious disease targets such as SARS-CoV-2 by qPCR and whole genome sequencing (WGS). The Greater Toronto Airports Authority (GTAA), operator of Toronto Pearson International Airport (Toronto Pearson), has been participating in WW surveillance since January 2022. As a major international airport in Canada and the largest national hub, this airport is an ideal location for tracking globally emerging SARS-CoV-2 variants of concern (VOCs). In this study, WW collected from Toronto Pearson's two terminals and pooled aircraft sewage was processed for WGS using a tiled-amplicon approach targeting the SARS-CoV-2 virus. Data generated was analyzed to monitor trends SARS-CoV-2 lineage frequencies. Initial detections of emerging lineages were compared between Toronto Pearson WW samples, municipal WW samples collected from the surrounding regions, and Ontario clinical data as published by Public Health Ontario. Results enabled the early detection of VOCs and individual mutations emerging in Ontario. On average, emergence of novel lineages at the airport ahead of clinical detections was 1–4 weeks, and up to 16 weeks. This project illustrates the efficacy of WW surveillance at transitory transportation hubs and sets an example that could be applied to other viruses as part of a pandemic preparedness strategy and to provide monitoring on a mass scale.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has had an impact globally on individuals and economies. Travel of individuals across borders, and especially modern air-travel by commercial airlines facilitated introduction of individuals carrying the virus to locations far from their point of infection, contributing to the rapid spread of SARS-CoV-2 worldwide (Chinazzi et al., 2020; Le Targa et al., 2022; Wells et al., 2020; Wu et al., 2020). While air-travel contributes to viral transmission across international borders, some evidence suggests that border closures enforced early in the pandemic did not reduce viral spread (Shiraef et al., 2022).

During the early SARS-CoV-2 pandemic, airport operations were critical to pandemic response for import/export of emergency personnel and medical supplies (*COVID-19 and the Aviation Industry*, n.d.; *"We Are Ready to Go to Work,"* 2021; News ·, 2020). Toronto Pearson International Airport (Toronto Pearson) processed 40% of Canada's air cargo prior to the pandemic and the average daily cargo activity more than doubled between 2019 and 2021 (*Fast Facts*, n.d.). The volume of cargo processed shows the importance of air cargo at Toronto Pearson locally, provincially and nationally during the pandemic (*Fast Facts*, n.d.). In addition to these more obvious functions during a pandemic, airports can also be an efficient and cost-effective point of wastewater surveillance for emerging or re-emerging pathogens arriving in a city, country, and region, while maintaining the anonymity of the travelers (Le Targa et al., 2022). When selecting WW sites for pathogen detection by sequencing to mitigate and/or track emerging

outbreaks in a region or country, WW from airports should be considered as an important public health activity for future pandemic preparedness and outbreak management.

Though WW surveillance is not a new technology for testing of infectious disease targets, the SARS-CoV-2 pandemic has provided an opportunity to modernize testing methods and target new pathogens which illustrates the utility of WW as a means of community-wide epidemiological surveillance (Child et al., 2023; Hrudey et al., 2022). Sewer shed sampling, including the influent of wastewater treatment plants (WWTPs), is a cost-effective and non-invasive strategy for capturing information on the presence and abundance of SARS-CoV-2 and the relative abundance of variants of concern (VOC) in defined communities, serviced by different WWTPs (Berry et al., 2022; Child et al., 2023; Hillary et al., 2021; Kitajima et al., 2020). WW samples are also able to capture asymptomatic, pre-symptomatic, or mild cases that may not be captured in clinical surveillance data (Berry et al., 2022; Government of Canada, 2021; Sah et al., 2021). Also, at a time with changing testing eligibility criteria, and significantly reduced clinical testing, WW signals provided a reliable and stable measure of community transmission (Berry et al., 2022).

SARS-CoV-2 evolution has resulted in a multitude of lineages and sub-lineages, some of which have increased transmissibility, immune evasion, or other advantages that make them of public health interest for surveillance (Nash et al., 2023; Pérez-Losada et al., 2020). The data returned from sequencing of WW samples is comprised of many individual viral genomes that must be disentangled from one another using bioinformatic tools, which use mutation frequencies to determine which lineages are present in each sample (Ellmen et al., 2021; Karthikeyan et al., 2022; Poon, 2022). Individual mutation frequencies used to define lineages can be surveyed on their own. This is useful when the mutations in question contribute to immune evasion or are indicative of emerging VOCs. The spike protein residue mutation R346T is an example of such a mutation, which is present in many BQ* and XBB* lineages (Cao et al., 2023; *covSPECTRUM*, n.d.).

As of March 2024, Toronto Pearson has direct flights to and from 61 countries which span North and South America, Africa, Asia and Europe (*Airlines and Destinations*, n.d.). Roughly a quarter of the number of weekly flights arrive from international destinations, while the other three quarters are split relatively evenly between destinations in the USA and elsewhere in Canada. Generally, Airlines serving countries on the continents of Africa and South America are few and are currently being routed through Terminal 1, while airlines serving countries on the continents of North America, Asia and Europe are more numerous, and are routed through both Terminals 1 and 3 (*Airlines and Destinations*, n.d.).

Sampling at Toronto Pearson through the WSI began in January 2022 making them the first Canadian international airport to serve as a regularly sampled WW surveillance site. Pearson Airport WW samples allowed the early detection of emerging VOCs in Ontario by 1–16 weeks compared to clinical testing, depending on the VOC. In this study examples are provided of early detection of emerging VOCs in southern Ontario during the SARS-CoV-2 pandemic and suggest that airport WW surveillance is a valuable tool for public health in pandemic preparedness as it can track populations at borders which can

allow lead time to implement public health measures, including masking and vaccination programs. To our knowledge, this is the first study to track lineages in Canadian airport terminal WW.

Methods

Site selection:

Toronto Pearson has two terminals (Terminal 1 and Terminal 3) which both serve domestic and international flights. WW taken from either Terminal has been contributed to by departing and arriving passengers on flights routed through that terminal, as well as terminal staff, in the 24-hour period preceding sample collection. Airplane lavatory sewage is emptied into trucks and carried to a triturator building at the airport, for disposal. Samples collected from this disposal point will be referred to here as pooled aircraft sewage and have been contributed to by most arriving aircraft in the 24-hour period preceding sample collection. Three samples per weekday were received from Toronto Pearson (pooled aircraft sewage, Terminal 1, and Terminal 3), and the WW collected at Toronto Pearson is treated at WWTPs in both Peel and Toronto.

City of Toronto, Regional Municipality of York and Regional Municipality of Peel, are all participants in the Ontario Wastewater Surveillance Initiative (WSI) coordinated and funded by the Ontario Ministry of the Environment, Conservation and Parks (MECP). In this program each participating region collects municipal WW samples from treatment plants and pumping stations for analysis at academic labs and/or the PHAC National Microbiology Laboratory Branch (Winnipeg, MB). The three universities involved with research here are part of the WSI program. Toronto Pearson samples are collected by a third-party company and sent by courier to the academic labs for analysis, supported by Public Health Agency of Canada and coordinated by Ontario WSI.

Sample collection:

This study includes data from samples collected over the period of June 1st 2022 to March 31st 2023. WW samples from two airport terminals at Toronto Pearson (Terminal 1 and Terminal 3) were collected over 24 h periods using autosamplers, which collect a series of grab samples over a 24 h period to create a composite sample. Pooled aircraft sewage was first collected, until October 2022, using passive torpedo samplers which were allowed to soak for 24 h each before being retrieved. After October 2022, an autosampler was installed in the aircraft waste triturator building to collect pooled aircraft sewage over 24 h in the same way samples from the Terminals were collected. Samples from municipal WWTPs were collected similarly by autosampler in the case of (sites 1–6 and 9) or by grab sampling (single timepoint collection) in the case of (sites 7 and 8), at pumping stations or pipes.

Library preparation and whole genome sequencing:

As part of the Ontario WSI, for each site included in this study WGS was conducted by one of three partners: University of Guelph, University of Waterloo, and University of Western Ontario. The following

section details which samples were processed by each lab, and how the procedures and reagents used differ.

One sample per weekday (Monday - Friday excluding statutory holidays) was collected from Toronto Pearson Terminal 1 and Terminal 3 for sequencing at University of Waterloo. One sample was collected for sequencing at University of Waterloo per week as well from each of the following municipal WWTPs, pumping stations, or pipes: Leslie Street Pumping Station - York Sewershed (Site 7), Warden and 407 - York Sewershed (Site 8), Humber AMF Pumping Station - York Sewershed (Site 9), Clarkson South-Peel Water Pollution Control Plant (Site 1), and G. E. Booth (Lakeview) Wastewater Treatment Plant (Site 2).

