In vitro and in ovo characterization of aquatic bird bornavirus 1 in avian species

by

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ABSTRACT

*In vitro* and *in ovo* characterization of aquatic bird bornavirus 1 in avian species

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Aquatic bird bornavirus 1 (ABBV-1) is associated with fatal inflammation of nervous tissue in avian species. Waterfowl act as the natural host, with populations of Canada geese existing as carriers in North America, however ABBV-1 has been detected from other non-waterfowl species. The objective of this thesis was to characterize the host range of ABBV-1 to determine the relative threat to the poultry industry. Studies were conducted to: 1) characterize ABBV-1 in continuous avian cell lines, 2) assess ABBV-1 replication kinetics in primary goose, duck, chicken and turkey fibroblasts, and 3) evaluate an *in ovo* model to assess ABBV-1 host restriction and pathogenesis. The results indicated that poultry species were semi-permissive to infection *in vitro* at decreased rates compared to natural hosts. Inoculation *in ovo* was ineffective in inducing virus spread and lesions in any species, making it unsuitable to study ABBV-1. This work will inform future research regarding ABBV-1.
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STATEMENT OF WORK PERFORMED

All the work described in this thesis was performed by me, under the guidance of Dr. Leonardo Susta and my advisory committee (Drs. Éva Nagy, Nicole Nemeth, and Csaba Varga) with the following exceptions:

The isolation of ABBV-1 from goose brain homogenate and subsequent sequencing of the virus described in Chapter 1 was done by Dr. Li Deng.

Characterization of growth in continuous cell lines, including the passaging of cells, titration, and RT-qPCR in Chapter 1 was done by Dr. John Pham.

The transmission electron microscopy in Chapter 1 was done by Bob Harris at the Advanced Analysis Centre.

The histopathology processing and slide preparation, including embedding in wax, sectioning, H & E staining, and IHC described in Chapter 4 was done by members the Animal Health Laboratory (AHL).
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LIST OF ABBREVIATIONS

ABBV-1 – Aquatic bird bornavirus 1
ABV – Avian bornavirus
AF – allantoic fluid
BoDV-1 – Borna disease virus 1
CAM – chorioallantoic membrane
CEF – chicken embryo fibroblasts
CnBV – canary bornavirus
CNS – central nervous system
DEF – duck embryo fibroblasts
DMEM – Dulbecco’s modified Eagle medium
DPI – days post infection
EsBV – Estrildid finch bornavirus
FBS – fetal bovine serum
FFU – focus forming unit
GEF – goose embryo fibroblasts
H&E – hematoxylin and eosin
HPI – hours post infection
IF – immunofluorescence
IHC – immunohistochemistry
ORF – open reading frame
PaBV – Parrot bornavirus
PBS – phosphate buffered saline
PDD – proventricular dilatation disease
PV – proventriculus/ventriculus
RNP – ribonucleoprotein
RT-qPCR – reverse transcriptase quantitative polymerase chain reaction
TEF – turkey embryo fibroblasts
TEM – transmission electron microscopy
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Chapter 1. Literature Review

1.1 Family Bornaviridae

1.1.1 History

Bornaviridae is a family of viruses capable of causing neurological infections in a variety of host species. Neurobehavioural disease in horses similar to what is now recognized as a bornavirus infection was first described in the 1700s as “hot-headed disease” (Zwick, 1927). After an outbreak of the disease in cavalry horses in the late 1800s, the condition was called “Borna disease” after the town in Saxony, Germany, where the outbreak occurred (Zwick, 1927). However, it was not until the late 1920s that a viral etiology was determined, by reproducing the disease in healthy horses upon inoculation of filtered (i.e., bacteria free) brain homogenates from diseased horses (Zwick, 1927). Complete characterization of the virus remained elusive until identification in 1994, when the virus was classified into a newly described family, Bornaviridae, within the order Mononegavirales (Briese et al., 1994). The virus, named Borna disease virus (BDV; now BoDV-1), remained the only virus within the family Bornaviridae until 2008 upon the discovery of a bornavirus from psittacine birds affected with proventricular dilatation disease (PDD), a severe chronic wasting disease (Honkavuori et al., 2008; Kistler et al., 2008). This bornavirus had considerable genomic sequence differences compared to BoDV-1 isolates from horses (Honkavuori et al., 2008; Kistler et al., 2008). Since this initial identification, researchers have identified multiple bornaviruses from avian hosts. In fact, an avian bornavirus (ABVs) species has been found to be highly prevalent and circulate freely among wild waterfowl in southern Ontario, the northeastern United States, and Europe (Guo et al., 2012; Delnatte et al., 2014; Thomsen et al., 2015).

1.1.2 Taxonomy and host range

The large sequence diversity that was introduced with the increasing number of recently discovered viruses required an improved taxonomical system at the species level of the family Bornaviridae (Kuhn et al., 2015). According to current classification by the International Committee on Taxonomy of Viruses (ICTV), the family Bornaviridae within the order Mononegavirales includes two genera, Orthobornavirus and the recently described Carbovirus.
The genus Carbovirus includes two species: Queensland carbovirus, and Southwest carbovirus, both isolated from carpet pythons. The genus Orthobornavirus (previously Bornavirus) encompasses 8 species: Elapid 1 orthobornavirus, Mammalian 1 orthobornavirus, Mammalian 2 orthobornavirus, Psittaciform 1 orthobornavirus, Psittaciform 2 orthobornavirus, Passeriform 1 orthobornavirus, Passeriform 2 orthobornavirus, and Waterbird 1 orthobornavirus (Amarasinghe et al., 2018; Maes et al., 2019). Species assignment was based on phylogenetic analyses, according to sequence similarities and differences between groups. Borna disease virus-1 (BoDV-1) and Borna disease virus-2 (BoDV-2) within the Mammalian 1 orthobornavirus species share 85% sequence similarity, whereas isolates of bornaviruses from avian hosts share 60-69% similarity with the mammalian bornaviruses (Kuhn et al., 2015). Greater overall sequence differences indicate a new species, whereas smaller differences indicate different genotypes within the same species (Kuhn et al., 2015). As of 2019, this new classification resulted in a total of 18 virus genotypes within 10 species (Amarasinghe et al., 2017). This taxonomy is also in agreement with segregation of the viruses based on the typical hosts (Amarasinghe et al., 2017). For example, parrot bornavirus 1 (PaBV-1) of the Psittaciform 1 orthobornavirus species is commonly isolated from parrots. A full list of currently recognized bornaviruses with corresponding genera and species is provided in Table 1-1.

Bornaviruses are capable of infecting a diverse spectrum of vertebrate hosts. Mammalian bornavirus infection is typically associated and most commonly observed in horses and sheep, although natural infection has been detected in a multitude of mammals, including monkeys, llamas, goats, deer, dogs, cats, and even humans (Kinnunen et al., 2013). Likewise, avian bornaviruses exhibit a broad host range across a wide variety of avian orders, including at least 80 species (Payne et al., 2011). Although ABVs appear to have some degree of host predilection, as reflected by the taxonomy (e.g., Psittaciform 1 orthobornavirus infection is mainly seen in Psittaciformes [Amarasinghe et al., 2017]), these viruses have been reported to cause natural infection in species other than their common hosts. For example, Waterbird 1 orthobornavirus infection in gulls, eagles and emus have been described (Ridgway et al., 1983; Weissenböck et al., 2009; Delnatte et al., 2011; Payne et al., 2012; Guo et al., 2015).
1.1.3 Morphology and replication

Bornaviruses are unique within the order Mononegavirales, as they replicate in the host cell nucleus, mediate differential use of transcription start and stop sites to produce both monocistronic and polycistronic mRNA transcripts, and take advantage of host cellular machinery for mRNA splicing (Briese et al., 1994). Since most studies regarding the morphology and genome organization of viruses in the family Bornaviridae focus on BoDV-1 of Mammalian 1 orthobornavirus as the type species, this section of the review will include those studies and report on any differences that may have been described specifically for avian bornaviruses.

Bornaviruses have enveloped virions with a round morphology and range in size from 80 to 103 nm (Zimmerman et al. 1994). As the classification in the order Mononegavirales implies, these viruses have a single, non-segmented, negative sense RNA genome (Briese et al., 1994). When compared to other viruses in the order Mononegavirales, the bornavirus genome is compact, approximately 9 kilobases (kb), and encodes 5 structural and one non-structural proteins. This is the result of multiple overlapping open reading frames (ORFs) produced by monocistronic and multiple polycistronic mRNA transcripts from three transcription-start sites and four transcription termination/stop sites (Briese et al., 1994; Cubitt et al., 1994). Additionally, the transcript encoding the L protein is alternatively spliced using host cellular machinery to remove one intron (Cubbitt et al., 1994). Similar to other viruses within the same order, the structural proteins, encoded by homonymous genes, include the nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G), and the RNA-dependent-RNA-polymerase (large [L]) (Cubitt et al., 1994). The N protein functions to encapsidate the full length of the genome; the P acts as a cofactor for the L, which has both transcriptase and replicase activity; the M protein acts to mediate interactions between the nucleocapsid and the viral envelope; and G is responsible for receptor recognition and viral envelope-host membrane fusion (Cubitt et al., 1994). The minimal replicative unit of bornaviruses is the ribonucleoprotein (RNP), consisting of the genome encapsidated in N monomers, in association with the P and L proteins (Cubitt and de la Torre, 1994). The non-structural X protein is translated from an alternative reading frame of the P gene mRNA (Schwardt et al., 2005). The X protein is multifunctional, and acts as a transcriptional regulator of replication, promoter of viral persistence, and modulator of the antiviral response (discussed below) (Poenisch...
et al., 2007). The layout of a typical bornavirus genome is reported in Figure 1-1, and a list of transcripts is provided in Figure 1-2.

The infectious cycle of bornaviruses starts with attachment and entry of virions into a host cell, a mechanism that is mediated by attachment of the G protein with the cognate receptor (Sauder and Staehler, 2003). The specific host cell receptor for bornavirus attachment remains unknown; however, the virus is highly neurotropic, and experiments conducted in rats have shown that BoDV-1 has a high specificity for neurons within the limbic system (de la Torre, 2002). Knockdown studies using oligodendrocytes assessing BoDV-1 entry have shown that gamma-aminobutyric acid receptor subunit alpha 3 (GABRA3), and serotonin receptor 1F (HTR1F) are necessary, confirming the neurotropism of bornaviruses but failing to explain the multitude of cell lines that are permissive to bornavirus infection in vitro (Clemente et al., 2010). Attachment of the virion initiates clathrin-dependent receptor-mediated endocytosis (Sauder and Staehler, 2003). As the pH of the endosome containing the virus decreases, a conformational change within the G protein mediates fusion between the host endosomal membrane and the viral envelope, releasing the RNP into the cytosol (Sauder and Staehler, 2003). The free RNP is then trafficked to the nucleus by host importins that recognize the nuclear localization signal (NLS) of the N, P, and L genes (Kobayashi et al., 2001; de la Torre, 2006; Tomoyuki and Tomonaga, 2013). In the nucleus, the L protein produces messenger RNA transcripts by using the transcription start/stop sites on the genome or by further RNA splicing (if necessary); transcripts are then exported out of the nucleus for translation (Sauder and Staehler, 2003). Assembly and release of bornavirus virions are poorly understood, however it seems that after translation the RNP is reassembled in the nucleus and exported out into the cytoplasm where the virions combine with the G and M proteins (which remain in the cytoplasm after translation) (Sauder and Staehler, 2003). Glycoprotein (G)-mediated cell-to-cell spread of virions is proposed to be the main method of within-host spread (Lennartz et al., 2016). Indeed, only very small amounts of virus can be recovered from the supernatant in vitro, while in the host the virus is almost exclusively found within cells (Lennartz et al., 2016).

Throughout the infectious cycle, bornaviruses remain highly cell-associated, and rather than causing cell lysis, infection is long-lasting and persistent (de la Torre, 2002; Tomonaga, 2002). Bornaviruses achieve persistence through at least three distinct mechanisms. First, the RNP
forms tight associations to core histones and assemble into viral factories (Matsumoto et al., 2012). During mitosis, factories disassemble and RNPs act as cellular components that become intertwined with host chromosomes and segregate into daughter cells, allowing for maintenance of nuclear infection (Matsumoto et al., 2012). Second, bornaviruses have tight regulation over their own transcription, reducing the amount of viral RNA present in the cell, making detection by the immune system difficult and promotes persistence (Ackermann et al., 2007). Regulation of viral RNA amount is mediated by the non-structural X protein, either through direct sequestration of the P protein or by triggering its oligomerization (Poenisch et al., 2004). Consequently, a lack of available P protein to act as a cofactor for the polymerase (L) significantly decreases the production of viral RNA (Perez et al., 2003; Ackermann et al., 2007).

