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Coxsackieviruses in Ontario, January 2005 to December 2011☆

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SUMMARY

Background: In 2010, there was an increase in enterovirus meningitis in the province of Ontario, Canada. Concurrently, there was also an increase in coxsackievirus A9-positive specimens in Alberta, Canada. This study aimed to describe the results of an investigation into the increase in coxsackievirus (A9 serotype) in 2010 in Ontario.

Methods: For the purpose of this study, we report on specimens tested by viral culture at Public Health Ontario Laboratory as part of routine laboratory testing from January 1, 2005 to December 31, 2011.

Results: Coxsackieviruses represented more than one third of enteroviruses detected, with A9 being the serotype most commonly identified. The most common specimen source in which A9 was isolated was cerebrospinal fluid, followed by nasopharyngeal swabs and stool. Patients in whom A9 was detected were older than individuals with any other coxsackievirus serotype.

Conclusions: The increase in enterovirus meningitis in Ontario in 2010 was likely due to an increase in A9 circulation. A9 was most commonly identified among children; however A9 may cause severe illness in both children and adults. Monitoring the circulation and epidemiology of enteroviruses can inform clinicians about circulating pathogens to optimize clinical testing and antibiotic use.

1. Introduction

Human enteroviruses can cause a wide range of disease severity, with febrile illness, irritability, lethargy, and hand, foot and mouth disease (HFMD) being the most common clinical presentations.[1,2] Due to the mild nature of these symptoms, disease incidence is often underestimated.[1] Severe clinical manifestations include aseptic meningitis, encephalitis, sepsis, hepatitis, and myocarditis.[3] The proliferation of enteroviruses occurs primarily in the respiratory and gastrointestinal tracts. Later, viremia may occur, resulting in spread to other body targets.[4] Fecal–oral spread represents the main transmission pathway of enteroviruses, although they can also be spread by respiratory routes.[3] Shedding in stool may last for up to 11 weeks, while shedding through the respiratory tract often lasts for 1 week or less.[5] Individuals of all ages are susceptible to enterovirus infection, however children and the immunocompromised are most vulnerable. These populations tend to shed the virus for longer periods, hence may play an important role in disease transmission.[1,6]

Coxsackieviruses are members of the *Enterovirus* genus in the family *Picornaviridae*, which have been categorized into 23 group A serotypes (A1–A22, A24) and six group B serotypes (B1–B6).[7,8] Most of the epidemiologic and clinical characteristics of coxsackieviruses are similar to other enteroviruses.[9] Coxsackievirus A9 (A9) has been associated with various illnesses, with generalized febrile exanthem being the most common.[10] A9 is the most commonly identified enterovirus associated with central nervous system infections.[8,11] Similar to other enteroviruses, A9 demonstrates seasonal circulation patterns, with a peak incidence occurring in the summer months in temperate climates.[1,10] A9 has been identified in sporadic disease occurrence as well as community outbreaks.[12–14] The incidence of A9 has been reported to be highest in infants and young children.[3,9,10]

In September 2010, an increase in the number of A9-positive specimens was reported by the Provincial Laboratory in Alberta, Canada.[15] During that timeframe, staff at Public Health Ontario were notified that three of Ontario's 36 health units were experiencing an increase in the number of cases of enterovirus

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meningitis and aseptic, presumed viral meningitis. An investigation was launched at Public Health Ontario Laboratory (PHOL) to determine if similar trends were being detected at our provincial public health laboratories and we discovered an increased number of A9 detections. The purpose of this study was to describe the results of the investigation to determine if the increase was associated with atypical findings.