In preparation for sequencing, the viral content of 20 mL of a WW sample was concentrated using Nanotrap Microbiome A Particles (Ceres Nanosciences) following the manufacturer's protocol "Manual Nanotrap® Wastewater Protocol using QIAGEN QIAamp® Viral RNA Mini Kit", section A. Viral Capture with the following modifications: whenever a magnetic stand is called for, the sample with beads was instead centrifuged at 8,000 × g at 4°C for 4 mins, and the pellet was resuspended in a small volume of remaining supernatant instead of water to transfer the sample to a smaller tube. The beads were resuspended in 700 µL of prepared Lysis Buffer (RLT + 2ME) for RNA extraction automated on a QIAcube connect using a RNeasy mini kit (Qiagen ID: 74116). RNA was then reverse transcribed using LunaScript RT SuperMix Kit (NEB #E3010) and amplified using Q5® High-Fidelity 2× Master Mix (NEB cat# M0492L) and the ARTIC V4.1 NCOV-2019 Panel of primers (IDT; 10008554, designed by the ARTIC network) resulting in ~ 400 bp amplicons. Amplicon libraries were prepared using the Illumina DNA Library Prep kit (20060059) with an additional 0.8× AMPure XP (Beckman Coulter) single sided bead cleanup prior to tagmentation. Paired-end (2x250 bp) sequencing of the libraries was performed using a MiSeq (Illumina).

One sample per day Monday-Friday (excluding statutory holidays) was collected from the triturator for sequencing at University of Guelph. Pooled aircraft sewage samples were prepared and sequenced as described previously (Lawal et al., 2022; Lawal & Goodridge, in preparation). Briefly, samples from passive samplers were preprocessed by submerging one of the embedded gauzes in 50 mL sterile PBS with 0.05% Tween 80 and antifoam reagent and stomached for 2 min. Viral particles in the filtrate from passive sampler and in the liquid samples from autosamplers were concentrated using Nanotrap Microbiome A particles (Ceres Nanosciences). RNA extraction was performed using the QIAamp Viral RNA extraction Mini kit (QIAGEN) carried out on the QIAcube (QIAGEN) instrument according to the manufacturer's extraction method. Complementary DNA was generated using the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher) and amplified using the same ARTIC V4.1 primers and protocols as above for the University of Waterloo. The amplicon libraries were prepared using the Nextera XT DNA library prep kit (Illumina). Paired end (2×150 bp) sequencing of the libraries was performed on the Illumina MiniSeq system. A maximum of 24 samples were loaded per run.

One sample per week was sequenced at the University of Western Ontario from each of the four Toronto WWTPs; Humber (Etobicoke) Water Pollution Control Plant (Site 3), Main (Toronto-Ashbridges Bay) Water Pollution Control Plant (Site 4), North Toronto Treatment Plant (Site 5), and Highland Creek (Scarborough)

Water Pollution Control Plant (Site 6). Ceres beads were not used to concentrate the virus, instead a 30 mL aliquot of the composite influent WW sample was centrifuged at 4200 × g at 4°C for 20 min. The supernatant was discarded, except for approximately 500 µl used to re-suspend the solids pellet. The re-suspended solids pellet was used for RNA extraction as described above for University of Waterloo. CDNA, PCR amplification and Library preparation was performed as described above for University of Guelph. Paired-end (2 x 300 bp) sequencing was performed using an Illumina MiSeq.

Data Processing and Frequency Prediction:

Fastq files for all samples were pre-processed using cutadapt to remove adapter sequences and minimap2 to map reads to a SARS-CoV-2 reference genome (NC_045512.2 – Wuhan-Hu-1) and generate coverage and mutation frequency data at all sites along the genome (Li, 2021; Martin, 2011). Mapped genomes were then processed using Alcov, a tool which uses unique and shared mutations to predict frequencies of SARS-CoV-2 lineages (Ellmen et al., 2021). Shared mutations are used to predict presence of sets of lineages while unique mutations are used to differentiate between individual sub-lineages. Mutations are only considered if the read depth at that location is greater than 10. Constellations defining each lineage in Alcov were built to include all mutations differing from the reference genome which occur in ≥ 90% of the specific lineage sequences on covSPECTRUM (*covSPECTRUM*, n.d.). Alcov was used to generate heatmaps and csv tables of predicted frequencies of lineages as well as mutation frequencies, from which the values presented in this article were extracted and plotted.

Data Transformation:

Predicted lineage frequencies output by Alcov are complex and are affected by genome coverage. Greater coverage of mutations which are unique to lineages in the mixed samples improves the ability of Alcov to make calls with more detail in sublineage separation. When fewer unique mutations are available, Alcov uses shared mutations to predict a parent lineage or will output an A-or-B statement in which more than one lineage is assigned to a frequency of occurrence in the sample because with the data available any of these listed lineages could account for the mutations detected.

To give the frequency of each lineage analyzed in this study (BQ*, BA.2.75*, BF*, XBB*, XBB.1.5*, XBB.1.16*, and XBB.1.9*), A-or-B statements were summed together and assigned to the parent lineage if all of their components were descendants of the lineage in question. For example, for the BQ* lineage calls, an Alcov call of “BQ.1 or BQ.1.1 or BQ.1.2” would have been added to the total BQ* frequency for that sample, but an Alcov call of “BA.5 or BA.5.3 or BE.1 or BQ.1” would not have been summed into the BQ* frequency lineage. The asterisk denotes the inclusion of descendant lineages, which do not overlap, apart from XBB* which includes all the lineages which make up XBB.1.5*, XBB.1.9* and XBB.1.16* as well as other XBB* lineages.

In Figs. 2–9, Terminals 1 and 3 at Toronto Pearson are grouped together as Airport (Terminal 1 & Terminal 3). Pooled aircraft sewage includes data from the passive torpedo samplers up until October 21, 2022, and the triturator building autosampler after this point. Clarkson South-Peel Water Pollution Control

Plant and G. E. Booth (Lakeview) Wastewater Treatment Plant are grouped together as “Peel (Clarkson & G.E. Booth)”. York Sewershed – Leslie Street PS, York Sewershed – Warden 407 and York- Peel OCF/Humber AMF are grouped together as “York (Leslie, Warden & Humber AMF)”. Humber (Etobicoke) Water Pollution Control Plant, Main (Toronto – Ashbridges Bay) Water Pollution Control Plant, North Toronto Treatment Plant, and Highland Creek (Scarborough) Water Pollution Control Plant are grouped together as “Toronto (North, Ashbridges, Humber & Highland Creek)”. Clinical data from Ontario are pulled from Public Health Ontario’s Weekly Epidemiological Summaries for SARS-CoV-2 genomic surveillance in Ontario (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2022a). Epi-weeks in this study begin counting at epi-week 1 at the beginning of each calendar year, which matches the reports that the clinical data was collected from.

Results

Airport WW can provide an early warning of emerging VOCs:

Emerging lineages and mutations of interest were tracked to compare first instances in airport WW, surrounding municipal WW, and clinical data where available. The lineages included in this study are BQ*, BA.2.75*, BF*, XBB*, XBB.1.5*, XBB.1.16* and XBB.1.9*. Summed detection data for each of these lineages is plotted in Figs. 2 through 8, and the first detections are compiled and compared in Table 1.

When all BQ* lineage predictions are compiled for all the sites (Fig. 2) results show that the first detection in epidemiological week (epi week) 33 is in an airport terminal. Then at epi week 34 the pooled aircraft sewage has a small signal which continues to be the main detection until the Ontario clinical data first reports BQ* in epi week 37 (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2022c). Surrounding municipal sites did not have a detection of BQ* until epi week 41 (York region).

BF* lineages were detected in the airport terminals in epi weeks 7 and 11 and then a few times in Peel (epi weeks 12 and 22) and once in Toronto in epi week 21 and multiple times in the airport terminals between epi weeks 21 and 24 before Ontario’s first clinical case was reported in epi week 23 (Fig. 4) (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2022a).

When XBB* lineages are analyzed as a group, detection occurred in Terminal and pooled aircraft WW during epi week 39, and then in municipal WW first in Toronto in epi week 45, though consistent detection does not begin in municipal WW until epi week 47. The first clinical detection in Ontario took place during epi week 41 (Fig. 5) (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2022c).

In addition to analyzing the XBB* lineages as a group, three specific sub-lineages of XBB were investigated: XBB.1.5*, XBB.1.16*, and XBB.1.9* as these seemed to be the most persistent. The first detection of an XBB.1.5* sub-lineage was in an airport terminal sample in epi week 39, far before the first municipal detection in a Toronto sample in epi week 47. There was another detection of XBB.1.5* in epi week 49, closely followed by the first clinical detection, also in epi week 49 (Ontario Agency for Health

Protection and Promotion (Public Health Ontario), 2023c, p. 202)). Consistent detection of XBB.1.5* in municipal and airport WW samples isn't observed until epi week 1 of 2023 (Fig. 6).