The third method by which bornaviruses establish persistent infection is immune evasion, which can be mediated in many different ways. The 5’ ends of both the genome and antigenome are trimmed to remove nucleotide triphosphates to leave behind a nucleotide monophosphate (Schneider et al., 2006). The lack of a triphosphate residue effectively shields the viral RNA from recognition by pattern recognition receptors (PRRs) (specifically retinoic acid-inducible gene-I; RIG-I), thus preventing the induction of type I interferon and other pro-inflammatory cytokines that would create an antiviral state within the cell (Schneider et al., 2006; Reuter et al., 2010). Furthermore, the X protein is involved in the inhibition of innate immune induction, having been shown to co-localize with and inhibit the mitochondrial antiviral signalling protein (MAVS). MAVS protein interacts with RIG-I and RIG-I-like receptors (RLRs) to trigger signalling cascades that promote cytokine induction leading to an antiviral state; therefore inhibition of MAVS action promotes persistence (Li et al., 2013). Lastly, the P protein acts as a competitive inhibitor of TBK-1 (Traf associated NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) [TANK] binding kinase-1) (Unterstab et al., 2005). Inhibition of TBK-1 blocks the induction of interferon-β and thus prevents antiviral responses (Unterstab et al., 2005).

1.1.4 Bornavirus infection in vitro

In cell culture, bornaviruses establish a non-cytolytic persistent infection with limited release of infectious virions and absence of cytopathic effect (CPE) (Tomonaga et al., 2002). Thus,
visualization of bornaviruses in vitro is dependent on indirect methods—frequently immunofluorescence—to detect viral antigens in the absence of CPE (Maclachlan & Dubovi, 2017). Immunofluorescence yields a typical staining pattern characterized by coarsely granular (i.e., speckled) nuclear reactivity, likely representing nuclear viral factories (Hirai et al., 2016). Growth in vitro is also commonly described as slow-growing, as experiments implementing immunofluorescence to track the spread of infection can vary from 15 days to two months or more to achieve 100% of cells infected in culture, depending on the host and cell type (e.g., neuronal, epithelial, etc.) (Bajramovic et al. 2003; Charlier et al. 2013; Rubbenstroth et al. 2014).

Bornaviruses are highly neurotropic in vivo and replicate in neurons, while also being found at lower levels in astrocytes and oligodendrocytes (Li et al., 2013). However, in vitro these viruses are capable of replicating in multiple cell types, with isolation/growth studies frequently being performed in epithelial and fibroblast cell lines (Bajramovic et al., 2003; Charlier et al., 2013; Rubbenstroth et al., 2014). In vitro studies with BoDV-1 frequently use rat neuronal cell culture as a model system for molecular research, as well as common cell lines including Vero cells (African green monkey kidney cells; epithelial) (Gonzales-Dunia et al., 1998), Madin-Darby canine kidney cells (MDCK; epithelial) (Compans et al., 1994), and mouse embryo fibroblasts (MEF; fibroblast) (Richter et al., 2007). ABVs likewise show broad cell type tropism in vitro, with growth studies being described in primary duck embryo fibroblasts (DEF) (Payne et al. 2011), continuous chicken embryo fibroblasts (DF1) (Rubbenstroth et al. 2013), continuous quail fibrosarcoma cells (QT6, fibroblast) (Rinder et al., 2009; Gray et al., 2010), and even in mammalian cell lines, such as Vero (Rubbenstroth et al., 2013).

1.1.5 Bornavirus-induced immunopathology

Bornaviruses are associated within the nucleus of neurons in both the central and peripheral nervous systems (Carbone et al., 1991). Experimental inoculation of rats with BoDV-1 indicates that the neuronal damage and resulting clinical signs are a consequence of T-cell mediated immunopathogenesis and not direct damage from viral replication (Stitz et al., 1989). Microscopic examination of diseased tissues indicates infiltration of immune cells into the central, peripheral, and autonomic nervous system. The makeup of this inflammatory population is largely composed
of CD8+ T-cells, with variable presence of CD4+ T-cells and macrophages (Stitz et al., 2002). When infected rats are administered immunosuppressive drugs (e.g. cyclosporine A; CSA), no clinical signs or lesions (e.g. encephalitis) manifest despite presence of infectious virus in the tissues (Stitz et al., 1989). Humoral immunity is not considered to have an important role in the pathogenesis, based on studies in which CSA administered rats had an antibody response similar to immunocompetent rats, despite absence of lesions (Stitz et al., 1989). Taken together, these results are supportive of T-cell mediated immunopathology of BoDV-1 infection in rats (Stitz et al., 2002). This notion is supported by studies of parrot bornavirus-4 (PaBV-4) infection in cockatiels, which failed to develop clinical disease when administered the same immunosuppressant drug (Hameed et al., 2018).

1.2 Avian bornavirus infection and proventricular dilatation disease

1.2.1 Etiology

Proventricular dilatation disease (PDD) is a severe, chronic wasting disease associated with birds in the order Psittaciformes (i.e., psittacine birds). Initially called “macaw wasting syndrome”, PDD has been recognized in psittacine birds since the 1970s (Ridgway et al., 1983). The disease name refers to the postmortem presentation of the proventriculus in affected birds, which is dilated due to the loss of muscular tone caused by destruction of autonomic neurons in the myenteric and gastric plexuses (Ridgway et al., 1983). In addition to gastrointestinal disease, affected birds may show neurological signs and behavioural abnormalities, similar to those described in mammals with Borna disease (Gregory et al., 1994). Despite early recognition of the disease, the etiological agent eluded identification until 2008, when a newly characterized species of bornavirus was determined to be the cause (Honkavuori et al., 2008; Kistler et al., 2008).

In 2008, molecular analysis of tissues from several species of psittacines (histologically confirmed with PDD) yielded positive results for bornavirus in a majority of samples (Kistler et al., 2008). Sampels derived from a white cockatoo (Cacatua alba), grey parrot (Psittacus erithacus), galah (Eolophus roseicapilla), Solomons cockatoo (Cacatua ducorpsii), blue and yellow macaw (Ara ararauna), and eclec tus parrot (Eclectus roratus). Through the use of a microarray system, RNA extracted from multiple PDD-affected organs (e.g., crop, proventriculus,
ventriculus, and brain) was reverse-transcribed and hybridized to a chip containing unique sequences from all virus families (Kistler et al., 2008). Following microarray chip analysis, the majority of cDNA transcribed from PDD-positive samples hybridized to Bornaviridae-specific virus sequences (Kistler et al., 2008). Given these result, the entire genome of a bornavirus isolate was sequenced and found sharing only 64% identity with known mammalian bornavirus isolates; thus a new species within the family Bornaviridae was discovered (Kistler et al., 2008). A similar study published at the same time corroborated these results through pyrosequencing of cDNA extracted from brain tissue from birds microscopically diagnosed with PDD (Honkavauri et al., 2008). Brain tissue in these cases originated from two blue-throated macaws (Ara glaucogularis) and a vinaceous-breasted Amazon (Amazona vinacea) (Honkavauri et al., 2008). Sequencing results confirmed the presence of bornavirus RNA in tissues from all confirmed PDD cases, while none was detected in the negative control tissues (Honkavauri et al., 2008).

Initially, the host range of avian bornaviruses was thought to be limited to psittacine birds. However, lesions consistent with infection and associated clinical signs have since been documented in a wide variety of avian taxa, leading to the discovery of multiple novel species of avian bornaviruses. Avian orders documented with PDD-like lesions and associated natural bornavirus infections include Psittaciformes (parrots, [e.g., African grey (Psittacus erithacus)]), Anseriformes (waterfowl [e.g., Canada goose (Branta canadensis)]), Charadriiformes (gulls, [e.g., ring-billed gull (Laurus delawarensis)]), Passeriformes (perching birds [e.g., common canary (Serinus canaria)]) and Accipitriformes (birds of prey [e.g., bald eagle (Haliaeetus leucocephalus)]), among others (Ridgway et al., 1983; Weissenböck et al., 2009; Delnatte et al., 2011; Payne et al., 2012; Guo et al., 2015).

1.2.2 Clinical signs

Birds are often infected for weeks, months or even years before developing clinical disease (Gregory et al., 1997; Gray et al., 2010). Some birds may not develop overt disease and instead are considered subclinical carriers (Delnatte et al., 2014). Initial, non-specific signs of PDD include weight loss, lethargy and depression (Doneley et al., 2007). Common clinical signs are associated with the gastrointestinal tract, and include regurgitation, dysphagia, and passing of
whole seeds in feces (Hoppes et al., 2010). The consequence of this is chronic maldigestion that leads to emaciation and eventual death (Hoppes et al., 2010). Central nervous system (CNS) disease is more common in some taxonomic groups (e.g., waterfowl) whereas gastrointestinal disease is the more common presentation in others (e.g., parrots) (Gregory et al., 1994; Delnatte et al., 2013). Clinical CNS disease may include tremors, seizures, ataxia or paralysis (Gregory et al., 1994). Inflammation in other organ systems is rare but has been described. For examples, blindness may result after the virus spreads through the optic nerve to the retina, and sudden death can occur after virus and associated inflammation spreads to cardiac ganglia (Steinmetz et al., 2008; Shivaprasad et al., 2010).

1.2.3 Postmortem lesions

Common gross observations of PDD include severe pectoral muscle atrophy, and marked dilation of the proventriculus and ventriculus with impaction of the proventriculus and intestines with undigested food (Gancz et al., 2009). The liver may also be diffusely pale due to chronic malabsorption (Gancz et al., 2009).

Non-purulent inflammation of the nervous system is a hallmark microscopic lesion of PDD. Specifically, lymphoplasmacytic infiltration within peripheral nerves that innervate the proventriculus and ventriculus is characteristic (Ritchie et al., 1998). In addition, lymphoplasmacytic infiltration as perivascular cuffing within the grey matter of the cerebrum, cerebellum as well as the spinal cord may be present in affected birds with CNS symptoms (Shivaprasad et al., 1995). In some cases, infiltration of similar inflammatory cells is seen in the heart and in the retina (Vice et al., 1992). On occasion, inflammation also extends to the parenchyma of tissues adjacent to the affected nerves and ganglia, such as adrenal glands, testes, and smooth muscle (Doneley et al., 2007).

1.2.4 Experimental reproduction of PDD in vivo

Multiple experimental inoculations of psittacine birds with ABVs have attempted to understand the pathogenesis of PDD. In one experiment, individual cockatiels (*Nymphicus hollandicus*) were challenged by administration of brain homogenate of an African gray parrot (*P.
erithacus) that tested positive for *Psittaciform 1 orthobornavirus* genotype parrot bornavirus 4 (PaBV-4) through multiple inoculations via the intramuscular, intraocular, intranasal and oral routes (Gancz et al., 2009). Challenged birds developed signs of PDD within 21 days post inoculation (dpi), including a rapid decrease in body weight associated with passing of undigested food in feces (Gancz et al., 2009). One bird showed neurological disease and behavioral changes, including paralysis and feather picking (Gancz et al., 2009). Postmortem analysis showed extensive dilatation of the proventriculus, which was impacted with undigested seeds, undigested seeds in the intestine, and hepatic lipidosis (consistent with chronic malabsorption) (Gancz et al., 2009). Infected cockatiels had extensive lymphoplasmacytic inflammation in the nervous tissue, including the myenteric ganglia, epicardial ganglia, and the cerebral grey matter (Gancz et al., 2009). Experimentally infected Patagonian conures (*Cyanoliseus patagonis*) showed similar pathology after challenge with duck embryo fibroblasts (DEF) persistently infected with *Psittaciform 1 orthobornavirus*, genotype PaBV-4 (Gray et al., 2010). Experimental infection of cockatiels (*N. hollandicus*) that were naturally infected (i.e., shedding) with bornavirus have been also performed. In this experiment, cockatiels were shown to be healthy carriers of PaBV-4 and were actively shedding the virus in their feces as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) (Payne et al., 2011). Despite evidence of previous infection, the cockatiels developed severe disease when inoculated with a homologous strain through the intramuscular route, albeit after an extended incubation periods (92 to 110 days) (Payne et al., 2011). Histopathology revealed extensive lesion development (lymphoplasmacytic inflammation, perivascular cuffing) throughout not only typically affected organs (e.g., gastrointestinal tract, brain, myenteric ganglia) but also other visceral organs, such as liver, pancreas, lungs, and heart (Payne et al., 2011). These results indicate a carrier status does not prevent clinical disease upon reinfection (Payne et al., 2011).