2. Methods

For the purpose of this study, we report on specimens tested by viral culture at PHOL as part of routine laboratory testing from January 1, 2005 to December 31, 2011. Viruses were isolated from various specimen types including nasal swabs, nasopharyngeal (NP) swabs, throat swabs, cerebrospinal fluid (CSF), auger suction (pharyngeal aspirates), endotracheal aspirates, stool, skin swabs, urine, and autopsy tissues. Primary screening for enteroviruses in viral culture was based on the appearance of an enterovirus-like cytopathic effect (CPE). Virus-infected cell cultures showing CPE were confirmed to contain enterovirus and serotyped using an indirect immunofluoresence assay (IFA). Rhesus monkey and WI-38 cell lines were used for respiratory specimens and African green monkey and MRC-5 cell lines for other specimen types. At PHOL, reagents were available to identify coxsackieviruses A9, A16, B1, B2, B3, B4, B5, and B6; echoviruses 4, 6, 9, 11, and 30; polioviruses 1, 2, and 3; and enteroviruses 70 and 71 (Light Diagnostics; Millipore Bioscience Division, Temecula, CA, USA). Untypeable isolates were then repassaged into fresh culture and examined by electron microscopy (EM). If after the second passage the isolate remained untypeable but was identified by EM as enterovirus, the result was reported at the genus level as enterovirus for non-respiratory specimens, and for respiratory specimens the result was reported as enterovirus-like. A selection of culture-negative CSF specimens from 2009 and 2010 were further evaluated by enterovirus real-time reverse transcriptase PCR (rRT-PCR) with primers targeting the 5′-untranslated region (5′-UTR). All enterovirus PCR-positive specimens were molecularly typed by nested RT-PCR targeting the viral capsid protein VP1 region and Sanger sequencing.

2.1. Statistical analysis

Two different laboratory information systems were in place at PHOL during the study period. Each viral culture database was first cleaned and then merged based on common variables including patient demographics, local health unit of residence, specimen source, test result, and date the specimen was received at PHOL. Only positive specimens were analyzed to describe seasonal trends and specimen sources, and patient-level analyses were performed to describe demographics and geographic distribution, counting only one positive specimen per patient per year.

Statistical analyses were performed using Stata/SE version 10.0 (StataCorp LP, College Station, TX, USA). The Chi-square or Fisher's exact test was used (the latter in the event of a small sample size) to determine the association between detected coxsackieviruses and month and year of detection, specimen source, patient age, sex, and health unit of patient residence.

Additional descriptive analysis and a mixed logistic regression model were performed on all patients who had at least one coxsackievirus detected and multiple specimens submitted, regardless of specimen result and episode for which the specimen was submitted, to compare the likelihood of specimens from various sources yielding a positive coxsackievirus result. Odds ratios (OR) with 95% confidence intervals (95% CI) were reported. Using this model, any subsequent specimen submitted within a 90-day period from first specimen submission was counted as part of the first episode. If the second or subsequent specimens were submitted more than 90 days from the previous specimen, this was considered as a separate episode. A lag period was calculated as the difference in days between the first and subsequently submitted specimen(s).

3. Results

3.1. Specimen information: yearly and seasonal trends

From January 1, 2005 to December 31, 2011, 941 (0.5%) specimens tested positive for enterovirus at PHOL, of which 334 (35.5%) specimens were coxsackieviruses (A9, A16, B1, B2, B3, B4, and B5) (Table 1). A9 was the most frequently identified serotype, identified in 103 (10.9%) of all enterovirus-positive specimens. In addition, echoviruses (4, 6, 9, 11, and 30) were identified in 132 (14%) of all enterovirus-positive specimens, with echovirus 6 the predominant serotype identified in 50 (5.3%) of all enterovirus-positive specimens. Enterovirus 71 was detected in 9 (0.9%) enterovirus-positive specimens and the remaining 466 (49.5%) enterovirus-positive specimens were not typeable and reported as enterovirus-like or enterovirus.

