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Quebec

Avian pathogens identification and genomic characterization: 2021 annual review of the Molecular Diagnostic Laboratory, Université de Montréal

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The poultry industry is an important part of Canadian agriculture and economy. For example, total egg production in 2020 in Québec and Ontario combined was ≥ 5.3 billion eggs produced by > 15 million laying hens. Annual egg production in these 2 provinces combined represents on average 60% of Canadian egg production (1,2). In recent years, the Canadian population has shown an ever-increasing craze for backyard birds as a source of eggs. These birds, therefore, need to be considered to efficiently control bird pathogens in commercial flocks. Furthermore, these birds may cause reappearance of old diseases that are generally well-controlled on commercial farms through biosecurity measures and vaccination programs, including Marek's disease, which is caused by an alphaherpesvirus (3). This emphasizes the importance of having an integrated vision to monitor and control avian infectious diseases. Furthermore, exotic and wild birds can carry several pathogens that can be problematic for commercial and backyard birds (e.g., *Avian influenza virus* and *Newcastle disease virus*). In addition, they can also carry zoonotic pathogens (e.g., *Avian influenza virus* and *West Nile virus*). In fact, a recent outbreak (December 2021) of an H5N1 highly pathogenic influenza A virus was reported in non-poultry birds in Newfoundland and Labrador, Canada (4). Presumably the origin of this outbreak was direct or indirect contact between domestic birds and wild influenza A virus positive birds. Unfortunately, this new outbreak seems to have spread to at least 2 other Canadian provinces (Nova Scotia and Prince Edward Island). Furthermore, since the beginning of 2022, outbreaks of this infectious agent were reported in commercial poultry, backyard or hobbyist flocks and wild birds in several states in the USA (5).

A census of avian pathogens identified in 2021 in Québec, Canada, by the Molecular Diagnostic Laboratory (MDL), Centre de diagnostic vétérinaire de l'Université de Montréal (CDVUM), a laboratory accredited by the American Association of Veterinary Laboratory Diagnosticians (AAVLD) is shown in Table 1. Note that the absence of some pathogens in this census does not provide proof of their absence in birds in Québec, as their detection may be the responsibility of other Québec veterinary diagnostic laboratories (e.g., university, government, and private laboratories).

Materials and methods

Samples submitted to MDL are very diverse and depend on the pathogen to be detected, as well as objectives or intentions of the veterinarian/pathologist. For example, feather follicles (Marek's disease virus), choana swabs *(Mycoplasma gallisepticum)*, tendons (avian reovirus), liver (adenoviruses), trachea *(Infectious laryngotracheitis virus)* and environmental swabs are submitted regularly. In addition, samples are pooled (e.g., mycoplasma infection monitoring) to enable testing of many birds at a reduced cost. More than 99% of the bird samples submitted to MDL were from Québec, (< 1% were from other Canadian provinces), with only Québec samples included in this census. Most pathogens were identified by polymerase chain reaction (PCR) or quantitative polymerase chain reaction (qPCR) diagnostic assays. However, a small portion (i.e., *Falcon aviadenovirus A* and *Pigeon torque teno virus*) were identified from clinical samples using next-generation sequencing (MiSeq, Illumina). The genetic diversity of some virus strains was determined by sequencing specific PCR amplicons using Sanger sequencing (Plateforme de séquençage et de génotypage des génomes du Centre de recherche du Centre Hospitalier

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Table 1. Identified avian pathogens in Québec in 2021 using molecular diagnostic methods.