In contrast, canaries experimentally infected with a *Passeriform 1 orthobornavirus* (canary bornavirus-2 [CaBV-2]), regardless of route of administration (intramuscular, subcutaneous, orally, nasal), did not develop clinical signs or gross or microscopic lesions consistent with PDD (Rubbenstroth et al., 2014). However, these birds had high viral titres in many tissues (e.g., brain, proventriculus, lung, heart, liver and duodenum) as measured by RT-PCR (Rubbenstroth et al.,
Furthermore, mallard ducklings inoculated through intraocular or intramuscular administration with PaBV-4 failed to develop clinical signs or lesions but were positive for bornavirus in tissues by RT-PCR (Gray et al., 2009).

1.2.5 Transmission and shedding

Multiple studies have documented bornavirus RNA in feces, which is suggestive of a fecal-oral route of transmission. Further, the highest concentration of bornavirus RNA is often found within feces (Hoppes et al., 2010). However, bornavirus RNA has also been detected in feathers, choanal and cloacal swabs, and even in the filters of air ducts of aviaries housing infected birds (Hoppes et al., 2010; de Kloet et al., 2011). This is consistent with infection in mammals, in which BoDV-1 transmission primarily occurs via the fecal-oral route (Sauder and Staehli, 2003). Transmission studies have revealed that uninfected rats were readily infected after exposure to feces and urine from BoDV-1-infected rats; infection in the former group was confirmed through immunohistochemical (IHC) reactivity for the virus within olfactory nerves (Sauder and Staehli, 2003). Avian bornaviruses are expected to be transmitted in similar ways. Furthermore, mallards that inadvertently consumed the droppings of infected parrots shed the virus in the feces but did not develop clinical signs (Hoppes et al., 2010).

Vertical transmission for ABV has not yet been confirmed. In the aforementioned experimental infection study of canaries with CaBV-2, birds were co-housed and allowed to breed after exposure to virus (Rubbenstroth et al., 2014). Resulting chicks were tested for CaBV-2 infection immediately after hatching by RT-PCR and IHC (Rubbenstroth et al., 2014). While the chicks showed no evidence viral infection by IHC, viral RNA was detected in tissues by RT-PCR (Rubbenstroth et al., 2014). In another experiment, yolk from one Canada goose egg (out of 53) tested positive for aquatic bird bornavirus (ABBV) through RT-PCR (Delnatte et al., 2014). Therefore, there is lack of evidence to support that vertical transmission is an important method of spread of ABVs.
1.3 Avian bornaviruses in waterfowl

1.3.1 Etiology

Despite the identification of bornaviruses from multiple avian hosts, PDD remains a disease associated with psittacines; however, PDD-like lesions had been observed in Canada geese (*Branta canadensis*) as far back as 1991 (Daoust *et al*., 1991). Postmortem examination of these infected geese included severe emaciation with non-suppurative inflammation of the nervous system (encephalitis and ganglioneuritis) (Daoust *et al*., 1991). Grossly, affected geese had serous atrophy of fat, severe atrophy of pectoral muscles, and dilated and impacted proventriculus with partially digested food (Daoust *et al*., 1991). Histologically, there was extensive lymphoplasmaacytic inflammation including perivascular cuffing in the proventriculus (Daoust *et al*., 1991). Proventricular impaction in geese is also a common gross finding with lead poisoning; however, lead levels detected in these affected geese were below those considered toxic for this species (Daoust *et al*., 1991). Thus, these findings appear to be suggestive of a viral disease; however, despite attempts, the researchers were not successful in isolating an infectious agent from the brain homogenates in cell culture (Daoust *et al*., 1991).

Soon after the identification of avian bornaviruses in 2008 in psittacine birds, waterfowl-specific avian bornaviruses were identified in 2009 and were later assigned the species name *Waterbird 1 orthobornavirus* (Payne *et al*., 2011; Delnatte *et al*., 2013; Kuhn *et al*., 2015). At the time of writing, the *Waterbird 1 orthobornavirus* species includes two genotypes: aquatic bird bornavirus 1 (ABBV-1) and aquatic bird bornavirus 2 (ABBV-2). Brain tissue from waterfowl species (Canada geese, mute swans [*Cygnus olor*], trumpeter swans [*C. buccinator*]) collected in Ontario from 1992-2011 from cases with a history or lesions consistent with PDD were tested for the presence of a bornavirus through immunohistochemistry (IHC) and RT-PCR (Delnatte *et al*., 2013). Results indicated the presence of bornavirus RNA or protein in a majority of cases (Delnatte *et al*., 2013).

1.3.2 Prevalence among wild birds in North America, and Northern Europe

The prevalence of *Waterbird 1 orthobornavirus* in North America and Europe among species of wild waterfowl has been investigated using different assays to detect virus or evidence
of viral infection. In general, these tests include IHC, RT-PCR (on cloacal swabs, feces) or tissues, and serology for anti-bornavirus antibodies (Delnatte et al., 2013). RT-PCR is most commonly used due to the simplicity of sample collection and efficiency of analysis. A survey of healthy Canada geese across multiple migratory flyways in the United States revealed a low prevalence of bornavirus shedding (3%; 12/409), as determined by RT-PCR of cloacal swabs (Payne et al., 2011). In the same study, when a subset of brain samples was analyzed in the same way, the virus prevalence was much higher (11/25, 44%) (Payne et al., 2011). This discrepancy may in part reflect that fact that bornavirus shedding from the cloaca is intermittent and thus may not accurately represent true infection status in all cases (Hoppes et al., 2013). A later study attempted to assess the prevalence of ABBV-1 through RT-PCR of the brains of mute swans from the northeastern United States (Guo et al., 2012). Results indicated that 23% (45/192) of swans were positive for ABBV; however, when considering populations of birds from specific geographical locations (e.g. Michigan, USA), prevalence was greater than 50% (Guo et al., 2012).

ABBV-1 prevalence among waterfowl (i.e., Canada geese, mute swans, and trumpeter swans) throughout Southern Ontario has been determined by IHC and RT-PCR on central nervous system (cerebrum, cerebellum, brainstem, optic lobes, and spinal cord) and peripheral nervous system (ganglia of proventriculus, ventriculus, esophagus, intestines, cloaca, and intestines) tissue. Some of these birds had pathology suggestive of PDD-like disease (e.g., gastrointestinal impaction, encephalitis, peripheral ganglioneuritis, etc.) (Delnatte et al., 2013). IHC confirmed infection with bornavirus in 23/41, 6/8, and 0/2 Canada geese, trumpeter swans, and mute swans, respectively. Likewise, with Guo et al. (2012, discussed above) there were populations where the proportions were considerably higher, with 18/27 of Canada goose samples from the Toronto Zoo testing positive (Delnatte et al., 2013). Infection was confirmed in 15/16 Canada geese and no mute swans, with the majority of positive birds originating from the Toronto Zoo (Delnatte et al., 2013). To further characterize the presence of ABBV and to validate the use of cloacal swabs testing as a mean of determining prevalence of infection, results from serology and RT-PCR on cloacal swabs collected from apparently healthy, free-ranging Canada geese, mute swans, trumpeter swans and mallards in southern Ontario were compared (Delnatte et al., 2014). Results revealed that a large proportion of (58%, 117/203) of these birds were seropositive for ABBV,
however, only a few birds had ABBV RNA detected by RT-PCR (≤10% of mute swans and 0% of mallards and trumpeter swans) (Delnatte et al., 2014).

Although there have been numerous investigations of the prevalence of ABBV-1 in North America, this virus has been less studied in other regions (Thomsen et al., 2015). A study of hunter-killed wild geese in Denmark evaluated presence of ABBV-1 RNA via RT-PCR analysis from brain tissues; evidence of ABBV infection was detected in three species (greylag goose [Anser anser], barnacle goose [B. leucopsis], pink-legged goose [Anser brachyrhynchus]), with an overall prevalence of 2.1% (7/333). Sequencing of these viruses showed high identity with a goose isolate (ABBV-1) from North America (Thomsen et al., 2015). However, none of the geese that were positive showed signs or neurological disease, indicating that they were healthy carriers (Thomsen et al., 2015).

1.3.3 Significance

As the previously mentioned studies have shown, the prevalence of ABBV-1 within free-ranging wild birds in Ontario and the United States is high—in some cases above 50% (Guo et al., 2012; Delnatte et al., 2014). Furthermore the host range of ABVs appears to be broad, as these viruses have been known to infect a wide variety of orders of birds outside of the commonly recognized reservoirs (i.e., Psittaciformes, Passeriformes and Anseriformes). Specifically, ABBV-1 has been found to cause disease in birds outside the order Anseriformes. A recent characterization of apparently healthy herring gulls (Larus argentatus), ring-billed gulls (L. delawarensis) and laughing gulls (Leucophaeus atricilla) (Order Charadriiformes) revealed ABBV-1 RNA in 9/439 brain homogenates, with 8/9 of the positive samples originating from the same location (Guo et al., 2015). Sequencing of the isolated virus indicated that it was closely related to ABBV-1 described in North American geese (Guo et al., 2015). One of the nine brains that tested positive also had lymphoplasmacytic infiltrates in the cerebrum consistent with ABV infection (Guo et al., 2015).

Tissues from a necropsied emu (Dromaius novaehollandiae [Order Casuariiformes]) from the Toronto Zoo, which presented with emaciation and neurological signs, tested positive for bornaviruses by both IHC and RT-PCR. Sequencing of the isolate revealed the virus was ABBV-
There have also been reports of ABBV-1 detected in a bald eagle (*Haliaeetus leucocephalus* [Order Accipitriformes]) (Payne *et al*., 2012). In addition, a Himalayan monal (*Lophophorus impejanus* [Order Galliformes]) died after exhibiting emaciation, proprioceptive abnormalities and ataxia. Brain from this bird tested positive for bornavirus, although the virus was determined to be a PaBV-4 genotype of the species *Psittaciform 1 orthobornavirus*, which commonly infects parrots (Bourque *et al*., 2015). This is a testament to the fact that bornaviruses affecting avian species seems to have a wide host range and readily spill over to other species, to which they are not epidemiologically associated. Collectively, these findings emphasize the lack of understanding of ABBV host restriction, and highlight the threat that ABBV may pose by infecting and becoming established in commercial poultry species (e.g., chickens, turkeys).

### 1.4 Research goals and objectives

#### 1.4.1 Gaps of knowledge

Gaps of knowledge permeate many aspects of the biology of ABBV-1 *in vitro* and *in vivo*. *In vitro* characterization of ABBV-1 is necessary in order to establish a reliable system for culture and titration, which is needed to enable further disease characterization and pathogenesis experiments in live birds. Additionally, the kinetics of virus growth in different primary and established avian cell lines could be used as an *in vitro* model to evaluate and study the molecular aspects of host restriction without the use of live birds (e.g., do cell lines from different avian species allow differential replication of ABBV-1?).

The pathogenesis of ABBV-1 in birds, including poultry and natural hosts such as geese and ducks, is not well characterized. Strains of ABBV-1 are capable of naturally infecting birds outside of the *Anseriformes* order, suggesting a wide range of possible hosts (Payne *et al*., 2012; Guo *et al*., 2015; Nielsen *et al*., 2017). Specifically, the ability of ABBV-1 to infect and replicate, as well as cause disease in and be shed by relevant poultry species (most notably chickens and turkeys) remains unknown. Additionally, the pathogenesis of ABBV-1 even in identified hosts (e.g., Canada geese and other waterfowl) has not been fully elucidated, as - to date - disease has not been reproduced through experimental inoculation of ABBV-1 in waterfowl. Characterization
of ABBV-1 host restriction and pathogenesis will improve our understanding of ABBV-1 epidemiology and potential surveillance strategies.

1.4.2 Hypotheses

1. ABBV-1 can be propagated in continuous cell lines from multiple avian species.
2. ABBV-1 has differential replication kinetics in primary cell lines from different avian species; this could model the susceptibility of those species to become infected \textit{in vivo}.
3. An \textit{in ovo} model is suitable to assess pathogenesis and growth of ABBV-1 in different avian species.

1.4.3 Objectives and proposed methods

Pursuant to the above hypotheses, the objectives of the proposed research can be broadly divided into two main areas: \textit{in vitro} and \textit{in ovo} characterization of ABBV-1.

A. The objectives relating to the \textit{in vitro} characterization include:

1. Evaluate the ability of ABBV-1 to grow in multiple continuous cell lines from avian species, and assess virus production in these lines
2. Assess viral replication kinetics in primary cell lines derived from representative poultry (chickens, turkeys) and waterfowl (ducks, geese) species (i.e., duck embryo fibroblasts [DEF], chicken embryo fibroblasts [CEF], goose embryo fibroblasts [GEF], turkey embryo fibroblasts [TEF]).