The distribution of coxsackievirus serotypes varied by calendar year ($p < 0.001$). Seventy-nine percent of A9-positive specimens were identified during 2007 and 2010; few A9 were identified in

Table 1
Enterovirus serotypes detected in viral culture by year the specimen was received at PHOL; January 1, 2005 to December 31, 2011, Ontario, Canada

Enterovirus	2005	2006	2007	2008	2009	2010	2011	Total serotypes
Coxsackievirus A9	2	3	21	3	8	60	6	103
Coxsackievirus A16	0	1	0	1	5	10	0	17
Coxsackievirus B1	0	1	15	2	14	3	4	40
Coxsackievirus B2	5	5	4	2	29	0	2	47
Coxsackievirus B3	4	6	0	1	3	0	3	17
Coxsackievirus B4	5	2	12	5	13	5	17	59
Coxsackievirus B5	24	1	3	2	5	7	9	51
Echovirus 4	0	0	0	3	2	1	4	10
Echovirus 6	5	4	2	4	0	31	4	50
Echovirus 9	0	8	4	1	25	7	2	47
Echovirus 11	0	2	0	2	4	0	2	10
Echovirus 30	11	0	0	1	3	0	0	15
Enterovirus	27	22	18	9	12	11	9	108
Enterovirus 71	0	2	2	0	1	2	2	9
Enterovirus-like	70	37	32	47	32	98	42	358
Total enteroviruses/year	153	94	113	83	156	235	106	941
Total specimens tested/year	18 807	19 137	19 145	26 163	47 820	21 621	26 843	179 536

PHOL, Public Health Ontario Laboratory.

Table 2
Percent positivity of all enteroviruses detected in cerebrospinal fluid specimens; January 1, 2005 to December 31, 2011, Ontario, Canada

Cerebrospinal fluid	2005	2006	2007	2008	2009	2010	2011	Total
Enterovirus-positive specimens	10	5	10	0	8	24	9	66
Total specimens tested	1347	1298	1202	1165	1087	995	990	8084
Percent positive	0.7	0.4	0.8	0.0	0.7	2.4	0.9	0.8

other years. Specifically, the number of A9-positive specimens in 2007 (21 specimens) and 2010 (60 specimens) were 4.8 times and 13.6 times, respectively, as high as the average number of A9-positive specimens for other years (average 4.4 specimens, range 2–8). The increase in coxsackievirus A9 in 2010 also corresponded with a four-fold increase in the detection of all enteroviruses in CSF (2.4%) by viral culture compared to the average detection during the rest of the period (0.6%) (Table 2). Testing of a selection of culture-negative CSF specimens by molecular methods identified 3/148 (2%) culture-negative specimens positive for enterovirus in 2009 and 19/245 (7.7%) culture-negative specimens in 2010. Molecular typing by VP1 sequencing was performed on 13 of the enterovirus-positive CSF samples and confirmed A9 in 10 specimens and echovirus 6 in two; one could not be typed. Molecular testing was not performed on any specimens from other study years.

Other coxsackievirus serotypes and the year in which they peaked included B5 (primarily identified in 2005), B2 (2009), and B4 (2011). Similar to other enteroviruses, the frequency of coxsackievirus detection was highest in the summer and fall months; this was true for all years ($p < 0.05$) (Figure 1). The incidence of A9 detection peaked in August–September, B2 in September, B1 and B5 in August, and B4 in November, for all years. While the identification of A16 peaked in July and B3 in November, counts for these two serotypes were low ($n < 20$), hence clear seasonal patterns could not be discerned.

Of all coxsackieviruses detected, 119/334 (36%) were isolated in NP swabs, 109/334 (33%) in stool, and 66/334 (20%) in CSF. The remaining viruses were detected in throat swabs, mouth swabs, skin scrapings, auger suction, nasal swabs, and urine (Figure 2). Specific coxsackievirus serotypes were more likely to be detected in certain specimen sources; this association was statistically significant ($p < 0.001$). The most common specimen source in which A9 was isolated was CSF (36/103; 35%), followed by NP swabs (29/103; 28%) and stool (25/103; 24%) (Figure 2). Stool was the most common specimen source in which B3 and B5 were identified, representing 8/17 (47%) and 27/51 (53%) of B1 and B3 isolates, respectively. The NP swab was the most common specimen source in which B1, B2, and B4 were isolated with 19/40 (48%), 21/47 (45%), and 27/59 (46%) of B1, B2, and B4 isolates, respectively, identified in NP swabs. A16 was mostly identified in NP swabs and skin scrapings, representing the specimen source in 4/17 (24%) A16-positive specimens each.