Pathogen categories	Names	Abbreviations	Diseases
Bacteria	*Clostridium perfringens*	*C. perfringens*	Necrotic enteritis
	Mycobacterium spp.	*M. avium, M. genavense, M. xenopi*	Avian mycobacteriosis
	Mycoplasma gallisepticum	*M. gallisepticum* (MG)	Chronic respiratory disease; infectious sinusitis
	Mycoplasma synoviae	*M. synoviae* (MS)	Infectious synovitis
Parasite	*Histomonas meleagridis*	*H. meleagridis*	Histomoniasis (blackhead disease; infectious enterohepatitis)
Viruses	*Aviadenovirus (Falcon aviadenovirus A)*	FaAdV	Hepatitis
	Aviadenovirus (Fowl aviadenovirus A to *E; formerly Fowl adenovirus A* to *E)*	FAdV	Adenoviral gizzard erosion, inclusion body hepatitis, and hepatitis hydropericardium syndrome
	Avibirnavirus (Infectious bursal disease virus)	IBDV	Infectious bursal disease (Gumboro disease)
	Avipoxvirus (Fowl and *Canarypox virus)*	Avian pox	Fowl pox
	Flavivirus (West Nile virus)	WNV	West Nile[a]
	Gammacoronavirus (Avian coronavirus)	IBV	Avian infectious bronchitis (includes the false layers syndrome)
	Gyrovirus (Chicken anemia virus)	CAV	Chicken infectious anemia
	Itovirus (Gallid alphaherpesvirus 1)	ILTV or GaHV-1	Infectious laryngotracheitis[a]
	Mardivirus (Gallid alphaherpesvirus 2)	MDV or GaHV-2	Marek's disease
	Orthobornavirus (Psittacine bornavirus)	PaBV	Proventricular dilatation disease
	Orthoreovirus (Avian orthoreovirus; formerly *Avian reovirus)*	ARV	Viral arthritis (tenosynovitis)
	Unclassified genus in *Anelloviridae* family *(Pigeon torque teno virus)*	PTTV	Undetermined, need further investigations

[a] Immediately notifiable diseases.

de l'Université Laval). Most PCR and qPCR diagnostic assays conducted by MDL are used to monitor infectious diseases and confirm the negative status of poultry flocks regarding various pathogens. Therefore, as expected, most of the results obtained by MDL are negative, since our domestic birds are generally healthy and without clinical signs. According to the origin of the clinical samples, the identified pathogens were classified into 3 categories: i) commercial and backyard flocks; ii) exotic and wild birds; and iii) all category origins. The first category was primarily composed of chicken samples but also included duck, goose, and turkey samples.

Results and Discussion

PCR/qPCR diagnostic assays

All avian pathogens detected in 2021 by MDL are listed in Table 1, including the official taxonomic classification, as well as names and abbreviations of the avian pathogens with their associated diseases. The number of positive cases for each pathogen is illustrated in Figure 1. Overall, 4, 1, and 13 distinct bacteria, parasites, and viruses, respectively, were detected (Figure 1). The most frequently detected pathogens by PCR/qPCR in commercial and backyard poultry categories in 2021 were: infectious bronchitis virus (IBV, *n* = 244 for all IBV strains; whereas *n* = 98 for DMV which is a specific IBV strain that appeared in 2017 in Québec and is involved in the false layers syndrome); *Avian reovirus* (ARV, *n* = 182); and *Infectious bursal disease virus* (IBDV, *n* =169). Interestingly, these 3 viruses were also the most frequently detected in 2020 by MDL, but in a lesser amount: IBV (*n* = 212 for all IBV strains and *n* = 60 for DMV strain), ARV (*n* = 139), and IBDV (*n* = 121) (6). The most frequent pathogens detected in 2021 in the exotic and wild birds were: *Psittacine bornavirus* (PaBV) (*n* = 5) and *West Nile virus* (WNV) (*n* = 5) (Figure 1). However, this report is an underestimate number of positive *West Nile virus* cases in the province in 2021, since most of the clinical samples were submitted to a government laboratory, the Laboratoire de santé animale du Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ). WNV was detected in 3 bird species: blue jay, goshawk, and sharp-shinned hawk (Table 2). The 2 most frequently detected pathogens in 2021 infecting all categories of birds (i.e., commercial, backyard, exotic, and wild) were: *M. gallisepticum* (*n* = 64) and Marek's disease virus (MDV, *n* = 55) (Figure 1); for these 2 pathogens, almost all positive cases were in commercial/backyard birds, except 3 (peacock, purple finch, and rhea) and 1 (peacock) cases, respectively (Table 2).