B. The objectives related to the \textit{in ovo} characterization include:

1. Assess the pathogenicity of ABBV-1 through inoculation of embryonated chicken, duck and turkey eggs by:
   i. Inoculating eggs through two different methods: chorio-allantoic and yolk sac;
   ii. Harvesting embryos from inoculated eggs at two time points during development: early and late;
   iii. Assessing viral replication through RT-qPCR and immunohistochemistry (IHC) in embryonic tissues;
   iv. Assessing lesion development in embryonic tissues though histopathology.
1.5 Figures and tables

Table 1-1: List of genera, species, and genotypes in the family *Bornaviridae*. Modified from Maes *et al.* 2019.

<table>
<thead>
<tr>
<th>Family <em>Bornaviridae</em></th>
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<tbody>
<tr>
<td><strong>Carbovirus</strong></td>
</tr>
<tr>
<td><em>Queensland carbovirus</em> jungle carpet python virus (JCPV)</td>
</tr>
<tr>
<td><em>Southwest carbovirus</em> southwest carpet python virus (SWCPV)</td>
</tr>
<tr>
<td><strong>Orthobornavirus</strong></td>
</tr>
<tr>
<td><em>Elapid 1 orthobornavirus</em> Loveridge’s garter snake virus 1 (LGSV-1)</td>
</tr>
<tr>
<td><em>Mammalian 1 orthobornavirus</em> Borna disease virus 1 (BoDV-1)</td>
</tr>
<tr>
<td><em>Mammalian 2 orthobornavirus</em> variegated squirrel bornavirus 1 (VSBV-1)</td>
</tr>
<tr>
<td><em>Passeriform 1 orthobornavirus</em> canary bornavirus 1 (CnBV-1)</td>
</tr>
<tr>
<td><em>Passeriform 2 orthobornavirus</em> estrildid finch bornavirus 1 (EsBV-1)</td>
</tr>
<tr>
<td><em>Psittaciform 1 orthobornavirus</em> parrot bornavirus 1 (PaBV-1)</td>
</tr>
<tr>
<td><em>Psittaciform 2 orthobornavirus</em> parrot bornavirus 5 (PaBV-5)</td>
</tr>
<tr>
<td><em>Waterbird 1 orthobornavirus</em> aquatic bird bornavirus 1 (ABBV-1)</td>
</tr>
<tr>
<td><em>Waterbird 2 orthobornavirus</em> aquatic bird bornavirus 2 (ABBV-2)</td>
</tr>
</tbody>
</table>
Figure 1-1: General arrangement of the Borna disease virus 1 genome.

Figure 1-2: Schematic of the start/stop sequences and transcripts produced in the Borna disease virus 1 genome.

S1, S2, and S3 represent transcription start sites and E1, E2, E3, E4, and E5 represent transcription termination sites. Numbers below the transcripts represent nucleotide position in the genome. Retrieved online from: https://veteriankey.com/bornaviridae-2/
Chapter 2. Characterization of an Ontario isolate of aquatic bird bornavirus 1 in immortalized avian cell lines

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2.1 Abstract

Aquatic bird bornavirus 1 (ABBV-1) can infect and cause neurological diseases in wild waterfowl; however, its replication in cell culture has only been documented in primary duck embryonic fibroblasts (DEF) and no other avian cells. In this study, ABBV-1 was isolated in DEF, and replication was further evaluated in immortalized avian cell lines, in order to determine a cell line most suitable for routine virus propagation. Virus spread and the amount of viral RNA, protein, and titre produced were assessed in immortalized duck embryonic fibroblasts (CCL-141), quail fibrosarcoma (QT-35) and chicken embryonic fibroblasts (DF-1). By passage three after infection, ABBV-1 established persistent infection in most CCL-141 and QT-35 cell cultures, but not in DF-1, where only few cells were infected. ABBV-1 RNA and infectious titre levels were highest in CCL-141, intermediate in QT-35, and lowest in DF-1. Unexpectedly, ABBV-1 N protein levels appeared to be similar or higher in QT-35 than in CCL-141, however the lower titre in QT-35 suggests inefficient viral assembly. In DF-1, infectious titre was not measurable, although low levels of viral RNA and N protein could be detected over multiple passages. CCL-141 yielded comparable titers to DEF, suggesting that CCL-141 can replace DEF in routine ABBV-1 propagation, allowing for improved consistency and standardization of virus production. The diverse replication efficiency of ABBV-1 in these cell lines offers opportunity for future studies of its replication mechanisms and host restriction.

**Keywords:** avian bornavirus, aquatic bird bornavirus, orthobornavirus, cell lines, waterfowl, poultry, goose, turkey
2.2 Introduction

The term avian bornavirus (ABV) encompasses a diverse group of viruses within the genus Orthobornavirus and family Bornaviridae, which naturally infect and are established in avian populations. There are five recognized species of ABV: Passeriform 1 orthobornavirus, Passeriform 2 orthobornavirus, Psittaciform 1 orthobornavirus, Psittaciform 2 orthobornavirus and Waterbird 1 orthobornavirus (Amarasinghe et al., 2017; Maes et al., 2019). The species Waterbird 1 orthobornavirus currently encompasses two genotypes, aquatic bird bornavirus 1 and 2 (ABBV-1 and ABBV-2). ABBV-1 was first identified in a retrospective post-mortem evaluation of Canada geese (Branta canadensis), trumpeter swans (Cygnus buccinator), and mute swans (C. olor) with neurological disease from Southern Ontario, Canada, using RT-PCR and immunohistochemistry (IHC) (Delnatte et al., 2011). Since then, the virus has been detected in emu and gulls, where clinical disease was apparent (Guo et al., 2015; Nielsen et al., 2018), as well as in subclinical wild geese (including Canada geese), mute swans and mallards (Payne et al., 2011; Delnatte et al., 2014; Thomsen et al., 2015). ABBV-1 is an enveloped negative-sense single-stranded RNA virus with a genome of approximately 9 kb, which contains six genes encoding for five structural proteins and one non-structural protein, arranged in order from the 3’ to 5’: nucleocapsid (N), non-structural X protein, phosphoprotein (P), matrix (M), glycoprotein (G), and RNA-dependent RNA polymerase (L) (Guo et al., 2013).

The isolation of viruses in cell culture is considered the “gold standard” for virus identification (Leland and Ginocchio, 2007), while the propagation of viruses in cell culture aids in the characterization of virus lifecycles and provides insight into innate host response to infection. Although primary cell cultures are recommended for isolation of avian viruses such as infectious bursal disease virus, Marek’s disease virus, Newcastle disease virus, and ABVs from infected tissues (Guo et al., 2014; OIE - World Organization for Animal Health, 2018), continued use of primary cultures for routine virus production presents multiple disadvantages. These include heterogeneity within and between batches due to the use of different animals each time a culture is made, limited in vitro life span before senescence ensues (Abbott et al., 1974; Beug and Graf, 1977), and constant access to readily available producer animals or eggs, which can lead to increases in time and costs of preparation. Obtaining virus-free eggs can be also challenging; for
example, ABV has been detected in the yolk of a Canada goose egg (Delnatte et al., 2014) and in embryos of commercial duck eggs (Hoppes et al., 2013) and of naturally infected psittacines eggs (Lierz et al., 2011; Kerski et al., 2012; Monaco et al., 2012). This problem is exacerbated when the virus is highly prevalent in the host population, which for ABBV-1, based on detection of antibody titres, can be up to 50% of certain waterfowl populations depending on location (Guo et al., 2012). Therefore, identification of immortalized cell lines that can be used to routinely propagate ABV would lessen the need for primary embryonic cultures during routine virus production and thus alleviate the drawbacks associated with primary cultures.

Many ABVs have been isolated using either primary embryonic cultures or immortalized cell lines, and all species contain at least one genotype that can replicate in cell culture. The current ABV invitrome, which is defined as the current collection of cell cultures known to support ABV replication (Bols et al., 2017), is briefly reviewed below and in Table 2-1. The species Passeriform 1 orthobornavirus contains three genotypes, canary bornaviruses 1-3 (CnBV-1-3), that are all capable of replicating in primary duck embryonic fibroblasts (DEF) and immortalized quail and chicken cell lines (Rubbenstroth et al., 2013). The Passeriform 2 orthobornavirus has one genotype, estrildid finch bornavirus 1 (EsBV-1), and it can also propagate in the same cell cultures that supported CnBV-1-3 (Rubbenstroth et al., 2014). Five genotypes within the Psittaciform 1 orthobornavirus species are parrot bornaviruses 1-4 and 7 (PaBV-1-4 and -7). PaBV-1 was isolated on DEF cultures (Gray et al., 2010), however propagation in immortalized cell lines is not documented. PaBV-2 can grow in DEF cultures (de Araujo et al., 2018) and in immortalized quail cell lines (Rinder et al., 2009; Reuter et al., 2010; Horie et al., 2015). PaBV-4 and PaBV-7 can both replicate in DEF cultures (Gray et al., 2010; Rubbenstroth et al., 2012) and in immortalized quail and chicken cell lines (Rinder et al., 2009; Horie et al., 2015; Rubbenstroth et al., 2012). However, PaBV-3 has not been isolated in cell culture. The species Psittaciform 2 orthobornavirus has only one genotype, PaBV-5, and it has only been isolated in DEF cultures to date (Guo and Tizard, 2015). PaBV-6 (Marton et al., 2015) and PaBV-8 (Philadelpho et al., 2014) genotypes have been identified but have not been assigned a species; these genotypes have not been isolated in cell culture. None of these ABV genotypes replicated in mammalian cell lines except for EsBV-1, which was only detected in Vero cells after 30 to 60 passages (Table 2-1). Although both
ABBV-1 and ABBV-2 have been isolated in DEF cultures (Payne et al., 2011; Guo et al., 2014), to date, there are no publications on the propagation of either ABBV-1 or ABBV-2 in immortalized cell lines.

The goal of this research was to characterize ABBV-1 replication in immortalized avian cell lines, in order to identify a suitable producer cell line that can be used to routinely propagate the virus. In this report, ABBV-1, isolated from an infected goose brain in DEF cultures, was shown to be capable of producing infectious virions in immortalized duck embryo (CCL-141) and quail (QT-35) fibroblasts cell lines. Infectious particles were not detected in immortalized chicken embryo fibroblasts (DF-1), although low-level viral gene and protein expression was suggested by reverse transcriptase (RT) quantitative (q) PCR and western blot. Of the three immortalized cell lines, the highest titre of ABBV-1 was recovered from the CCL-141 duck embryo cell line and the titre level was comparable to the titre from infected DEF.

2.3 Materials and methods

2.3.1 Cells

Four cell culture systems were used. One primary cell line of duck embryo fibroblasts (DEF) derived from Pekin duck embryos (King Cole Duck Ltd., Whitchurch-Stoufville, Ontario) using standard protocols (Schat and Sellers, 2008), and three immortalized cell lines: duck embryo fibroblasts (CCL-141) (Wolf et al., 1974), chicken embryo fibroblasts (DF-1, CRL-12203) (Schaefer-Klein et al., 1998), both obtained from the American Type Culture Collection (ATCC), and quail fibrosarcoma cell line (QT-35) (Moscovici et al., 1977). For routine propagation, DEF, CCL-141, QT-35 and DF-1 cells were grown in maintenance media (Dulbecco's Modified Eagle Medium [DMEM; Corning, ThermoFisher, Mississauga, Ontario] supplemented with 10% fetal bovine serum [FBS; Hyclone, ThermoFisher] and 1% Penicillin-Streptomycin-Amphotericin B [PSA; Hyclone, ThermoFisher]). For passaging, cells were washed with phosphate buffer saline (PBS, Hyclone, ThermoFisher) and dissociated with either Trypsin-EDTA (Hyclone, ThermoFisher) diluted at 0.05 to 0.125% in PBS or 1x TryPLE (Gibco, ThermoFisher). DEF cells were passaged every two to three days and the immortalized cell lines every five to eight days, at a split ratio of 1:2 to 1:3. All cultures were incubated at 37°C in an atmosphere of 10% CO₂.
method of cell propagation was the same also for cells that were persistently infected with ABBV-1 (see below).

2.3.2 Isolation of ABBV-1 in DEF

ABBV-1 was isolated from the brain of a naturally infected Canadian goose (*Branta canadensis*), a kind gift from Dr. Dale Smith of the Ontario Veterinary College. Virus isolation was carried out as previously described (Guo *et al.*, 2014), with minor modifications. Briefly, goose brain was homogenized and 20 µL were used to directly infect DEF (passage 2, approximately 75% confluence) in a 12-well plate (Nunc, ThermoFisher), to reach a final 1:10 dilution in DMEM with 1% PSA without serum. The inoculum was kept until DEF cells were passaged into a 6-well plate (Nunc, ThermoFisher) with maintenance media. Confirmation of persistent infection was carried out at the Animal Health Laboratory (Guelph, Ontario, Canada) by performing RT-qPCR for ABBV-1 M gene (Delnatte *et al.*, 2013) on cell pellets (1,000,000 cells); uninfected DEF were negative.