3.2. Patient information: demographics

Between January 1, 2005 and December 31, 2011, 310 patients tested positive for any coxsackievirus. The mean and median age of patients in whom a coxsackievirus was detected ($n = 299$) was 7.6 and 2 years, respectively, with a range of 10 days to 80 years. Coxsackieviruses were most frequently detected in children; 201/310 (65%) patients were less than 4 years old and 93/310 (30%) were less than 12 months old. Ninety-four (30%) patients tested positive for the A9 serotype. A9 was the most common serotype detected across all age groups except for those aged 1–4 years, for whom B4 was the most common serotype (Figure 3). Patients in whom A9 was detected were older than individuals with any other coxsackievirus serotype identified; mean and median age was 12.3 and 4 years, respectively ($p < 0.01$). The age of patients in whom A9 was detected in 2010 was not different compared to those who had A9 isolated in any other year ($p > 0.05$).

Of the 290 coxsackievirus-positive patients for whom sex was reported, 174 (60%) were male and 116 (40%) were female; this did not achieve statistical significance. A similar sex distribution was observed among patients in whom A9 was detected; 57/87 (66%) were males and 30/87 (34%) were females.

More than half of the patients (153/310) in whom any coxsackievirus was identified were from Toronto (78/310; 25%), Peel (28/310; 9%), Halton (27/310; 9%), and Niagara (20/310; 7%) health units; however this was congruent with their respective proportionate populations in Ontario. Other patients were randomly distributed across Ontario. A similar distribution in health unit of residence was observed among A9 patients during all years, as well as during the increase in 2010 ($p > 0.05$).

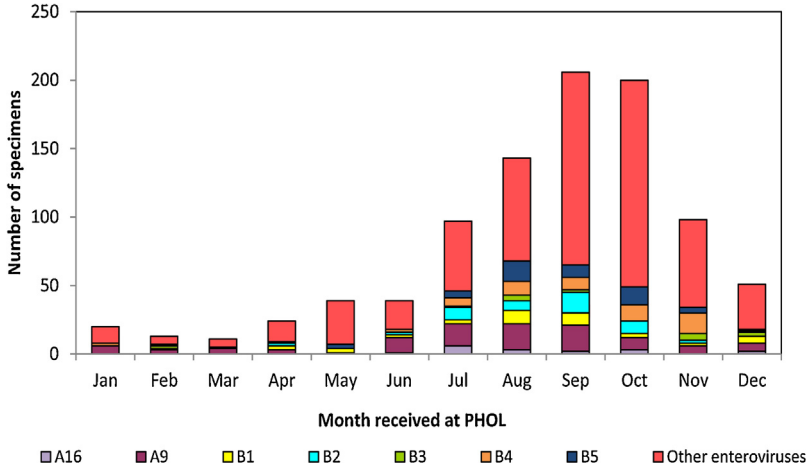

Figure 1. Coxsackievirus serotypes and other enteroviruses detected by month the specimen was received; January 1, 2005 to December 31, 2011, Ontario, Canada.