The first detections of XBB.1.16* were in pooled aircraft sewage during epi week 9, followed by detection in the airport terminals and the first clinical detection in epi week 10 (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2023a). The first detection in municipal WW follows shortly after, in epi week 11 (Fig. 7).

For XBB.1.9*, the first detections are all in the pooled aircraft sewage samples from epi weeks 2–6. The first clinical detection in Ontario doesn't take place until epi week 5, whereas the first detection in municipal WW is not until epi week 9 in Peel Region (Fig. 8) (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2023b, p. 24).

Initial detections for each lineage analyzed here are summarized in Table 1.

Table 1
Summary of first detections

SARS-CoV-2 Lineage	Epi week of first detection in WW at Toronto Pearson International Airport and surrounding municipalities					Epi week of first clinical case	Lead time
	Pooled Aircraft	Airport Terminals	Toronto municipal	Peel Region	York Region		
BQ*	2022–34	**2022–33**	2022–44	2022–42	2022–41	2022–37	4 weeks
BA.2.75*	2022–31	**2022–28**	2022–44	2022–50	2022–34	2022–29	1 week
BF*	2022–21	**2022–7 (11)**	2022–21	2022–12	2022–23	2022–23	16 weeks (12 weeks)
XBB*	**2022–39**	**2022–39**	2022–45	2022–48	2022–50	2022–41	1 week
XBB.1.5*	2023–1	**2022–39 (49)**	2022–47	2023–1	2023–1	2022–49	10 weeks (0 weeks)
XBB.1.16*	**2023–9**	2023–10	2023–11	2023–12	-	2023–10	1 week
XBB.1.9*	**2023–2**	2023–6	-	2023–9	2023–9	2023–5	3 weeks

Table summarizing first detection for each lineage in all 5 WW sample groups and in clinical. Detections in are recorded as "year – epi week number". Lead time was calculated from the first WW detection (which is bolded in each row) to the first clinical detection. If the lineage was not detected before March 30th, 2023, the corresponding cell has a single dash instead of an epi-week. In cases where the initial

detection was more than two weeks ahead if any other detection in this study, the second detection is also considered in this table and the values are placed in brackets.

Mutations of interest provide an early warning signal for lineage groups:

Over the pandemic, there were periods where it was increasingly difficult to track specific SARS-CoV-2 lineages in WW due to convergent evolution of mutations in the spike protein's receptor binding domain. During this time, some WW surveillance programs followed certain important mutations. These mutations were deemed important for a variety of reasons, but the one we chose to examine here (S:R346T) is a mutation that aids SARS-CoV-2 lineages that have this mutation to escape antibodies, including the monoclonal antibodies used for treatment of COVID-19 (Cao et al., 2023). The S:R346T mutation is present in several lineages of SARS-CoV-2 that were circulating during the period of this study. The first instance of this mutation is a very low frequency detection in a York Region sample in epi week 23, followed by several higher detections in pooled aircraft sewage and samples from both Toronto and Peel Region in epi weeks 25–26 (Fig. 9). There is no information about the first clinical detection of this mutation in Ontario.

Discussion

Toronto Pearson is the largest international airport in Canada, hosting 40 airlines that fly to more than 155 cities worldwide (*Fast Facts*, n.d.). In 2019, 50.5 million passengers travelled through the airport. There are nearly 50,000 people employed at the airport (Schwartz et al., 2022). The diversity and number of people served by Pearson make it an extremely valuable site to monitor as part of joint efforts between Public Health Agency of Canada and the Ontario WSI program.

In this study we have several examples of initial detections of SARS-CoV-2 lineages from WW samples collected from Toronto Pearson (Terminals or Pooled Aircraft Sewage) ahead of a municipal wastewater detection with a lead time ranging from one week (BA.2.75*, XBB*, XBB.1.16*) to 4 weeks (BQ*) typically and up to 16 weeks (BF* lineages) before a clinical sample is detected in Ontario. A one-week lead time was more common. The lineages and mutations followed in this study were chosen based on interest during the sampling program as identified by PHAC, Ontario Public Health Units, GTAA, or by media coverage.

Transient nature of airport WW and accuracy of first detections:

Some considerations should be made for the transient populations contributing to the airport sampling sites. Terminals provide information on both the transient population of travelers (people who may or may not be terminating their journey in Southern Ontario, but rather connecting onward to other airports nationally or internally) and the more consistent population of airport staff and associates who work in close proximity to the travelers. Pooled aircraft sewage on the other hand mostly represents the transient populations made up of travelers and mobile aircraft staff. In both cases, the transient populations cause multiple signal spikes which drop again before a new lineage gains a foothold in Ontario and we begin to

see more consistent detection of a lineage in municipal WW. Each spike could be a single case or a few cases passing through the airport, and there is no way to know where the person or persons carrying the virus will end up. The result is that each initial detection, even if isolated, should be treated as valuable data if it is going to serve as an early warning signal.

It is not typical to place confidence in single detections from WW surveillance due to the nature of the sample type, being comprised of many different individual viral particles and with each particle contributing a partially degraded genome, necessitating frequency prediction tools to 'detangle' the sample into individual lineages and there are limits to the accuracy of such tools. For example, a benchmarking publication which included Alcov concluded that calls at frequencies less than 5% should be interpreted with caution due to high background noise in WW samples (Sutcliffe et al., 2023). As a result, in municipal WW surveillance patterns and trends in data especially in slowly emerging lineages lend confidence to the predictions. Initial detections in this study are predicted at 5% or higher (apart from BA.2.75), but there are other factors that can inform lower confidence in single-point signal spikes, such as the possibility that diagnostic mutations were missed. For this reason, in the cases (BF*, XBB.1.5*) where the initial detection was greater than 2 weeks ahead of any other detection considered in this study (other WW collected from the sites included in this analysis or Ontario clinical) the second detections should be considered as well when calculating lead time gained (bracketed values in Table 1). If WW sequencing in airports were to be used to inform pathogen impact on public health and inform policy changes in future pandemics, recommendations from data collectors to the recipients of the data would have to be well balanced to account for differences between single and multiple detections with corresponding interpretations weighted accordingly.

It is valuable as well to be able to track the national entry of pathogens and their emergence in the community through airport coupled with municipal WW surveillance amidst limited clinical testing to inform how detections at entry points translate to transmission in the surrounding regions. The long timespan between terminal and municipal WW detections in some instances (e.g. BQ*, Fig. 2) suggests that in some cases variants may not have been quick to spread after initial introduction to the local population. The transmissibility and infection rates of Sars-CoV-2 differ due to many factors (i.e. by variant and by age demographics and vaccination rates of individuals), and not all viral detections directly translate to continued transmission (Koelle et al., 2022). After initial detection of emerging lineages, the signal in Toronto Pearson WW generally continues to fluctuate, with higher peaks in frequency than the municipal WW from the surrounding regions. Toronto Pearson WW then would not be useful to monitor subsequent local trends in VOC dominance past the initial community introduction, which is the key role of municipal WW testing in SARS-CoV-2 surveillance.

Unique mutations influence the ability to accurately call specific sub-lineages

XBB lineages provide a showcase of how frequency predictions using WW sequencing are affected by availability of unique mutations, despite the ability of prediction tools including Alcov to use shared mutations as well to make lineage predictions. XBB, as a recombinant of BA.2.10.1 and BA.2.75 sub-

lineages (BJ.1 and BM.1.1.1 respectively), has a distinct mutation constellation compared to BQ* and BF* lineages, which are descendant from BA.5 and other BA.5 sub-lineages which were in circulation around the time of its emergence in Ontario (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2022c, p. 21; *TAG-VE Statement on Omicron Sublineages BQ.1 and XBB*, n.d.). Despite high transmissibility and associated rapid spread, XBB* was detected in Toronto Pearson WW a week ahead of the first clinical case (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2023d). XBB.1.5 has only three nucleotide mutations (C44T, T17124C, T23018C) differentiating it from XBB.1, with only one of these resulting in a coding change (T23018C yields S:F486P), making it less distinct for picking out of a mixed sequencing sample (*covSPECTRUM*, n.d.). WW data collected from Toronto Pearson was unable to provide more than one early detection compared to clinical sequencing in the case of XBB.1.5 likely due to our inability to confidently predict it when present in samples at low abundance as it is difficult to differentiate from other XBB* signal. XBB.1.5* was also prevalent in the United States of America before emergence in Ontario so it is likely that early transmission occurred across the Canada-USA border land crossings, which would affect lead time (Parums, 2023). XBB.1.16* has 12 nucleotide changes (C11750T, C11956T, T12730A, A14856G, G18703T, A22101T, C22995G, T23018C, G27915T, T28297C, A28447G, C29386T) compared to XBB.1, and XBB.1.9* has five nucleotide changes (G5720A, C12789T, T23018C, G27915T, T28297C) compared to XBB.1 which makes these two groups of XBB sub-lineages slightly easier to differentiate and thus detect in WW (Table 1) (*covSPECTRUM*, n.d.). In addition to the differences in number of unique mutations, viral shedding across lineages/sublineages may not be the same (Prasek et al., 2023). For example, one lineage might be shed for a longer period of time than other lineages. This in turn might affect the proportion and amplification of new variants in wastewater (Prasek et al., 2023).