2.3.3 Sequencing of ABBV-1

The identity of the isolated virus was further confirmed by full-genome sequencing and assembly of the ABBV-1 genome. Based on available ABBV-1 genome sequences from GenBank, a total of 18 overlapping primer pairs (Appendix 1) were used to reverse transcribe and amplify the virus genome from nucleotide 80 to 8,957. Briefly, RNA was extracted from infected DEF using the Roche High Pure RNA Isolation Kit (Roche Diagnostics, Indianapolis, IN), reverse transcribed with M-MuLV reverse transcriptase (NEB, Whitby, ON) and amplified with Q5® High-Fidelity DNA Polymerase (NEB, Whitby, ON), according to the manufacturer’s instructions. The PCR cycle conditions were: 98°C for 1 min, followed by 40 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 75 s, then a final extension of 72°C for 5 min. Genomic ends were amplified by 3’ and 5’ RACE PCR, as previously described (Li *et al.*, 2005). Briefly, the 3’ end of viral RNA was ligated with adaptor DT88 using T4 RNA ligase (Invitrogen, ThermoFisher) and cDNA synthesis of the ligation product was conducted using primer DT89 and primer 22-R (Supplementary Table 1). The 5’ end was amplified using a poly-C tailing approach (Albuquerque-Silva *et al.*, 1998) with primer 19-F (Appendix 1) followed by poly C-tailing using dCTP and terminal
deoxynucleotidyl transferase (TdT; NEB, Whitby, ON). Further PCR amplification of C-tailed cDNA was performed using the 5’ RACE abridged anchor primer (AAP) and primer 20-F (Appendix 1). Amplified genomic regions were resolved on agarose gel, excised, and subjected to Sanger sequencing (Advanced Analysis Centre, Genomics Facility, University of Guelph). Partial sequences were assembled using Geneious, version 8.0 (Biomatters Inc., Newark, New Jersey, USA).

2.3.4 Infection of immortalized cell cultures with ABBV-1 and comparison as a producer cell lines

Cultures of CCL-141, QT-35 and DF-1 in T25 cm² flasks were infected by incubating cells with a total of 5 mL maintenance media containing 6.13x10⁵ focus forming unit [FFU]/mL of ABBV-1 (stock produced from infected CCL-141 by freeze and thaw – see below). Five days post infection (dpi), cells were passaged at a 1:3 ratio using regular maintenance media (passage (p) 1). Presumably infected cultures were then passaged every 5 to 8 days, for a total of 13 passages. In order to assess the suitability of producer cell lines, at defined passage intervals, some of the cells were harvested for either immunofluorescence (p1-3), RNA extraction and RT-qPCR (p2-7), virus isolation and titration (p8-13), protein extraction and western blotting (p11-13), or processed for transmission electron microscopy (TEM) imaging (p12).

2.3.5 Harvesting and titration of ABBV-1 from infected cell cultures

A cell-free virus stock from persistently infected cell lines (both primary or immortalized) was harvested using a freeze and thaw method. Briefly, cells were detached from the flasks with trypsin and centrifuged at 300 x g for 5 min. After centrifugation, cells were re-suspended in 5 mL of 2% FBS/DMEM (for T25 cm² flasks) and 10 mL of 2% FBS/DMEM (for T75 cm² flasks) and subjected to three cycles of freezing (-80°C) and thawing. The mixture was centrifuged again at 2000 x g for 5 min to pellet cellular debris. The supernatant was collected, aliquoted, and stored at -80°C.

The amount of infectious virus in the stock was determined in one of the four cell lines (DEF, CCL-141, QT-35, DF-1, depending on the experiment - see below) through limiting dilutions in 96-well plates, and calculated using the Spearman-Karber method of 50% tissue
culture infectious dose (TCID\textsubscript{50}). Two hundred microliters of ten-fold serial dilution of virus suspensions in 2% FBS/DMEM were added to each well and incubated for five to seven days. After incubation, immunofluorescence (see below) was performed and wells were scored positive or negative for presence of virus by observation under a Zeiss Fluorescent Microscope (Axio Observer.A1). The titers were reported in TCID\textsubscript{50}/mL and converted to FFU/mL by multiplying by 0.69 (Luria et al., 1978).

In order to test if PCR could be used as a more sensitive method to calculate virus titer in cases of low-level virus replication, a RT-qPCR based variation of the TCID\textsubscript{50} assay (Gustafsson et al., 2012) was used, where titration was done on 24-well plates instead of 96-well plates and each well was scored for presence of ABBV-1 using RT-qPCR (see virus titration by RT-qPCR below) instead of immunofluorescence staining.

2.3.6 Immunofluorescence (IF)

Immunofluorescence (IF) was conducted to evaluate ABBV-1 infection of persistently infected cells, as well as to determine infection of single wells in 96-well plate format for TCID\textsubscript{50}. To detect infection, a rabbit monospecific antibody (Ab) (Pacific Immunology, Ramona, CA) against a peptide spanning residues 332 to 354 of the ABBV-1 N protein (Cys-KEAQLARYRRREVTRGEDGAHLS) was used at 1 µg/ml. The secondary Ab was a goat antibody against rabbit IgG conjugated with AlexaFluor488 (ThermoFisher) used at 1 µg/ml. Briefly, supernatant was removed from the plates/wells, cells were washed with twice with PBS and fixed with 1:1 methanol-acetone mixture for 20 min at -20°C. Cells were then washed twice with PBS and blocked for 1 to 3 hrs with 5% goat serum diluted in PBS-T (PBS with 0.1% Tween-20). After blocking, cells were incubated with the primary Ab diluted in blocking buffer for 1 to 3 h at room temperature or overnight at 4°C. Cells were then washed three times with PBS-T and finally incubated with the fluorophore-conjugated secondary Ab in blocking buffer at room temperature in dark chamber for 1 h. Cells were then washed three times, incubated with DAPI (0.5 µg/ml, diluted in PBS) for approximately 5 min and observed using a fluorescent microscope (Zeiss Axio Observer.A1). Uninfected cells that underwent the same IF procedure were used as a control.
2.3.7  Protein extraction, quantification, and western blot analysis

To determine virus presence and relative amounts of viral N protein, western blot was conducted on persistently infected cell lines. Briefly, cells were washed with PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate). After 30 min the cell lysates were centrifuged at 10,000 × g for 15 min at 4 °C to remove cellular debris, and supernatant were collected. Protein concentration was inferred by spectrophotometry using the Pierce BCA Protein Assay Kit (ThermoFisher). Cell lysates were then incubated for 10 min at 95 °C on a hot plate with 5% (v/v) β-mercaptoethanol to denature proteins, followed by separation on a 10% SDS-PAGE at 120 V for 1.5 h, and transferred to a polyvinylidene difluoride (PVDF) membrane with 1x Towbin buffer at 25 V for 1 h (semi-dry transfer, BioRad). Membranes were then blocked with 5% skim milk in PBS-T at 4°C overnight and subsequently incubated with either anti-ABBV-1-N Ab diluted 1 µg/ml or mouse monoclonal anti-beta actin diluted 1 µg/ml (ThermoFisher, Mississauga, Ontario) in blocking buffer at room temperature (RT) for 1 hour with constant shaking. After being washed three times with PBS-T, membranes were incubated at RT for 1 hour with 0.5 µg/ml dilution of either goat anti-rabbit or goat anti-mouse IgG secondary Abs conjugated to horseradish peroxidase (ThermoFisher, Mississauga, Ontario). After three washes with PBS-T membranes were incubated with the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher, Mississauga, Ontario) and detected with a BioRad ChemiDoc MP Imaging System until signal developed (Image Lab 6.0.1 software).

Semi-quantitative analysis to determine the relative N protein expression levels between all samples was performed using the BioRad Image Lab 6.0.1 software. For each sample, the intensity of the N bands was normalized the one of the β-actin (housekeeping protein); mean normalized values were compared between cell cultures.

2.3.8  Virus titration by RT-qPCR

RT-qPCR was performed on cell cultures infected with ABBV-1 to quantify viral RNA levels over multiple passages. RNA from samples was extracted with RNeasy Mini Kit (Qiagen, Toronto, ON) according to manufacturer’s protocol. Purified RNA was reverse transcribed and
amplified using a Luna Universal Probe one-step RT-qPCR kit (NEB, Whitby, ON) with primers and probes targeting the ABBV-1 N gene (forward primer, 5’-ATG CAC TTG CAC TCT TAG AC-3’; reverse primer, 5’-TCC CCA TAA AAC CTC CCA AC-3’; probe, 5’-6-FAM-CCC TGC CCG CAG AGA GAA ATT CCA T-BHQ-3’). The cycling conditions were as follows: 55°C for 10 min reverse transcription; 95°C for 1 min initial denaturation, and 40 cycles of 95 °C for 10 s denaturation and 60 °C combined annealing and extension. Negative threshold values (Ct value) was scored as being above 35.

2.3.9 Transmission electron microscopy

Transmission electron microscopy was done to visualize ABBV-1 virions in the persistently infected CCL-141 cell line. Approximately 10^6 cells CCL-141 from passage 12 were fixed overnight at 4°C (4% paraformaldehyde and 2.5% glutaraldehyde in 0.5M HEPES buffer), washed 3 times in HEPES buffer (15 minutes each), and then treated with 1.0% osmium tetroxide for 2 hours. After 3 washes in HEPES buffer, cells were incubated in 2% Uranyl Acetate for 2 hours, washed 3 times with HEPES buffer, and dehydrated through baths with increasing ethanol concentrations (one time in 25%, 50%, 75%, and 95% ethanol and 2 times in 100% ethanol with 20 min changes). Subsequently, cells were infiltrated with a graded series of LR White resin/ethanol (one time in 25%, 50%, and 75%, and twice in 100% resin), embedded in pure resin in airtight capsules, and let to polymerize at 60°C overnight. Sections, 100 nm in thickness, were cut using the Leica Ultracut microtome and mounted on 100 mesh copper grids with formvar and carbon coating. Samples were post stained with Reynold's lead citrate and Uranyl acetate. Imaging was done using the Philips CM 10 transmission electron microscope, and employing the SIS/Olympus Morada camera and iTEM software.

2.3.10 Statistical analysis

Means of N protein optical density (normalized to B-actin) and infectious titre between groups (i.e., cell cultures) were compared using the analysis of variance (ANOVA) test followed by a Tukey post-hoc test with multiple comparisons, as implemented in the GraphPad Prism 8.0 software (GraphPad, San Diego, CA). Differences were considered significant for p < 0.05.
2.4 Results

2.4.1 ABBV-1 isolation and identification in primary duck fibroblasts

To isolate ABBV-1 for subsequent propagation and evaluation in immortalized cell lines, DEF were inoculated with homogenized goose brain. At passage 8 and 10, infection was confirmed by RT-qPCR for the M gene, with Ct values of 19.57 and 17.17, respectively. No virus genetic material was detected in uninfected control DEF. The decrease in Ct values from passage 8 to 10 suggested that the virus was actively replicating. A confluent T75 flask of infected DEF cells yielded, after three cycles of freezing and thawing, approximately 2.09 x10^4 FFU/mL, as determined by limiting dilutions.

An antibody against ABBV-1 N protein was raised by immunizing rabbits with a synthetic peptide. The affinity purified antibody confirmed infection of DEF by detecting an approximately 40 kDa protein band on western blot (WB), consistent with the N protein (Fig. 2-1A). The antibody also detected ABBV-1 in infected DEF (p 7) (Fig. 2-1C), with no signal in uninfected cells (Fig. 2-1B). Immunoreactivity in the nucleus appeared strong and coarsely stippled, and in the cytoplasm was weak with a finely stippled reactivity (Fig. 2-1C). Taken together, data from RT-qPCR, titration of cell lysate, western blot analysis and IF demonstrated that DEF supported the replication of ABBV-1 and became persistently infected. However, no cytopathic effect (CPE) was seen.

Full genome sequencing of the virus was done to identify the ABV species and genotype of this virus. The assembled full genome of the virus was 9006 nucleotide in length, and a Basic Local Alignment Search Tool (BLAST) search showed that it was 97% and 99% identical to ABBV-1 isolate AF-168 from Germany (Rubbenstroth et al., 2016) and 062-CQ from Texas (Guo et al., 2013), respectively.