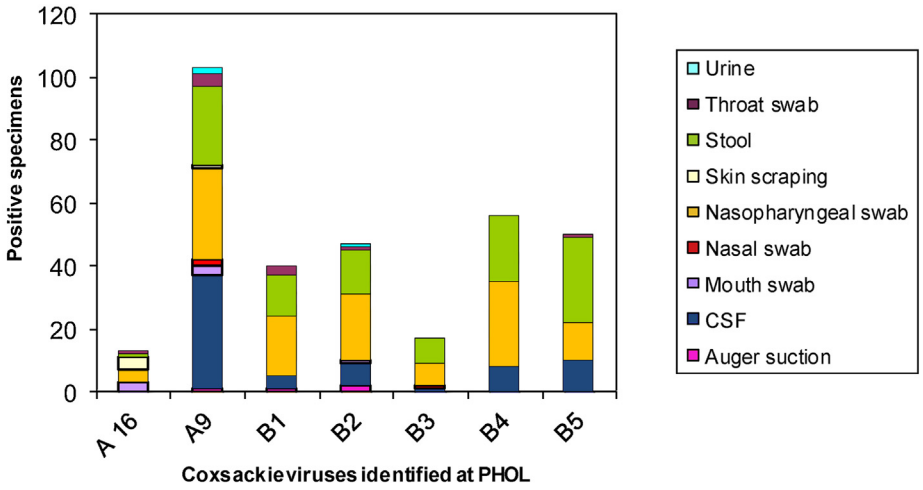

Figure 2. Coxsackievirus serotypes by specimen source; January 1, 2005 to December 31, 2011, Ontario, Canada.

3.3. *Patient information for those with multiple specimens submitted*

Eighty-two (27%) of the 310 patients in whom coxsackieviruses were detected had more than one specimen submitted, with a range of one to six specimens submitted per patient, for a total of 197 specimens; coxsackieviruses were detected in 106 of these specimens. However, not all specimens were submitted at a single point in time. Fifty-seven (69%) of the 82 patients had multiple specimens submitted as part of a single episode, 22/82 (27%) had specimens submitted during two separate episodes, and 3/82 (4%) patients had specimens submitted as part of three or more episodes. Of all multiple specimens submitted as part of the same episode (n = 160 specimens), 125 (78%) specimens were the first submitted specimen or had a lag time of 0–2 days from the first submitted specimen and 35 (22%) specimens had a lag time of more than 2 days (range 0–71 days). In addition, 64/82 (78%) patients had multiple specimens submitted during the same episode and 18/82 (22%) patients had a single specimen submitted for different episodes. The most common combination of specimen types collected for multiple submissions during the same episode was NP swab/stool specimens submitted for 12/64 (19%) patients, followed by CSF/stool specimens in 7/64 (11%) patients. Interestingly, not all specimens submitted as part of the same episode yielded a positive result. For example, off 12 patients who had both an NP swab and stool collected, only the NP swab yielded a positive result in two patients and only stool yielded a positive result in another two patients; both specimen sources were positive in eight patients. Among patients who had CSF/stool specimens collected, only CSF yielded a positive result in one patient, only stool in four patients, and both specimen sources in two patients. For three patients, specimens collected from the same body site on the same day or within a 0–2-day lag period did not yield the same test results. One patient had two CSF specimens submitted on the same day and only one was positive for coxsackievirus. The second patient had two stool specimens submitted 1 day apart and only the earlier specimen yielded a positive coxsackievirus result. The last patient had two NP specimens submitted 2 days apart and only the earlier one yielded a positive result.

A mixed logistic regression model was run to compare results of multiple submissions for the same patient episode (n = 160) adjusted for lag period and specimen source. We found that second and subsequent specimens submitted within 0–2 days from the first specimen submitted were more likely to yield a positive result than those submitted after 2 days (OR 3.13, 95% CI 1.33–7.37). Among patients who had more than one specimen collected within the same episode, stool specimens were more likely to yield a positive coxsackievirus result than NP swab specimens (OR 4.12, 95% CI 1.68–10.08). This relationship was not significant for CSF ($p > 0.05$). When comparing positive results for specimens collected for different episodes, no patient had a coxsackievirus isolated more than once during the entire study period.