Terminal and Aircraft Sewage are Most Valuable When Used Together:

We would expect initial detection of emerging lineages which originate outside of Canada to be in the pooled aircraft sewage more often due to the contributing populations being more transient, but in this study emerging VOCs were more frequently detected in terminal WW first. The pooled aircraft sewage was collected using passive samplers from January until October 21, 2022, and many of the VOCs in this study emerged before this time period. While passive samplers were being used, the coverage for pooled aircraft sewage samples was not ideal (median BOC = 48), which could be contributing to this (Lawal & Goodridge, in preparation). The later detection of many lineages by pooled aircraft WW is also confounded by a possible reluctance of passengers to use the airplane lavatory, in favor of waiting until landing at the airport, or that the lavatories may not be needed on shorter flights (Jones et al., 2023). After October 21st 2022 when an autosampler was used for pooled aircraft sewage as well, Breadth of Coverage greater than a depth of 5 (BOC) (median BOC = 93) was on average higher than in Terminals (median BOC T1 = 79 and T3 = 81) likely due to the triturator sewage being less dilute. In the examples of two XBB sub-lineages, XBB.1.9 and XBB.1.16 (Figs. 7 and 8), which emerged in Ontario after the switch in collection method, the pooled aircraft sewage samples have earlier signals and in the case of XBB.1.16 provide the only early detection. On the other hand, in the example of the S:R346T mutation, pooled aircraft sewage had an earlier detection even though this was during the period when passive samplers

were in use at the triturator. Whether the differences in performance between the two sample types are due to the timing of sampling method change or dilution of the sample by local staff members, both sample types will be valuable to collect in future pandemic preparedness surveillance programs as they provide insight into different population types.

Target selection as a limitation:

Pandemic preparedness through WW surveillance at airports will also require knowledge of a potential threatening virus prior to its arrival in Canada. In the current study, tiled amplicons designed specifically to capture only SARS-CoV-2 are used to sequence the SARS-CoV-2 particles found in WW. This strategy works well for catching emerging VOCs because once the amplicons have been sequenced, the assembled genomes can be re-analyzed looking for new lineages retrospectively as new VOCs become concerning in other parts of the world. Even so, this strategy cannot identify new lineages. New lineage prediction could be accomplished using bioinformatics approaches such as non-negative matrix factorization (NMF) in the future (Ellmen et al., in preparation).

If the surveillance is extended to other viral targets, sequencing strategies will have to be developed for each different target ahead of their potential spread into Canada for the data to be available early enough to serve as an early warning for public health decision making. This could mean developing and implementing more tiled assays for virus families that are an ongoing concern (RSV, Influenza) or those that have caused outbreaks in recent history i.e. Mpox (Maloney et al., 2022; Nash et al., in preparation). Alternatively, deep shotgun metagenomic sequencing could be performed to collect data without a target for detection of potentially novel viral threats. It should be noted that shotgun sequencing of viral RNA is resource intensive as viral reads are often less abundant than bacterial reads, requiring a large amount of sequencing space and/or rigorous pre-selection or rRNA depletion to capture the viral community in detail (Nash et al., 2023; Pérez-Losada et al., 2020).

Turn-around time to reporting as a limitation:

During data collection for this project, results of sequencing for airport sampling sites were reported to airport personnel, MECP, and PHAC on a bi-weekly basis but not to surrounding municipalities due to privacy agreements while piloting the Toronto Pearson WW sampling program. It is important to note the turn-around time of sequencing from sample-receipt to reporting is 1–2 weeks. This means that in the above examples if data were available to municipalities or in a centralized hub, the lead time to react to a first detection with policy decisions in preparation for a new VOC import after notification by the data collectors would be less than the raw lead times given in the table which are based on the sample collection dates. In the case of detections that occurred only a week prior to the clinical detection, the notification would not precede testing of infected individuals, but the data would at least be available to the public health unit that the VOC is present earlier than clinical reports.

Once a clinical case has occurred in a region which will be reported as a given lineage, the region will still have to wait 1–4 weeks before sequencing data from the clinical sampling for that case is made

available to the PHUs (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2023b, 2023a). After a variant enters a community/region, it is valuable information for the PHUs to be made aware of, so the reporting turnaround times of WW surveillance in general are valuable while PHUs await clinical testing results. Airport WW surveillance reports would be an even earlier datapoint to inform PHUs of emerging lineages even if the lead time to the variant's detection in the region by clinical testing is not very long.

Conclusions

WW collection at Toronto Pearson provides an example of the early warning capabilities of WW surveillance at points of entry into Canada. In this study a lead time of 1–4 weeks was typically observed between the first detection of an emerging SARS-CoV-2 lineage in WW collected from one of three sampling locations at Toronto Pearson and the first Ontario clinical detection of the same lineage. This was always a longer lead time than between municipal WW and clinical detections. WW surveillance at points of entry such as Toronto Pearson could provide an early warning signal for pathogen entry into surrounding regions, contingent on turnaround time to reporting. This study demonstrates early detection by amplicon sequencing of SARS-CoV-2, but similar strategies could be used to set up surveillance programs for other viral pathogen targets. WW surveillance at points of entry such as airports should be considered when designing programs for pandemic preparedness.

Declarations

Competing Interests

Trevor Charles is the founder and a shareholder of the company Metagenom Bio Life Science Inc., which provides genomic sequencing services to academic and industry clients, including for pathogen surveillance in wastewater and food production systems. Brittany Maxwell, Steven Thomas and Marcos Zambrano are employees of the Greater Toronto Airports Authority which is the operator of Toronto Pearson Airport.

Author Contribution

AKO wrote most of the written sections, coordinated partner inclusion, and supervised the WW data collection at University of Waterloo. JJK transformed the data to create all figures and figure legends and provided writing and in-depth revisions to the manuscript. OUL and RG led WW data collection at University of Guelph and Western respectively. OUL and AIA provided Materials and Methods sections for their respective Universities' WW data. BM, LC, CB, AQ, KA, MP, RS, MDF, NCK, and AAF were all part of an in-depth revisions process to ensure that we are using the expertise of our partner organizations. AKO, RG, AIA, YC, SRP, HH, SOA, SK, SN, VRP, FR, and MJP performed in-lab experiments to collect the data at one

of the three Universities involved in the project. BM, LC, CB, AQ, KA, MP, RS, ST, MZ, VF, EG, MV, and MH are all responsible for the coordination of collection and availability of samples from their affiliation (Pearson Airport or Municipality). CL, EJA, LG and TCC are principal investigators of the academic labs involved in this study. They have provided supervision and guidance for the data collection and have contributed to the manuscript during revisions. All authors have read the manuscript and agree to publish.

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Data Availability

The raw sequencing data can be viewed on the Sequence Read Archive (SRA) under bio project PRJNA1088471.