2.4.2 Characterization and comparison of ABBV-1 replication in immortalized chicken, duck, and quail cell lines

The replication of ABBV-1 in immortalized chicken (DF-1), duck (CCL-141), and quail (QT-35) fibroblast cell lines was compared qualitatively and quantitatively. Qualitatively, infected
cultures at early passages were observed by phase contrast light microscopy to identify possible CPE, and by IF to determine virus spread in the cell population and localization of virus in individual infected cells. Quantitatively, the efficiency of virus replication in each cell line was measured by three methods: RT-qPCR targeting the N gene to compare viral RNA levels, western blotting to determine N protein expression, and titration (TCID<sub>50</sub>) of virus form cell lysate. Throughout all the passages, no morphological differences between the infected and uninfected cells were observed by phase contrast light microscopy for any of the cell lines. The other results are described in the sections below.

2.4.3 ABBV-1 spread and localization in cell culture

Early replication and spread of virus in cell populations (CCL-141, QT-35 and DF-1) over the first three passages post infection were monitored by IF. The time between each passage ranged from 5 to 8 days, and all three cultures were passaged on the same day. In DF-1, there were only few infected cells at all passages. In contrast, in CCL-141 and QT-35 the number of infected cells increased between passages, with the highest increase of positive cells from passage 2 to 3 (Fig. 2-2) and by the third passage, the majority of the cells appeared infected (Fig. 2-2). Pattern and distribution of immunoreactivity was similarly to what seen in DEF, with strongest signal in the nucleus. Nucleus-associated speckled or punctuated staining was stronger in CCL-141 than in QT-35.

2.4.4 ABBV-1 N gene expression in cell lines

Viral gene expression in cell lines was monitored for up to seven passages after infection by RT-qPCR. For DF-1, the Ct value was 25.00 at passage 2 and remained between 23.00 and 25.00 Ct over five passages, up to passage 7 (Table 2-2), suggesting stable, low-level viral gene expression or genome replication. For CCL-141, the Ct value was 20.52 at passage 2, reached 16.86 at passage 3 and continued to decrease until reaching 15.92 at passage 7, the last measured time point (Table 2-2). The biggest decrease in Ct value was observed from passage 2 to 3. For QT-35, the Ct value was 19.88 at passage 2, decreased to 17.68 at passage 3 and continued decreasing to passage 5; however, after passage 5, the Ct value increased at passage 6 and 7,
indicating some fluctuations in the amount of ABBV-1 N gene expression in this cell line. No viral RNA was detected in uninfected control cells for any cultures.

2.4.5 ABBV-1 N protein expression in cell lines

To compare the magnitude of viral N protein production in each cell line, a western blot with semi-quantitative analysis of the band optical densities was performed on passage 11-13. The time between each passage ranged from 4 to 6 days, and all cultures were passaged on the same day. Prominent N protein bands were detected in lysates from infected CCL-141 and infected QT-35 (Fig. 2-3A). N protein bands were absent in lysates from infected DF-1, however when the exposure time was increased, bands became apparent (Fig. 2-3A). Semi-quantitative assessment of N protein expression was compared by evaluating the optical densities of the bands on the membrane. The average N protein expression level of each cell line was determined relative to β-actin (Fig. 2-3B), and the ratio for each cell line compared to each other. The normalized N protein levels in infected CCL-141 and QT-35 were significantly higher than in infected DF-1 (p < 0.05). While the average N protein expression level of infected QT-35 was higher than infected CCL-141, this difference was not statistically significant (p > 0.05).

2.4.6 ABBV-1 infectious titre production in immortalized cell lines compared to DEF

The production of infectious ABBV-1 titre was measured at different passages using three variations of TCID₅₀ assays described below. In the first variation, the virus collected from each cell line was titred on the same (homologous) cell line using the TCID50 assay; this method was performed for virus collected from passage 8, 12 and 13. According to this method, no measureable infectious titre was detected for DF-1 (Fig. 2-4). For CCL-141 cells, the average ABBV-1 titre from all passages was 4.36 x 10³ FFU/mL per 1,000,000 cells, and for QT-35 cells, the average titre was 1.15 x 10² FFU/mL per 1,000,000 cells (Fig. 2-4).

In the second variation, the virus collected from each cell line was titred on the same batch of primary DEF using the TCID50 assay. In this case, DEF were used as a reporter cell line to standardize the viral titre produced from different producer cells, in order to assess if titration in the same or a different cell line then the producer cells might impact virus titration. No measureable
infectious titre was detected on cell lysate from infected DF-1 when titred on DEF (Fig. 2-4). For virus collected from CCL-141 and QT-35 cells, the average ABBV-1 titre on DEF was $9.33 \times 10^3$ FFU/mL and $4.47 \times 10^1$ FFU/mL per 1,000,000 cells, respectively (Fig. 2-4). For comparison, virus that was collected from persistently infected DEF had an average titre of $7.76 \times 10^3$ FFU/mL per 1,000,000 DEF (Fig. 2-4).

In the third variation, the virus collected from each cell line was titred on the same producer cell line and each well scored using RT-qPCR. RT-qPCR was used to detect low level virus replication in each well of the TCID$\text{}_{50}$ assay that may be below the threshold of IF detection. Virus collected from passage 8 of infected DF-1, CCL-141 and QT-35 was used as positive controls. For DF-1, ABBV-1 titre was not detectable, confirming the TCID$\text{}_{50}$ based on IF results. For infected CCL-141 and QT-35, titres were $1.62 \times 10^2$ and $2.34 \times 10^1$ FFU/mL per 1,000,000 cells, respectively.

Overall, there was no statistically significant difference in the amount of virus detected when the virus was titred on either the producer cell lines or on DEF. Regardless of the detection method, infected CCL-141 cells produced higher titre than infected QT-35 cells, but no infectious titre was detected from infected DF-1 cells.

2.4.7 ABBV-1 visualized in persistently infected CCL-141 by transmission electron microscopy

TEM was used to locate ABBV-1 virions in persistently infected CCL-141 cells, which produced the highest viral titre of the three immortalized cell lines. Virus-like particles with approximately 100 nm diameter and bound by a double-layered membrane were observed in multiple cells of the persistently infected CCL-141 cultures (Fig. 2-5A,B).

2.5 Discussion

An ABBV-1 isolate from Ontario, Canada was isolated in DEF and its replication was characterized in immortalized avian cell lines, DF-1, CCL-141, QT-35. The initial virus isolation was done in DEF because these cells were previously shown to support ABBV-1 replication (Murray et al., 2017; Payne et al., 2011). ABBV-1 replicated in CCL-141 and QT-35 cells,
producing detectable infectious titre, while in DF-1, the virus maintained low-level RNA and protein that was detectable by RT-qPCR and western blot, respectively, but did not yield infectious virus particles as determined by TCID\textsubscript{50} assays. No cytopathic effects were observed in infected cells; however, immunofluorescence staining showed ABBV-1 replication in both the cytoplasm and nuclei of cells but with strongest intensity in the nuclei. Nuclear-localized replication is a characteristic feature of both avian (Ouyang et al., 2009; Raghav et al., 2010; Guo et al., 2014) and mammalian bornaviruses (Briese et al., 1992; Cubitt and Torre, 1994; Pyper et al., 1998), which is a unique feature among other viruses in the \textit{Mononegavirales} order whose replication occurs in the cytoplasm.

Primary DEF showed to be able to become infected, support replication and release of infectious particles of ABBV-1. This is consistent with what is reported in the literature (Table 2-1) in that most reports successfully documented propagation of multiple species of ABVs in DEF. Some, but presumably all, of the DEF used in previous reports (Rubbenstroth et al., 2012, 2013 and 2014) were derived from Pekin duck (\textit{Anas platyrhynchos domesticus}), as in this study. Our results showed that the titre of ABBV-1 from DEF was approximately 2.09 x 10\textsuperscript{4} FFU/mL; unfortunately other publications working with ABBV-1 do not document virus titres from DEF, making comparison impossible (Payne et al., 2011; Guo et al., 2013; Murray et al., 2017). For other avian bornaviruses, DEF grew PaBV-2 and PaBV-4 at 6.5x10\textsuperscript{5} FFU/mL (Hameed et al., 2018) and to 8.0x10\textsuperscript{4} FFU/mL (Gray et al., 2010; de Araujo et al., 2018). However, these titres cannot be directly compared with our results as in those studies PaBV-2 and PaBV-4 were sonicated after freezing and thawing, possibly leading to slightly higher titres.

Of the three immortalized cell lines examined, CCL-141 supported ABBV-1 replication best, followed by quail QT-35 regardless of whether the virus was titred in the homologous producer cell lines or DEF. The additional use of DEF as a reporter cell line in the TCID\textsubscript{50} assay minimizes inherent biological differences between the immortalized cell lines that can influence the result of the TCID\textsubscript{50} assay. These differences include, but likely not limited to, possible variations in availability of cellular receptors for the virus, rate of viral gene and protein expression, and efficiency of virion assembly between the cell lines. In infected CCL-141, average infectious titre was 4.37 x 10\textsuperscript{3} FFU/mL on CCL-141 or 9.33 x 10\textsuperscript{3} FFU/mL on DEF, which is
similar levels to those produced by infected DEF (7.76 x 10^3 FFU/mL) in this report. Therefore, CCL-141 appears to be a suitable candidate to be used as a replacement for DEF for routine propagation of ABBV-1. The quail cell line, QT-35 supported ABBV-1 replication but the infectious titre was significantly lower than the one produced from either CCL-141 or DEF.

The difference in the capacity of CCL-141 and QT-35 to support ABBV-1 replication could be due to either the differentiation stages of the cell lines, permissiveness of fibroblastic cells *in vitro*, or naturally evolved preference for duck cells as waterfowl are natural ABBV-1 hosts. CCL-141 line was developed from fibroblasts of duck embryos (Wolf *et al*., 1974), while QT-35 was developed from quails between 6 to 24 days old (Moscovici *et al*., 1977). Other ABVs also replicate better in embryonic cell lines, even from the same host species. Quail-derived embryonic cell line, CEC32 (Kaaden *et al*., 1982), supported replication of PaBV-2, PaBV-4, PaBV-7 and EsBV-1 better than quail QM7 myogenic cell line from a young bird (Antin & Ordahl, 1991; Rinder *et al*., 2009; Rubbenstroth *et al*., 2012; Rubbenstroth *et al*., 2014). Additionally, for EsBV-1, replication was better in embryonic DF-1 chicken cell line (Schaefer-Klein *et al*., 1998) than in chicken epithelial hepatocellular carcinoma LMH cell line from 20 days old chicken (Kawaguchi *et al*., 1987; Rubbenstroth *et al*., 2014). Variable susceptibility of cell lines to support virus growth could also depend on variability in the efficiency of the antiviral response, including the interferon (IFN) pathway; for instance it has been shown that exogenous IFN-α suppressed PaBV-2 and PaBV-4 replication in CEC32 and QM7 cell line (Reuter *et al*., 2010; Reuter *et al*., 2016).

With regards to the role of cell differentiation in supporting various ABV replication, in the current and multiple previously mentioned studies, fibroblasts cultures (DEF, CEC32 and CCL-141) supported ABV replication best while myogenic QM7 and epithelial hepatocellular carcinoma LMH performed worse. However, since CCL-141, QT-35 and DF-1 cultures in this study are all fibroblasts, it appears that cell differentiation alone is not enough to dictate different levels of support for virus growth. The reason why CCL-141 yielded the highest titer is possibly due to the susceptibility of natural hosts from which the cell cultures derived. Natural ABBV-1 infection has been described in ducks, but not quails or chickens. For example, ABBV-2 has been isolated from ducks (Guo *et al*., 2014), and ducks naturally and indirectly exposed with ABVs developed serum antibody and shed viral RNA in feces (Gray *et al*., 2009; Delnatte *et al*., 2014).
However, to date, there have been no reports of either natural or experimental ABV infection of quail, although the potential does exist for quail to be infected with ABV. In fact, previous *in vitro* studies successfully propagated multiple ABV genotypes in quail cell lines (*Table 2-1*), as also confirmed in the present study, where ABBV-1 grew in QT-35. In addition, quail experimentally infected with BoDV-1 produced high amount of antibodies against the virus (Ludwig and Bode, 2000), suggesting that this species maybe susceptible to ABBV-1 infection.