Taxonomic classification of circoviruses was recently revised following the discovery of several new circoviruses. In that regard, the *Gyrovirus* genus of chicken anemia virus (CAV), previously classified into the *Circoviridae* viral family, was reclassified into the *Anelloviridae* viral family in 2017 (7). Until 2011, CAV was the only member of the *Gyrovirus* genus. However, discovery of several new gyroviruses in various hosts, including humans, chicken, and sea birds (8–11), has greatly increased the diversity of gyroviruses. Based on bioinformatics, the MDL CAV PCR diagnostic assay may cross-react to varying degrees with

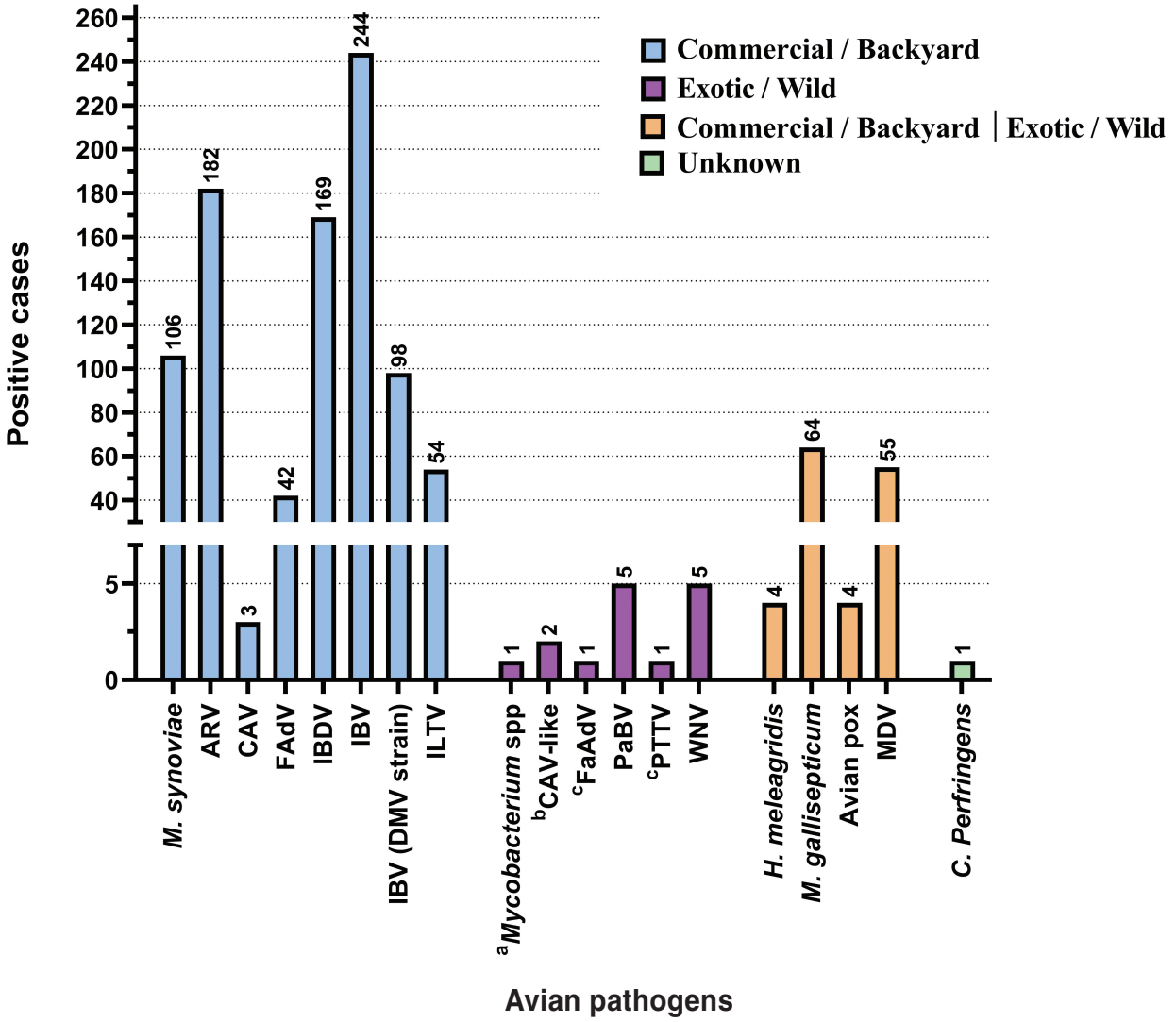

Figure 1. Avian pathogens detected by PCR/qPCR in 2021 from Québec submitted bird samples. Pathogen names and abbreviations are described in Table 1.