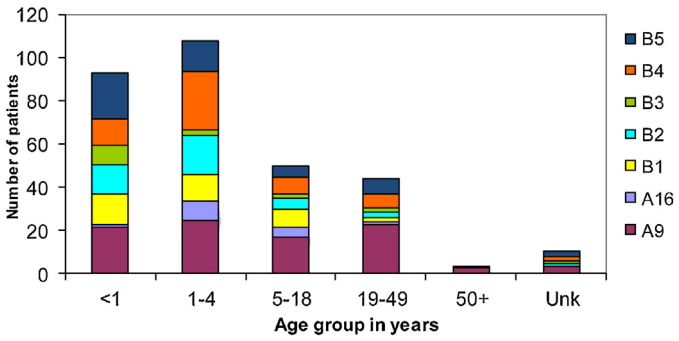

Figure 3. Number of patients with coxsackievirus detected, by serotype and age; January 1, 2005 to December 31, 2011, Ontario, Canada.

4. Discussion

Monitoring the epidemiology and types of circulating enteroviruses over time is important for the early identification of new serotypes or more virulent strains and the detection of disease clusters associated with specific serotypes; our study included 7 years of laboratory surveillance data. Coxsackieviruses represented more than one third of enteroviruses identified, with A9 being the most common serotype, followed by B4, B5, and B2. A9 was responsible for the increase in enterovirus meningitis in Ontario. Concurrently, A9 was reported to be associated with the increase in aseptic meningitis in Alberta, Canada.[15] Globally, various coxsackievirus serotypes have been reported to circulate in different geographic areas. For example A16, B3, B5, and A10 were reported to be the most dominant serotypes in Taiwan from 1999 to 2006, and B5, B2, and A9 were the most common coxsackievirus serotypes in the USA during 1970–2005.[3,16]

In our study, the circulation of coxsackieviruses in general and the A9 serotype in particular demonstrated yearly variability. A higher frequency of A9-positive samples was identified in 2007 and 2010, with the highest frequency in 2010. The increased detection did not appear to be related to an increase in testing, as

the number of specimens tested in each of these 2 years was lower than the annual average number of specimens tested for the entire study period; 19 145 and 21 621 specimens versus 25 647 specimens, respectively. The A9 serotype has been reported previously in the USA as a serotype with endemic patterns of circulation, described as circulating at low levels with a few distinct peaks.[16] It is unclear why some serotypes dominated in specific years, but population immunity to specific serotypes in addition to the emergence of new lineages or re-emergence of genotypes that circulated in the past are believed to influence circulation patterns.[16] Genomic sequencing of a set of isolates from the A9 outbreak in Alberta in 2010 found that A9 displayed signs of recombination and genetic divergence in the VP4 region, but the sequence was conserved in the antigenic regions of the VP1, VP2, and VP3 genes. This finding supports the theory that the increase was not as a result of the emergence of a new A9 mutant, but likely occurred due to a decline in herd immunity against this serotype.[15] All coxsackieviruses including A9 primarily circulate in the summer and fall; this has been well established.[3,10,17]

Coxsackieviruses were most commonly isolated in NP swab specimens, followed by stool and CSF. A9 was primarily isolated in CSF and the same pattern was observed during the peak in 2010. The increased detection of enteroviruses in CSF supports the observation that there was an increase in enterovirus meningitis in Ontario, Canada in 2010. CSF followed by respiratory specimens and stool have been reported elsewhere as the most common specimen sources in which coxsackieviruses were detected. However, these surveillance data have some limitations due to incompleteness of reporting and variations between testing procedures between laboratories, which may affect the results.[16] Isolation from CSF confirms disease etiology and likely represents severe disease due to a central nervous system infection, however virus identification from this source is impacted by the frequency of use of such an invasive procedure.[18] Other studies have reported CSF, throat swabs, and stool specimens as the most common sources for the detection of B serotypes; however these studies reported only positive results and not positive isolation rates.[17] In our study, only 3% of all coxsackieviruses and 4% of A9 were detected from throat swabs. Similar to other enteroviruses, the detection of coxsackieviruses in non-sterile sites may confirm the presence of the virus, but may not necessarily be the causative organism of acute infection because children and the immunocompromised shed the virus from stool for many weeks after disease onset.[1,6]