The level of ABBV-1 N protein produced by QT-35, as determined by western blot, appeared to be slightly higher than production in CCL-141. This was unexpected, as QT-35 produced less infectious virions per cell unit compared to CCL-141. It is possible that an excessively high level of N protein in QT-35 might have affected assembly and replication of infectious virions, by altering the optimal stoichiometric interactions between virus components. While this remains to be experimentally verified, previous research supports these hypotheses. Studies with BoDV-1 showed that high N protein levels can sequester the P protein (Poenisch *et al.*, 2007), preventing interaction with the X protein, ultimately reducing the functionality of the RNP complex and the overall efficiency of virus replication (Schwemmle *et al.*, 1998). Similarly for measles virus, which also establishes persistent infection, overexpression of N protein suppresses replication by interaction with the P protein (Doi *et al.*, 2016). An alternative explanation, as observed with vesicular stomatitis virus, is that at excessive levels the N protein aggregates, preventing optimally interaction with the viral genome (Wertz *et al.*, 1987).

Host restriction *in vivo* may also be reflected *in vitro*, as the literature suggests that chicken-derived cells do not support ABV replication as well as duck or quail cells. For example, replication of CnBV-1, -2, -3, PaBV-2, -4, and -7 in either DF1 or LMH cultures, or both, were lower than in quail cell lines (Rinder *et al.*, 2010; Rubbenstroth *et al.*, 2012; Rubbenstroth *et al.*, 2013). PaBV-1 and PaBV-4 did not replicate in chicken embryo fibroblasts but replicated in DEF (Gray *et al.*, 2010). This trend is confirmed in the present study, as infection of DF-1 with ABBV-1 resulted in no detectable infectious virions after 13 passages, regardless of the detection method. Low sensitivity of the TCID$_{50}$ assay coupled with IF was ruled out as a potential reason for lack of infectious titre detection, as the same method coupled with RT-qPCR to score wells confirmed the initial result. However, maintenance of constant low amounts of viral RNA and protein levels
were recorded over at least seven and three passages, respectively. This suggests that DF-1 is capable of becoming infected by ABBV-1 but the amount of viral RNA and proteins produced is insufficient to support virion assembly or the amount of infectious virions produced is too limited to establish infection levels detectable in the TCID₅₀ assay.

Overall, this study isolated an ABBV-1 virus from a Canada goose in Ontario, Canada, and documented replication of the virus in CCL-141 and QT-35, expanding the repertoire of cell culture systems that can be used to propagate the virus. Of the immortalized cell lines, CCL-141 supported ABBV-1 replication best with titre level similar to that of DEF. DF-1 did not support detectable ABBV-1 titre production, but the virus maintained a low level of gene and protein expression in the culture. Future continual passaging and monitoring of infected DF-1 would be of interest to assess if the amount of virus would increase after many subsequent passages, as this phenomenon has been previously observed with VERO cells persistently infected with EsBV-1 (Rubbenstroth et al., 2014).
### 2.6 Figures and tables

Table 2-1: Review of previous studies documenting growth of avian bornaviruses (ABVs) in cell culture.

<table>
<thead>
<tr>
<th>ABV genotype*</th>
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<td>CnBV-3</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>EsBV-1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes^z</td>
</tr>
<tr>
<td>PaBV-1</td>
<td>Yes</td>
<td></td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>PaBV-2</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
*See introduction for taxonomy*

“Yes” indicates detectable replication in cell line

“No” indicates no detectable replication in cell line

Empty cells indicate no data available

DEF – primary duck embryo fibroblasts; CEF – primary chicken embryo fibroblasts; QEF – primary quail embryo fibroblasts; CEC32 – quail embryo fibroblasts; QM7 – quail smooth muscle cells (derivative of QT6); QT6 – quail fibrosarcoma cells (6 to 24 days old bird); DF1 – chicken embryo fibroblasts; LMH – chicken hepatoma cell line; VERO – African green monkey kidney cells; MDCK – Madin-Darby Canine Kidney; C6 – rat glial cells

$^z$ 45+ passages

<table>
<thead>
<tr>
<th>PaBV-4</th>
<th>Yes</th>
<th>Yes</th>
<th>Yes</th>
<th>Yes</th>
<th>No</th>
<th>Yes</th>
<th>No</th>
<th>Gray et al., 2010; Rinder et al., 2009; Horie et al., 2015</th>
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<tr>
<td>PaBV-5</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Guo &amp; Tizard, 2015</td>
</tr>
<tr>
<td>PaBV-7</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td>Rubbenstroth et al., 2012</td>
</tr>
</tbody>
</table>
Figure 2-1: Detection of ABBV-1 from persistently infected DEF using western blotting and immunofluorescence (IF).

DEF cultures were exposed to homogenate from goose brain and monitored for potential ABBV-1 replication using western blotting and IF. A) Western blot showing detection of ABBV-1 N protein in lysates from infected DEF by affinity purified anti-N protein antibody. N protein was not detected in uninfected control DEF culture. Beta-actin was detected in both infected and control DEF cultures. B) and C) IF of control and infected DEF cultures, respectively, using an affinity purified antibody against the N protein (green signal). Nuclei were counterstained with DAPI (blue signal).
Figure 2-2: Detection of ABBV-1 replication, by immunofluorescence (IF) in DF-1, CCL-141, and QT-35 at early passages post-infection
(Figure 2-2 cont’d) IF of ABBV-1 infected DF-1, CCL-141, and QT35 at passages 1, 2 and 3 post infection. The time between each passage ranges between 5 to 8 days, and subcultivation was done on the same day for each infected cell line. Blue color shows DAPI staining, and green signal demonstrates N protein expression in cells. The forth column shows a zoomed inset from the passage 3 picture of the third column. Speckled or punctate staining in the nucleus was more readily observed in infected CCL-141 (forth column) than the other cell lines. Scale bar represents 100 µm.
Table 2-2: Detection of ABBV-1 N gene RNA in cell lines by RT-qPCR over multiple passages

<table>
<thead>
<tr>
<th>Infected cell lines at multiple passages (p)</th>
<th>Mean Ct*</th>
<th>Standard Deviation</th>
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<tr>
<td>DF-1 ABBV-1 p2</td>
<td>25.00</td>
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<tr>
<td>DF-1 ABBV-1 p3</td>
<td>24.96</td>
<td>0.13</td>
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<tr>
<td>DF-1 ABBV-1 p4</td>
<td>24.84</td>
<td>0.10</td>
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<td>DF-1 ABBV-1 p5</td>
<td>24.47</td>
<td>0.24</td>
</tr>
<tr>
<td>DF-1 ABBV-1 p6</td>
<td>23.71</td>
<td>0.08</td>
</tr>
<tr>
<td>DF-1 ABBV-1 p7</td>
<td>24.73</td>
<td>0.08</td>
</tr>
<tr>
<td>DF1 Control</td>
<td>Negative</td>
<td>na</td>
</tr>
<tr>
<td>CCL-141 ABBV-1 p2</td>
<td>20.52</td>
<td>0.11</td>
</tr>
<tr>
<td>CCL-141 ABBV-1 p3</td>
<td>16.86</td>
<td>0.13</td>
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<td>16.61</td>
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<td>0.02</td>
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<td>0.12</td>
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</tr>
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<td>QT35 ABBV-1 p2</td>
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<tr>
<td>QT35 ABBV-1 p3</td>
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<td>16.33</td>
<td>0.08</td>
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<tr>
<td>QT35 ABBV-1 p6</td>
<td>16.68</td>
<td>0.04</td>
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<td>QT35 ABBV-1 p7</td>
<td>18.24</td>
<td>0.08</td>
</tr>
<tr>
<td>QT35 Control</td>
<td>Negative</td>
<td>na</td>
</tr>
</tbody>
</table>

“Ct” cycle threshold.
“Negative” indicates the sample did not cross threshold (i.e., value > 35).
*Indicates the Ct values average and standard deviation for each passage were calculated from 3 replicates.
Figure 2-3: Relative level of N protein expression in infected DF-1, CCL-141, and QT-35

Western blot and semi-quantitative analysis of relative N protein level expression in cell lysates collected from cultures of persistently infected DF-1, CCL-141, and QT-35 at passages 11, 12 and 13. A) Western blot showing detection of intense N protein bands in infected CCL-141 and QT-35 cultures and faint N protein bands in infected DF-1 cultures. Beta-actin protein bands were detected with strong intensity in all samples. ABBV-N bands were seen from DF-1 cell lysates after long exposure. B) Semi-quantitative analysis of N protein bands relative to beta-actin bands. Different letters indicate significant differences (p<0.05). Relative N protein expression in CCL-141 and QT35 was significantly higher than in DF-1.
Figure 2-4: Production of infectious ABBV-1 titre in immortalized cell lines compared to DEF

The amount of infectious ABBV-1 produced in each cell line was measured using TCID$_{50}$ and compared to DEF. Virus collected from each infected cell line was titrated on the same uninfected cell line (homologous titration), and on DEF. Titres were log transformed prior to statistical analysis. When comparing between the different immortalized cell lines, infected CCL-141 produced the highest titre of ABBV-1 and at a similar level to infected DEF. There was no significant difference in titre levels when virus from each cell line was titred on the naïve homologous cell line or on DEF. For the immortalized cell line, highest ABBV-1 titre was produced from infected CCL-141, followed by infected QT-35. Infected DF-1 cell line did not produce detectable titre. Different letters indicate significant differences (p<0.05).
Figure 2-5: Visualization of ABBV-1 virions in infected CCL-141 by transmission electron microscopy.

Persistently infected CCL-141 cultures were imaged using TEM. (a) Spherical, approximately 100 μm, viral-like particles were observed at the periphery of the cell. (b) Higher magnification of viral-like particles. Red arrows indicate viral-like particles – membrane bound with surface decorations and electron-dense material inside.
Chapter 3. Growth kinetics of aquatic bird bornavirus 1 in primary embryonic fibroblasts of goose, duck, chicken, and turkey

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Running head: Growth of ABBV-1 in avian cell lines
3.1 Abstract

Aquatic bird bornavirus 1 (ABBV-1) is an infectious agent associated with development of a fatal wasting disease of waterfowl, caused by chronic meningoencephalitis and ganglioneuritis. Although waterfowl species appear to act as the natural host of ABBV-1, the virus has been sporadically isolated from other avian species, showing the potential for a broad host range. To evaluate the host specificity and the risk of ABBV-1 infecting and becoming established in commercial poultry species, we assessed the ability of ABBV-1 to grow in primary fibroblasts from chicken, turkey, duck, and goose embryos. Fibroblasts were infected with a low (multi-step growth curves) or high (single-step growth curves) multiplicity of infection (MOI), and the ability of ABBV-1 to infect and spread in cells was assessed by immunofluorescence, whereby virus growth was measured by percentage of infected cells over time. Multi-step growth curves indicated that ABBV-1 replicated and spread in goose and duck embryo fibroblasts, establishing a population of persistently infected cells, while it was unable to do so in chicken and turkey fibroblasts. Single-step growth curves showed that all cell types could be infected, however ABBV-1 established a population of persistently infected cell only in goose and duck fibroblasts. These data indicate that although chicken, turkey, duck and goose embryo fibroblasts are permissive to ABBV-1, infection and establishment of a persistent infection appeared to be more efficient in duck and goose cells. This indicates that replication in chickens and turkeys is possible, although future work into pathogenesis and the innate immune response is needed.

Keywords: ABBV-1, host range, primary cell lines, in vitro characterization, growth curves, immunofluorescence
3.2 Introduction

Aquatic bird bornavirus 1 (ABBV-1) is an infectious agent responsible for causing a chronic and eventually fatal meningoencephalitis and ganglioneuritis in several species of migratory waterfowl (e.g., Canada geese, trumpeter swans, mute swans) throughout Canada and the United States (Delnatte et al., 2013; Murray et al., 2017). ABBV-1 is a genotype of the species Waterbird 1 orthobornavirus, in the genus Orthobornavirus, family Bornaviridae and order Mononegavirales (Maes et al., 2019). Within the genus, viruses that are isolated from avian hosts are termed avian bornaviruses (ABVs). These viruses have a non-segmented, negative-sense, single-stranded RNA genome, which is roughly 9kb with six genes that encode for five structural proteins and one non-structural protein: nucleoprotein (N), non-structural protein (X), phosphoprotein (P), matrix (M), glycoprotein (G), and RNA dependent RNA polymerase (large; L) (Guo et al. 2013).