[a] A non-specific PCR diagnostic assay that can detect various species of the *Mycobacterium* genus was done as described (27). The sequencing of the PCR amplicon must be carried out to identify the *Mycobacterium* species. In birds, there are several species of *Mycobacterium,* including *avium, genavense*, and *xenopi*.
[b] The CAV PCR diagnostic assay can cross-react with other closely related anelloviruses.
[c] These viruses were detected by next-generation sequencing.

other genomic closely related gyroviruses and perhaps, albeit to a much lesser extent, other members of *Anelloviridae*. Some veterinarians will request to apply the CAV PCR diagnostic assay on clinical samples to detect circoviruses in exotic and wild birds. However, it is viruses genetically related to anelloviruses that should be detected. Consequently, when a CAV PCR positive result is obtained from an exotic/wild bird, the virus species must be confirmed by sequencing the viral genome. As illustrated in Figure 1, two CAV-like cases were confirmed in gulls (Table 2). Exotic/wild birds deemed positive for various pathogens are described in Table 2. Avian pathogens were identified in at least 12 exotic/wild bird species (Table 2).

Viral strains identification and viral genome sequencing

For some of the identified pathogens, veterinarians requested MDL to use more advanced microbial genome molecular analyzes to characterize the strains, genotypes, serotypes, virulence factors, etc. Some of the resulting data are used for strain selection to design autogenous vaccines. The following is an overview of our results on viral genomic characterization, based on sequencing partial or all genomes of avian viruses.

When requested by veterinarians, IBV strains are identified by sequencing a portion of the viral S gene. However, MDL has developed 2 types of IBV qPCR diagnostic assays: the first

Table 2. Exotic and wild birds positive for various pathogens.

Birds	*Mycobacterium* spp.	*M. gallisepticum*	*H. meleagridis*	Avian pox	CAV-like[a]	FaAdV	MDV	PaBV	PTTV	WNV
American kestrel						1				
Blue jay										1
Conure[b]	1							5		
Goshawk										1
Gull					2					
Peacock		1					1			
Pheasant			1							
Pigeon									1	
Purple finch		1								
Rhea		1								
Sharp-shinned hawk				1						3
Wild turkey				1						

[a] The CAV PCR diagnostic assay may cross-react with closely related viral genomes of other anelloviruses.
[b] Includes parrots and budgerigars.

detects all genotypes of IBV strains (IBV qPCR), whereas the second was specifically designed to detect only IBV DMV-like strains (IBV DMV qPCR) (Figure 1). In 2021, following bioinformatics analysis of the IBV S gene, 60, 32, 4, and 4% of Québec strains were genotyped as DMV, Mass41, Conn46, and CA1737 strains, respectively.

The IBDV strains are characterized by sequencing the VP2 viral gene. Two types of IBDV strains were identified in Québec in 2021: the 105_Pennsylvania type (genogroup 2; 93.33%) and Del-E (6.67%).

The ARV strains are characterized by sequencing a portion of the S1 viral genome segment, the σC gene. As usual, ARV viruses have high genomic diversity and, therefore, several viral strains are identified each year. Unfortunately, there is no consensus regarding ARV strains classification nomenclature, complicating comparisons among laboratories. In 2021, 6 clusters were identified in Québec based on the Lu et al (12) classification, as follows: vaccine 1733-like (10.53%), cluster 2 (57.89%), cluster 4 (5.26%), cluster 6 (15.79%), and 2 unclassified clusters (5.26% each).

The FAdV strains are characterized by sequencing the hexon viral gene. In 2021, all sequenced FAdV strains were clustered into the 8b type (classified as *Fowl aviadenovirus E* viral species).