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Figures

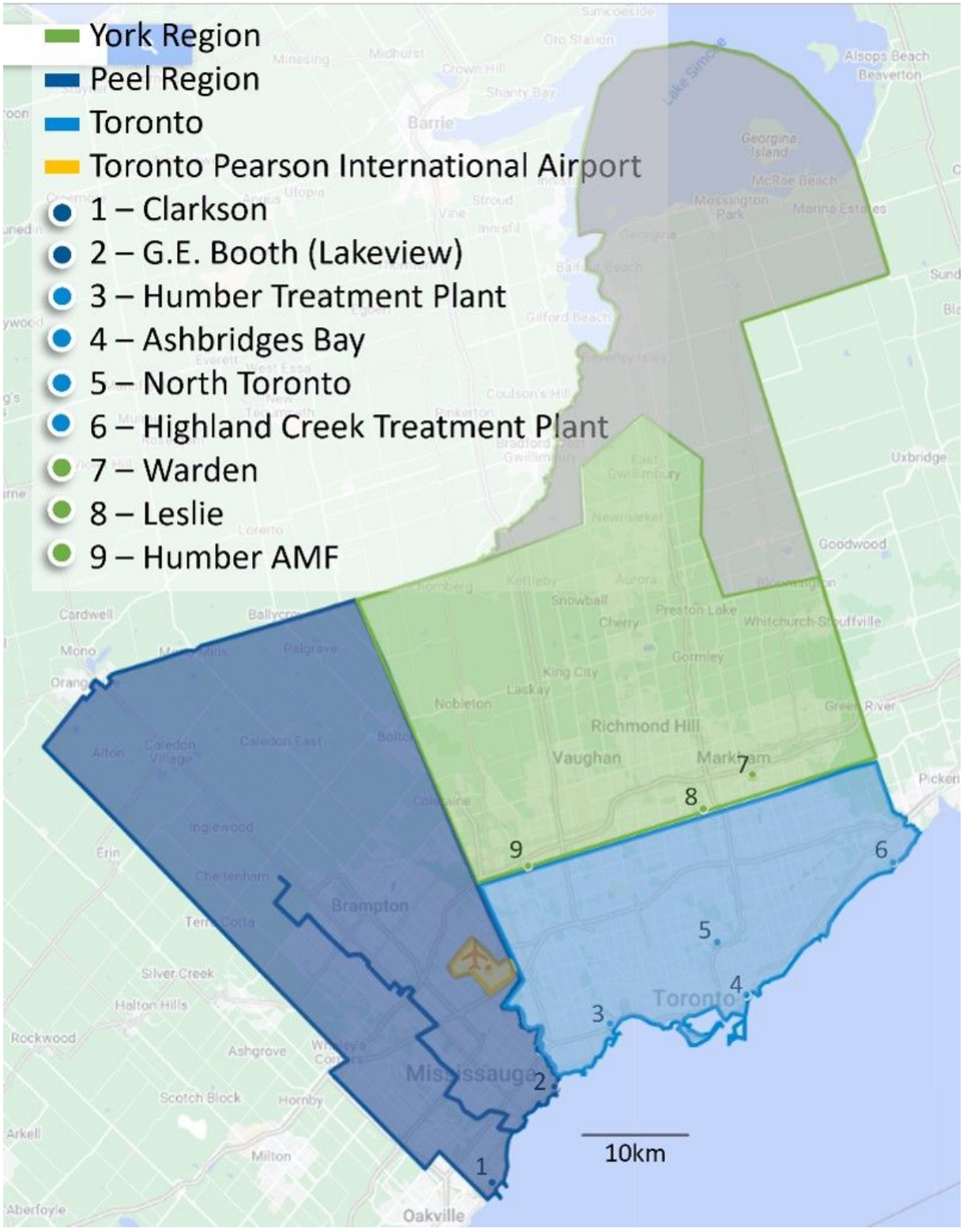

Figure 1

Map of sewersheds and sampling sites in the regions surrounding Pearson Airport.

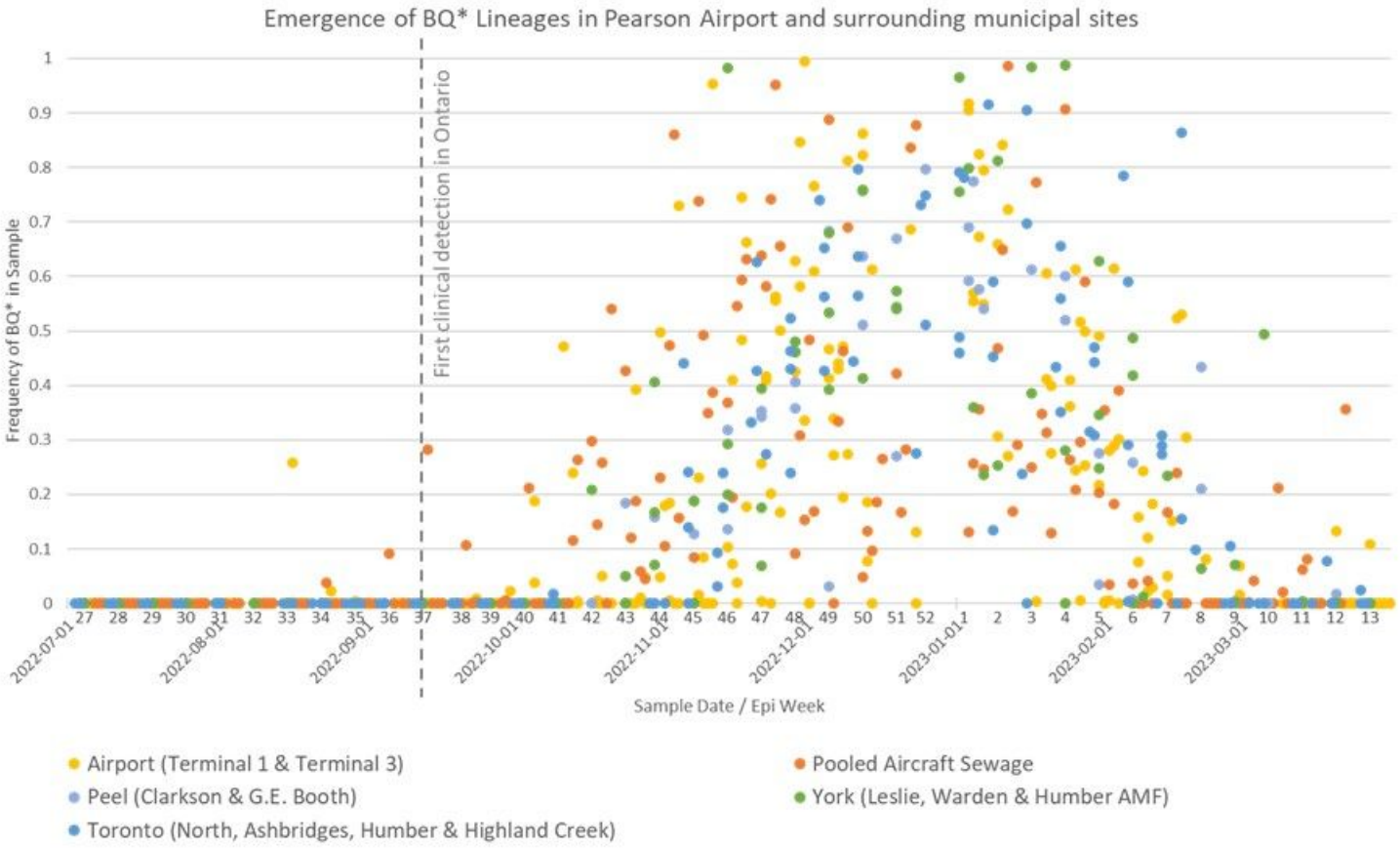

Figure 2

Frequency of BQ* lineages in WW samples from Toronto Pearson and surrounding municipal sites in Toronto, York, and Peel regions. Each point represents a single WW sample. Grey dashed line represents the date of the first clinical sequence for BQ* in Ontario.