Consistent with previous reports, coxsackieviruses were most frequently detected among infants and children; however, patients in whom the A9 serotype was detected were older than patients who had any coxsackievirus isolated.[3,9,10,16] The wide age distribution of patients in whom A9 was detected in addition to the fact that A9 was primarily found in CSF supports that A9 may be the cause of severe disease across all age groups.[17] However, this may reflect a testing bias, as children are more likely to be tested for enterovirus when they experience milder illness, while adults only tend to undergo laboratory testing when they present with more severe illness.[17]

Some published studies have reported a male predominance in patients with coxsackieviruses, but statistical significance was not determined.[3,9,16] In our study, the highest proportions of individuals in whom any coxsackievirus or the A9 serotype was detected were male; however, this did not achieve statistical significance.

When more than one specimen was submitted for a patient, specimens with a shorter lag period (0–2 days) were more likely to yield a positive coxsackievirus result compared to those submitted more than 2 days after the previous specimen. This highlights the importance of early submission of specimens for viral detection. In addition, the specimen source in which coxsackieviruses were most likely to be detected was stool, followed by NP swab. This may be due to the fact that like enteroviruses, coxsackieviruses are shed for longer periods in stool than through respiratory pathways, or to the fact that physicians collect more stool and NP specimens from people presenting with enterovirus-like symptoms, hence allowing more opportunity for detection.[19] There was no difference in the yield of positive results between CSF and NP swab. The date of symptom onset was not consistently reported by specimen submitters, so our model could not adjust for symptom onset to specimen collection times.

Our study has a number of limitations. The coxsackieviruses reported may not represent all circulating coxsackieviruses. Most people who have a coxsackievirus infection manifest mild illness and do not seek medical care, and thus do not undergo laboratory testing.[2] PHOL uses viral culture to test for enteroviruses, which are known to be difficult to grow in this medium.[19] While PHOL performs the majority of testing for enteroviruses in Ontario, other community and hospital laboratories perform these tests, with many using molecular testing rather than culture. Therefore, the numbers reported here do not represent the total number of enteroviruses identified in all Ontario laboratories. However, the use of molecular testing and sequencing in a subset of CSF specimens did detect more enteroviruses in 2010 but also confirmed that A9 was the predominant enterovirus causing aseptic meningitis in 2010. Additionally, PHOL does not test for all coxsackievirus A serotypes, therefore we are unable to report on the incidence of all serotypes. Specifically, we were unable to appreciate the role of A6 in causing atypical HFMD, the occurrence of which was recently reported in the USA.[20] We were unable to report overall percent positivity for coxsackieviruses because specimens tested by viral culture may have been collected for reasons other than enterovirus-related symptoms. Hence our denominator data are not specific to enterovirus-related disease. Finally, PHOL does not collect detailed clinical information and we were unable to ascertain if disease severity was caused by coxsackieviruses or another etiology.

In conclusion, this study documented historical trends in Ontario of enteroviruses and coxsackievirus A9 in particular. In 2010, there was an increase in enterovirus meningitis in the province of Ontario, Canada, which was likely due to random yearly variability of A9 circulation, which also increased in 2010. Similar to other coxsackieviruses, A9 was most commonly identified among children, but may cause severe illness in both children and adults. Early specimen collection is recommended to optimize virus detection. Monitoring the circulation and epidemiology of enteroviruses can inform clinicians about circulating pathogens to optimize clinical testing and antibiotic use.

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Conflict of interest: Adriana Peci, Anne-Luise Winter, Alexandre Marchand-Austin, Romy Olsha, Nino Lombardi, and Jonathan Gubbay have no conflict of interest to declare.

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