Our diagnostic/research team's is currently collaborating with Dr. Mohamed Faizal Abdul-Careem, University of Calgary, for genomic and pathogenesis characterizations of Canadian Infectious laryngotracheitis virus (ILTV) strains (13,14). Recently, we reported that Canadian ILTV strains, which belong to 2 genotypes, had significant differences in their virulence potency in infected chickens, including virus transmission and disease severity (14). The entire viral genome of 2020 and 2021 ILTV Québec strains were recently sequenced using next-generation sequencing, and bioinformatics analyses are underway.

Viruses of the genus *Aviadenovirus* infect various species of birds (15). Aviadenoviruses targeting the liver, e.g., fowl aviadenoviruses (FAdV), cause diseases such as inclusion body hepatitis in chickens (16). However, falcon aviadenoviruses (FaAdV) have been less characterized than FAdV. Until recently, only 2 to 15% of the FaAdV genome sequence present in various falcon species, such as Northern aplomado falcon *(Falco femoralis)* and American kestrel *(Falco sparverius)*, have been reported (17,18). A liver tissue sample from an American kestrel that died in August 2021 in Québec, Canada, with a necrotizing hepatitis associated with basophilic intra-nuclear inclusion bodies suggestive of an adenovirus infection, was analyzed by next-generation sequencing. The coding-complete genome of a FaAdV strain was sequenced and submitted to a public nucleotides (nt) databank (GenBank accession number OM367996) (19). The viral genome sequence obtained was 39 008 nt. The closest full-length genome sequence in GenBank was the *Fowl aviadenovirus E* strain HUNG6 (GenBank accession number MK572853). However, the 2 coding-complete genomes shared only 46.95% nt identity using MAFTT alignment (Geneious Prime software) (19). To our knowledge, this is the first FaAdV coding-complete genome sequence reported worldwide.

Like CAV, *Torque teno viruses* (TTV) are members of the *Anelloviridae* viral family (20) and can infect a wide host spectrum (21). Identification of the first pigeon TTV (PTTV) was reported 10 y ago in China (22), but pathogenesis data are scarce. Interestingly, a multifocal hepatic necrosis was recently reported during an outbreak in Australian domestic pigeons *(Columba livia)* coinfected with *Pigeon aviadenovirus 1* (PiAd-1) and PTTV, without clearly being able to elucidate the exact role of PTTV in this outbreak (23). In October 2021, a juvenile female pigeon was found dead at L'Ange-Gardien, Québec, Canada. The bird was emaciated and had a fungal infection of the air sacs (aspergillosis). In addition, a lymphoid depletion of the bursa of Fabricius with necrosis, fibrinous deposition, and presence of large basophilic intracytoplasmic and intranuclear inclusions, were detected histologically. Although the CAV PCR diagnostic assay conducted on the bursa of Fabricius was negative, next-generation sequencing of this tissue yielded a complete viral genome of a PTTV strain (CDVUM-2021-2594812) that was deposited to a public databank (GenBank accession number

OM654319). The complete viral genome sequence was 1577 nt; the closest full-length genome sequence in GenBank was the PTTV strain ZJDY discovered from a pigeon liver in China in 2012 (GenBank accession number KF477319). The 2 complete viral genomes shared 99.03% nt identity. However, no other DNA virus, such as adenovirus or circovirus, was detected by next-generation sequencing in the bursa of Fabricius. To our knowledge, this is the first PTTV whole genome sequence reported in Canada.

Conclusions

A total of 1041 molecular diagnostic assay positive results were obtained in 2021 (Figure 1) for the pathogens listed in Table 1. This represents an identification rate of 2.85 avian pathogens per day in 2021 at MDL, emphasizing the importance of monitoring infectious diseases in all bird species (domestic and wild) to ensure animal welfare and public health. Furthermore, several pathogens are transmitted among bird species, and from birds to humans (3). In addition, the impact of an avian pathogen infection may vary among bird species. For example, *Duck atadenovirus A* infections were reported in ducklings in 2019 and 2020 in Québec (6,24). Although typically asymptomatic in infected ducks, this virus is highly pathogenic in chicken layers and is the etiological agent of the egg drop syndrome (25), an immediately notifiable chicken disease in Canada (26). Therefore, monitoring of infectious diseases in all types of bird species regardless of their living environment (commercial, backyards, exotic, and wild, inside, outdoor, zoo natural enclosure, etc.) needs to be promoted across Canada.

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