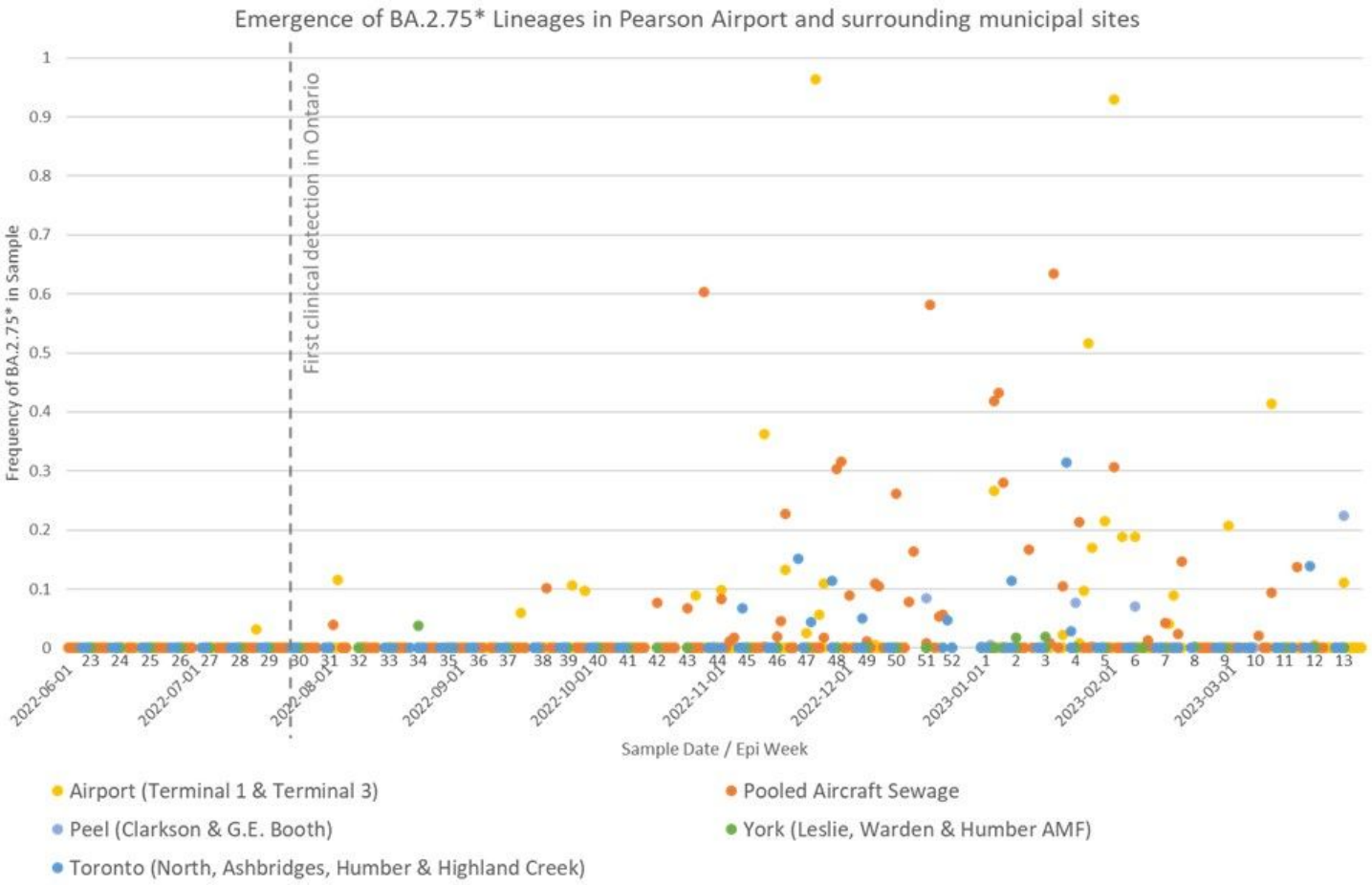

Figure 3

Frequency of BA.2.75* lineages in WW samples from Toronto Pearson and surrounding municipal sites in Toronto, York, and Peel regions. Each point represents a single WW sample. Grey dashed line represents the date of the first clinical sequence for BA.2.75* in Ontario.

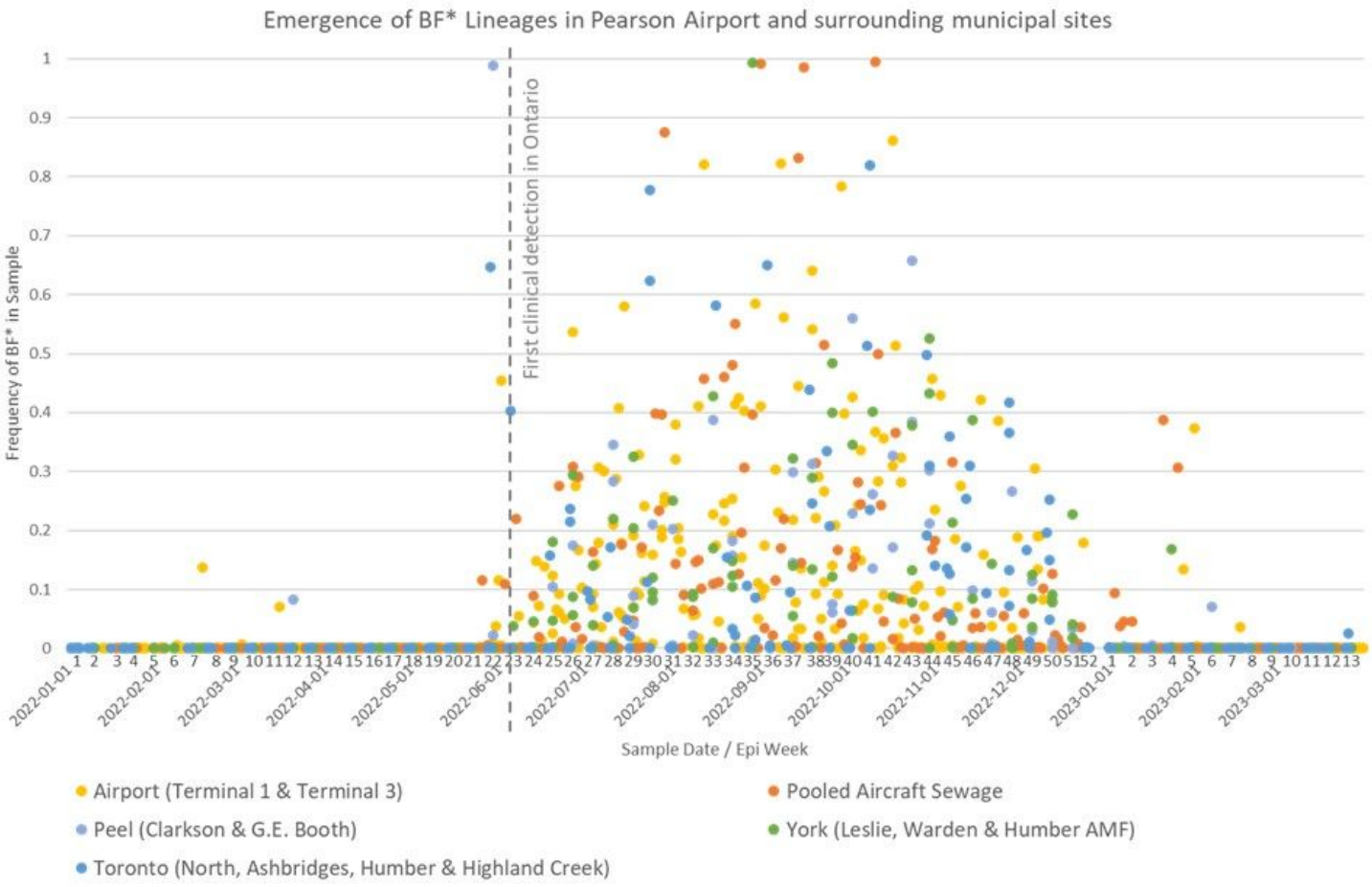

Figure 4

Frequency of BF* lineages in WW samples from Toronto Pearson and surrounding municipal sites in Toronto, York, and Peel regions. Each point represents a single WW sample. Grey dashed line represents the date of the first clinical sequence of BF* in Ontario.

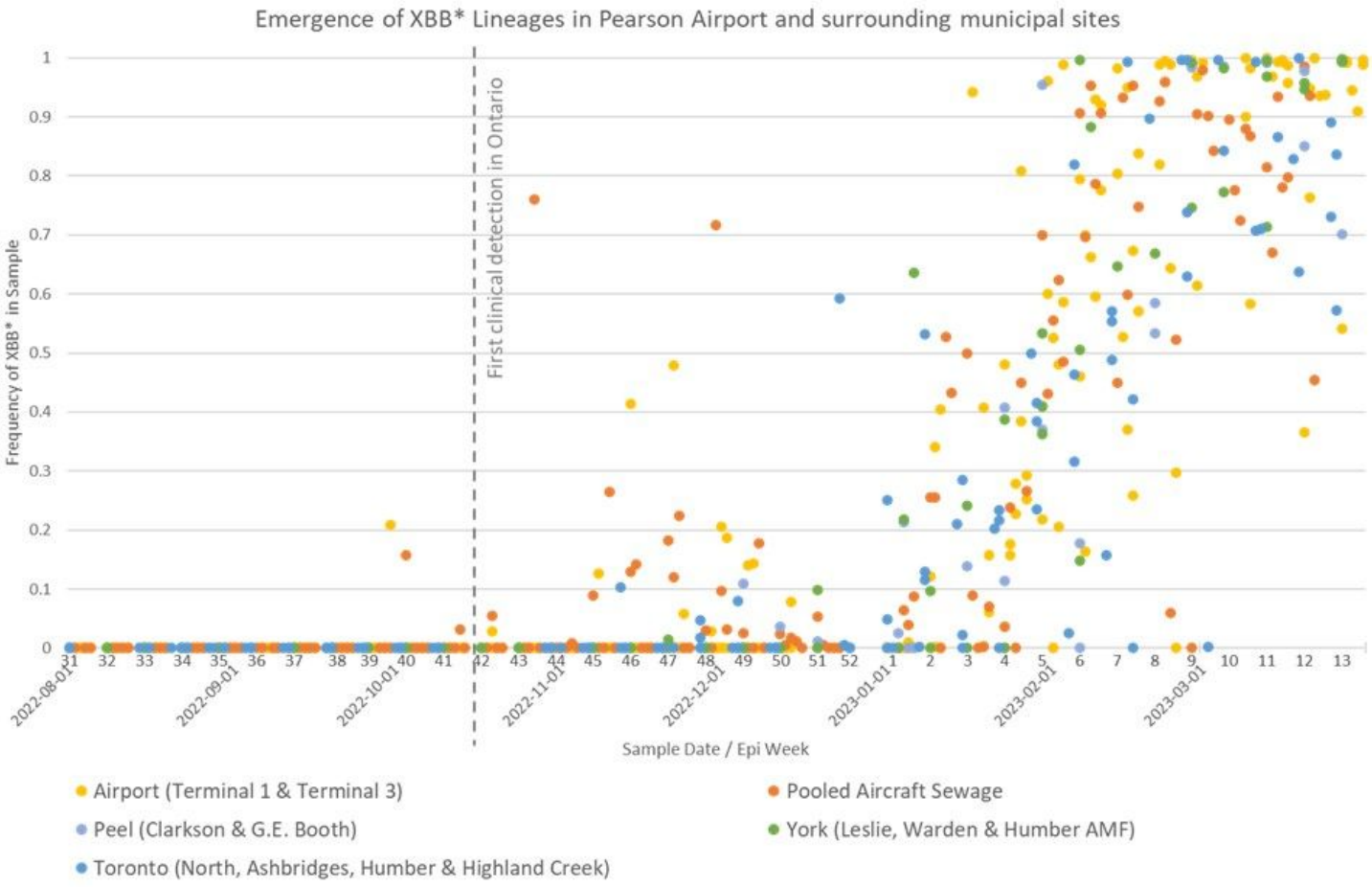

Figure 5

Frequency of XBB* lineages in WW samples from Toronto Pearson and surrounding municipal sites in Toronto, York, and Peel regions. Each point represents a single WW sample. Grey dashed line represents the date of the first clinical sequence of XBB in Ontario.

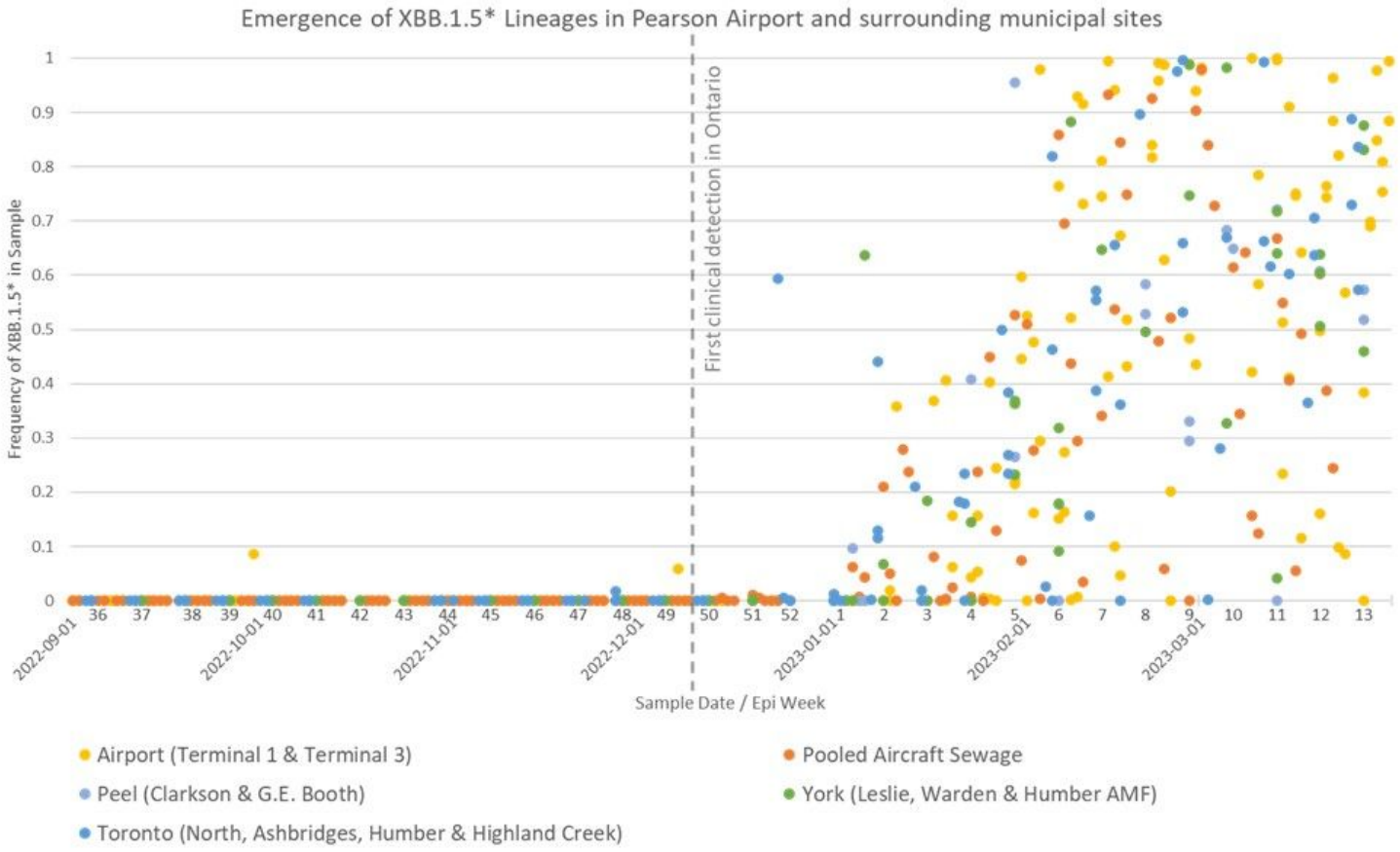

Figure 6

Frequency of XBB.1.5* lineages in WW samples from Toronto Pearson and surrounding municipal sites in Toronto, York, and Peel regions. Each point represents a single WW sample. Grey dashed line represents the date of the first clinical sequence of XBB.1.5* in Ontario.

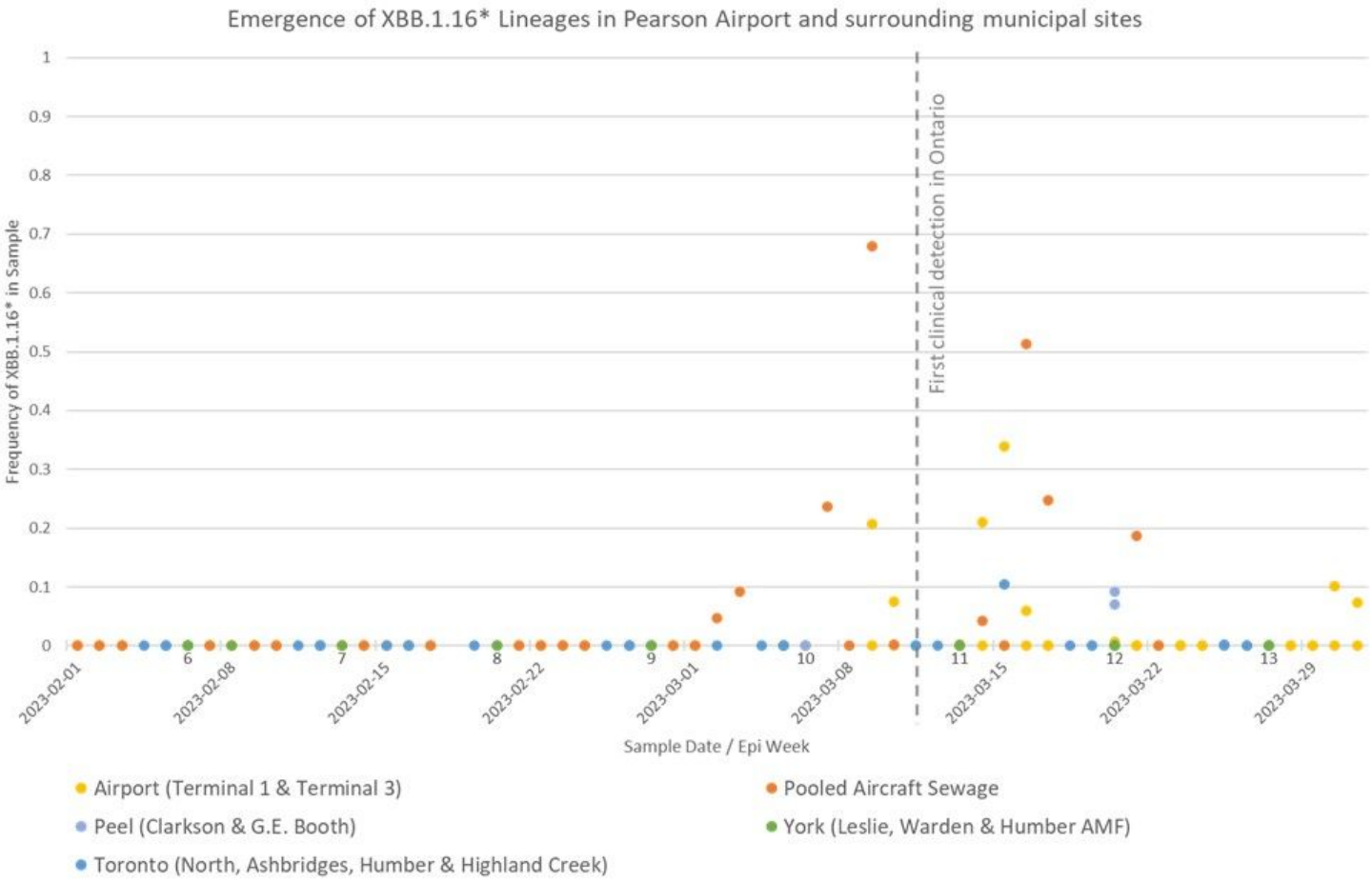

Figure 7

Frequency of XBB.1.16* lineages in WW samples from Toronto Pearson and surrounding municipal sites in Toronto, York, and Peel regions. Each point represents a single WW sample. Grey dashed line represents the date of the first clinical sequence of XBB.1.16* in Ontario.

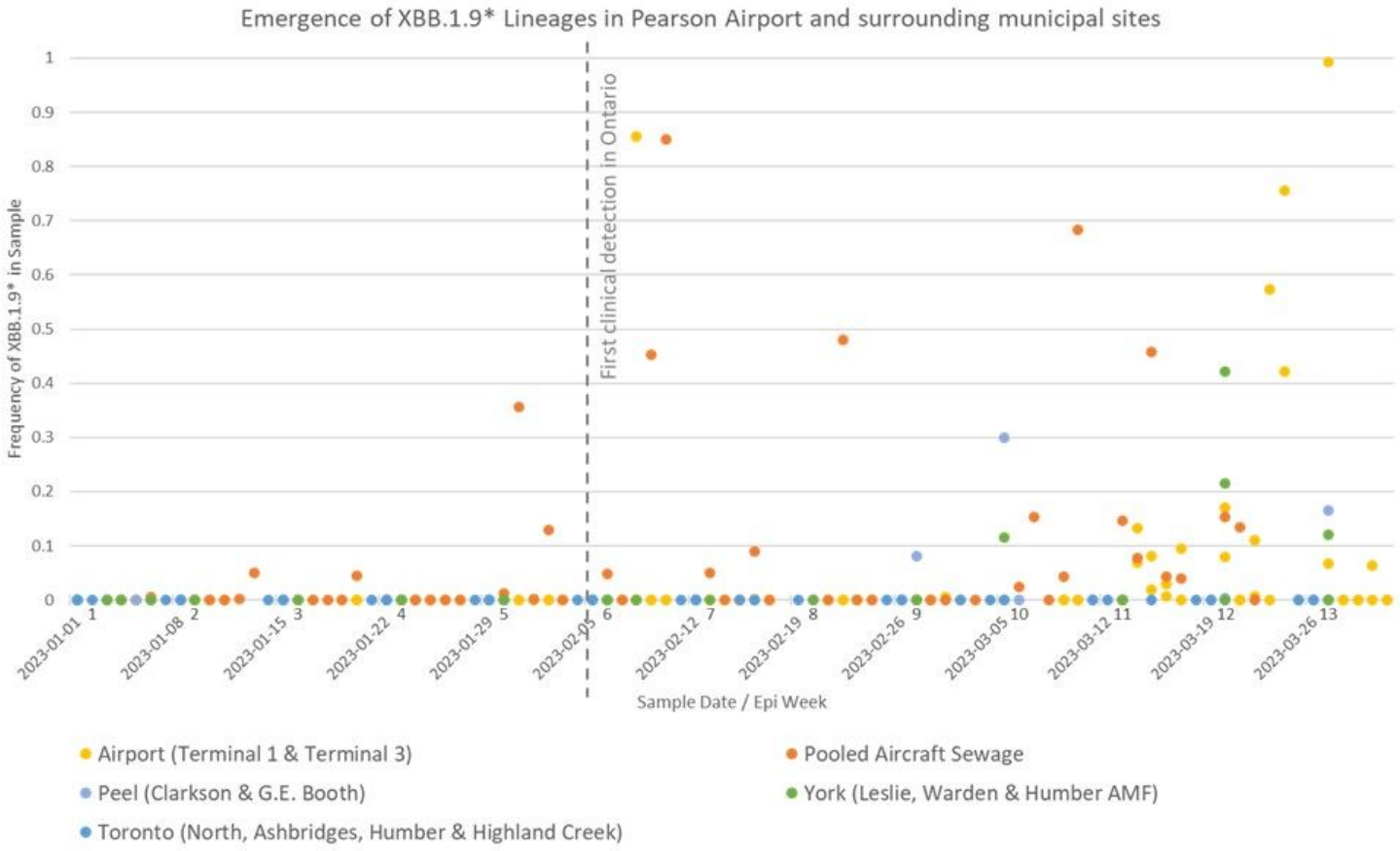

Figure 8

Frequency of XBB.1.9* lineages in WW samples from Toronto Pearson and surrounding municipal sites in Toronto, York, and Peel regions. Each point represents a single WW sample. Grey dashed line represents the date of the first clinical sequence of XBB.1.9* in Ontario.

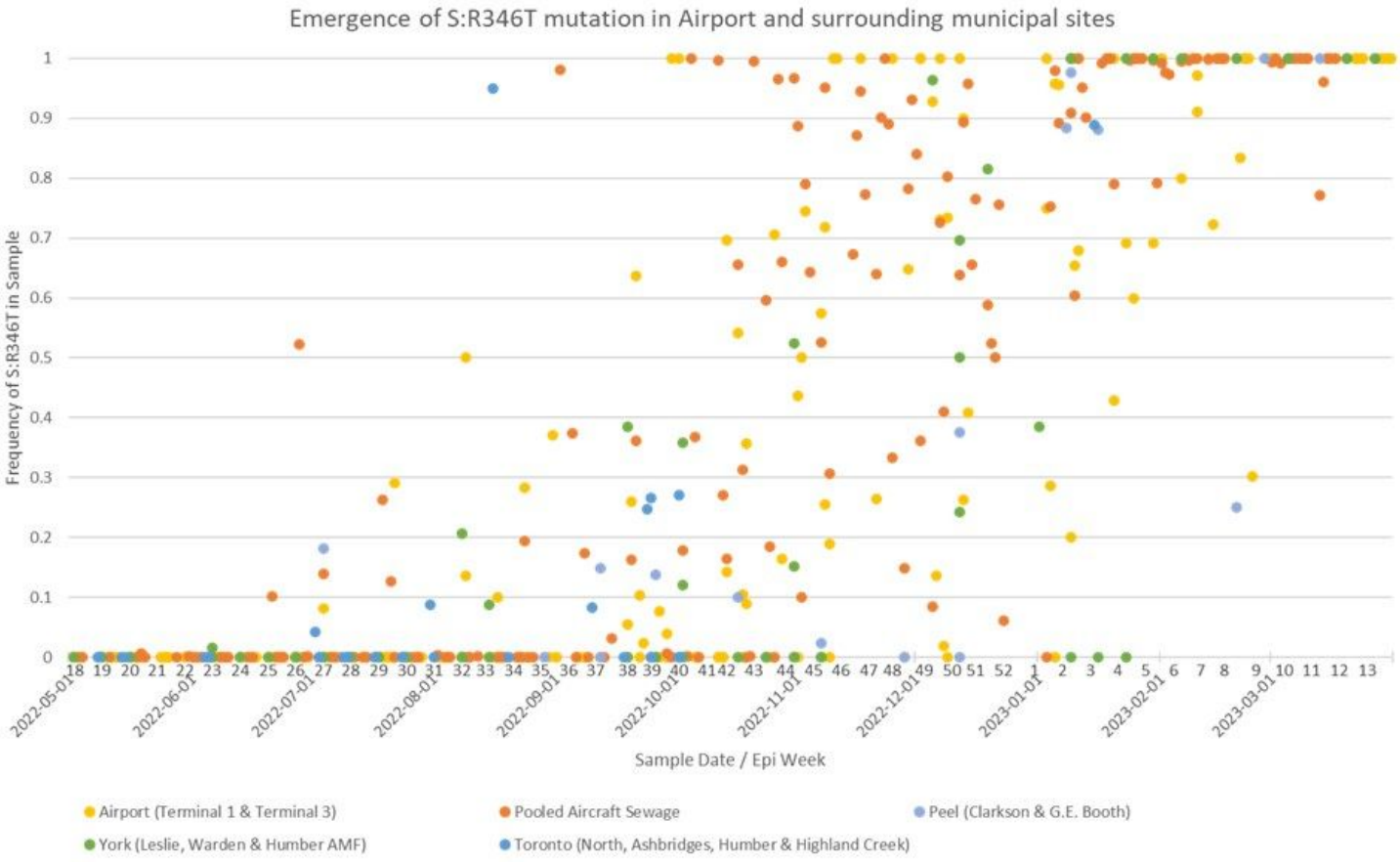

Figure 9

Frequency of the alert mutation S:R346T in WW samples from Toronto Pearson and surrounding municipal sites in Toronto, York, and Peel regions. Each point represents a single WW sample.