Although distinctly neurotropic in vivo, ABBV-1, and bornaviruses in general, are able to replicate in a variety of cell types in vitro. Mechanisms of replication have been elucidated mostly through research into Borna disease virus-1 (BoDV-1), of the species Mammalian 1 orthobornavirus, the type species of the family Bornaviridae. Virus enters the cell via receptor-mediated endocytosis after the G protein recognizes an unknown host receptor (Sauder and Staehler, 2003). The ribonucleoprotein (RNP; a complex of viral RNA encapsidated with N monomers, in association with P and L proteins) is released into the cytoplasm after pH-induced G conformational changes causes fusion of the viral envelope with the endosomal membrane (Sauder and Staehler, 2003). The free RNP is then trafficked to the nucleus, where genome replication and gene transcription are initiated (de la Torre, 2006). Bornaviruses do not cause a lytic replication cycle, and induce persistent infection through multiple mechanisms, including inhibition of the host immune response, negative regulation of transcription by the viral X protein, and tight associations of the RNP with host chromatin, which mediates transfer of viral RNP to daughter cells during mitosis (de la Torre, 2002; Tomonaga, 2002; Matsumoto et al., 2012). There is limited release of mature, infectious virions in vitro, rather the virus infects new cells by cell-to-cell spread that is mediated by the G protein (Tomonaga et al., 2002; Matsumoto et al., 2012). Infected cells do not display cytopathic effect (CPE), and cannot be distinguished from uninfected
cells by light microscopy alone (Tomonaga et al., 2002). Therefore, visualization of infection in vitro is dependent on immunoassays—frequently immunofluorescence (IF)—to detect viral antigens in the absence of CPE (Maclachlan & Dubovi, 2017).

Given the low virus release in the supernatant, IF is also frequently used to course the growth of bornaviruses in cell lines, in order to evaluate the proportion of infected cells over time. Evaluation of growth characteristics in multiple cell lines have been described for BoDV-1 (Charlier et al. 2013), as well as a handful of ABV genotypes, including estrildid finch bornavirus-1 (EsBV-1), parrot bornavirus-2 (PaBV-2), and canary bornaviruses (CnBV-1, 2, and 3) (Rubbenstroth et al., 2013 and 2014; de Araujo et al., 2018). However, it should be noted that most of these reports focus on assessing the suitability of different cell lines for virus isolation, and IF is used to quantify the number of infected cells at a single time-point post-infection, and not the spread or growth of virus over time. In cases where the latter is done, the curves show that bornaviruses are generally slow-growing and can take up to a month before all cells in culture are infected, although this varies with the cell type (Rubbenstroth et al. 2014). While ABBV-1 is commonly isolated using primary duck embryo fibroblasts (DEF), to the author’s knowledge, no quantitative analysis of ABBV-1 growth in any cell lines (either primary or continuous) have been described.

While waterfowl act as the natural reservoir, ABBV-1 infection is not limited to these species. Since identification in 2009, ABBV-1 has been sporadically isolated from birds taxonomically distinct from waterfowl including bald eagles (Payne et al., 2012), gulls (Guo et al. 2015), and emus (Nielsen et al., 2017). Similarly, other ABVs that affect psittacine birds (e.g., Psittaciform 1 orthobornavirus 1 genotype Parrot bornavirus-4; PaBV-4) have caused disease in avian species from different taxa, such as Galliformes (Bourque et al. 2015). Although this evidence suggests that ABVs have the potential for a broad host range, the ability of ABBV-1 to become established in agriculturally important species of poultry (i.e., chickens, and turkeys) is currently unknown.

In this study, we hypothesized that primary cell lines can be used as a model to predict ABBV-1 host restriction in common poultry species, by measuring the ability of the virus to
replicate and persist in primary embryonic fibroblasts of chickens, turkeys, ducks, and geese. Replication kinetics of ABBV-1 were determined using multi-step and single-step growth curves. Primary cell lines were chosen as more physiologically relevant when compared to continuous cell lines. Characterization of host restriction \textit{in vitro} is a first step in understanding the ability of ABBV-1 to infect poultry species, and better appraise the relative risk that this virus poses for commercial poultry.

3.3 Materials and methods

3.3.1 Eggs

Fertilized White Leghorn chicken, Hybrid turkey, Pekin duck, and Emden goose eggs were purchased from the Canadian Food Inspection Agency (Ottawa, Ontario, Canada), Hendrix Genetics (Kitchener, Ontario, Canada), King Cole Ducks Ltd. (Whitchurch-Stoufville, Ontario, Canada), and a local producer (Ontario, Canada), respectively. Chicken and turkey eggs derived from specific pathogen free (SPF) flocks. Eggs were incubated at 37.5°C at 60-75% humidity and rotated every 2 hours in an egg incubator (GQF Manufacturing, Savannah, Georgia, United States).

3.3.2 Cells

Finite chicken, turkey, duck, and goose embryo fibroblasts (CEF, TEF, DEF, GEF, respectively) were produced from fertilized eggs, using standard protocols (Schat and Sellers, 2008). Briefly, at 11 days of embryonation (DOE) for chickens, and 13 DOE for turkey, duck, and geese, a single, viable egg was removed from the incubator and decontaminated by submersion in 70% ethanol for 5 minutes. Sterile scissors were then used to open the egg at the level of the air sac and the embryo was removed and placed onto a petri dish using sterile forceps. Using a scalpel, the head wings, legs, and internal organs were removed, and the trunk was rinsed several times with sterile phosphate buffered saline (PBS; Fisher BioReagents, ThermoFisher) to remove any blood. The remaining portion of the embryo was minced into small pieces using scissors and transferred into a flask with 1X trypsin (Hyclone, ThermoFisher) at 37°C with stirring for 10 minutes. The supernatant was then filtered through a 40 µm cell strainer (Corning, Durham, North Carolina, USA) and fetal bovine serum (FBS; Wisent, St. Bruno, Quebec, Canada) was added to a final concentration of 5% (v/v) to inactivate the trypsin. The mixture was centrifuged at 350 x g
for 5 minutes to pellet cells before being re-suspended in maintenance media (Dulbecco’s modified Eagle’s medium [DMEM; Wisent, St. Bruno, Quebec] with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin/amphotericin B [PSA; Hyclone]). A total of 2.0x10^6 cells were counted using Countess II (Life Technologies, ThermoFisher, Mississauga, Ontario, Canada) and plated onto a T-75 flask (Nunc, ThermoFisher) for routine passaging in maintenance media.

### 3.3.3 Virus stocks

Stock of ABBV-1 was harvested from persistently infected GEF (ABBV-GEF). ABBV-GEF were routinely passaged in maintenance media using T-175 flasks (Nunc, ThermoFisher), in order to create a large stock of cells. Approximately 48 hours before planned virus harvesting, media was removed and replaced with fresh maintenance media supplemented with 10 mM sodium butyrate (Alfa Aesar, ThermoFisher), a compound that has been shown to increase BoDV-1 titres by up to 10-times (Pauli and Ludwig, 1985). After 48 hours of incubation, media was replaced with a hypertonic buffer (maintenance media supplemented with 250mM MgCl₂ [specs] and 10mM HEPES [specs]) for 2 hours to promote release of virus into supernatant. Virus containing supernatant was then collected and filtered through a 0.45 µm filter, and incubated overnight at 4°C with half a volume (2:1 ratio) of polyethylene glycol 8000 (PEG8000; Fisher BioReagents, ThermoFisher) solution supplemented with 0.4M sodium chloride (Fisher BioReagents, ThermoFisher, Mississauga, Ontario, Canada), with constant shaking. After incubation, the mixture was centrifuged at 4000 rpm for 15 minutes at 4°C, and the virus pellet was re-suspended in PBS with 1% FBS, at a ratio of 1:600 of final resuspension volume to originally harvested virus supernatant. Virus stock was titrated using a 50% tissue culture infectious dose (TCID₅₀) assay (Chapter 2), aliquoted, and stored at -80°C. The TCID₅₀ titer was calculated using the Spearman-Karber calculation (Ramakrishnan, 2016) and converted to focus forming units (FFU) multiplying by 0.69 (Luria et al. 1978).

### 3.3.4 Multi-step ABBV-1 growth curve

A schematic representation of the methods used for the multi-step growth curve is shown in Figure 3-1. A total of 0.1 x10⁶ CEFs, TEFs, GEFs, and DEFs were each plated into separate wells of a 12-well plate (Nunc, ThermoFisher) (n = 5 wells for each species) and let to adhere
overnight. Immediately prior to infection, the number of cells from one well for each species was counted using a hemocytometer, in order to calculate the right amount of virus for the selected multiplicity of infection (MOI; amount of FFU per cells). The remaining four wells were rinsed twice with PBS and replenished with 0.5 mL of maintenance media. Three wells from each species \((n = 3)\) were then infected with ABBV-1 at a MOI of 0.01 (i.e., 1 FFU of virus per 100 cells). One of the remaining four wells was treated with an equivalent volume of PBS as a negative control. Cells were allowed to incubate with virus (or PBS) for one hour. Infectious inoculum was then removed, cells were rinsed 3 times with PBS, and replenished with maintenance media. Cells were first split two days after initial infection and then split every other day for the remaining course of the experiment at a 1:3 ratio, with 2/3 passaged into 6-well plates (Nunc, ThermoFisher) for maintenance over a 21 day duration. The remaining 1/3 was plated in 24-well plates (Nunc, ThermoFisher) and let to adhere overnight before detection of virus-infected cells by IF, as described below. Overall, IF was carried out over 10 passages, corresponding to 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 days post-infection (dpi).

### 3.3.5 Single-step ABBV-1 growth curve

A schematic representation of the methods used for the single-step growth curve is shown in Figure 3-2. A total of 0.05 x \(10^6\) CEFs, TEFs, GEFs, and DEFs, were plated in separate wells of a 24-well plate (Nunc, ThermoFisher, Mississauga, Ontario, Canada) \((n = 21\) for each species) and let to adhere overnight. Immediately prior to infection, the number of cells from one well from each species was counted using a hemocytometer, in order to calculate the right amount of virus for the selected MOI. The remaining 20 wells were rinsed twice with PBS and replenished with 250 µL of maintenance media. Fifteen \((n = 15)\) wells were infected with ABBV-1 at an MOI of 5 (i.e., 5 FFU of virus per one cell), while the remaining five were inoculated with an equivalent volume of PBS (negative control). Cells were allowed to incubate for one hour before inoculum was removed, then were rinsed 3 times with PBS, and replenished with 0.5 mL of maintenance media. At 0, 1, 2, and 4 dpi, IF was done on four wells (three infected with ABBV-1 plus one PBS inoculated negative control) from each species, as described below, to detect the percentage of infected cells at each time point. The remaining four wells (3 infected and one control) that were not used at these time points were passaged and split in a 1:3 ratio, where 2/3 was maintained in
6-well plates (Nunc, ThermoFisher, Mississauga, Ontario, Canada), and 1/3 was split into a 24-well plate for further IF detection of virus at 6, 10, 14, and 21 dpi.

3.3.6 Immunofluorescence (IF)

Cells in each well were rinsed twice with PBS before being fixed and permeabilized with ice-cold methanol-acetone (1:1 ratio) for 20 minutes at -20°C. Cells were then rinsed three times with PBS, blocked one hour at room temperature in blocking buffer (10% [v/v] normal goat serum [NGS; Sigma-Aldrich, Oakville, Ontario] in PBS with 0.1% [v/v] Tween-20 [PBST]), and incubated overnight at 4°C with primary rabbit antibody diluted to 1 μg/mL in blocking buffer. The primary rabbit antibody was affinity purified and monospecific against a synthetic 22-amino acid peptide (Cys-KEAQLARYRRELREVTRGEDGAHLIS) representative of a region near the C terminus of the N protein of ABBV-1 (Pacific Immunology). Cells were then rinsed three times with PBST before incubation with secondary goat-anti-rabbit AlexaFluor488-conjugated antibody (ThermoFisher) diluted in 1 μg/ml in blocking buffer for one hour at room temperature in the dark. Cells were rinsed 3 times with PBST and counterstained with DAPI (ThermoFisher) diluted 0.5 μg/mL PBST for 10 minutes, before rinsing with PBST.

Positive IF reaction was visualized under a fluorescent microscope (Zeiss Observer.A1, Toronto, Ontario) using the same exposure time. Three pictures were taken per well at a 10x objective with two channels (DAPI and FITC). For each picture, total number of cells was counted using the DAPI (blue) channel, and the number of infected cells was determined by counting immunoreactive cells using the FITC channel (green). Therefore, considering 3 wells for each time point, the final number of infected cells over the total number of cells per time point was determined by averaging the data from 9 pictures. Cell counting was assisted using ImageJ software (National Institute of Health; NIH).

Higher magnification pictures of ABBV-1 infected GEF, DEF, CEF, and TEF at 6 days after infection at an MOI of 5 were visualized with confocal laser scanning microscopy. At 5 dpi 0.02 x10^6 cells from each species were seeded into chambered microscope slides (Falcon, Corning, New York, USA) and let to adhere overnight. The following day (6 dpi) virus was detected using the IF protocol described above. A coverslip with mounting buffer (90% v/v glycerol, 20mM Tris
pH = 8) was used to seal the cells and ABBV-1 in the cells was visualized using a Leica DM 600B microscope coupled with a Leica TCS SP5 laser system.

### 3.3.7 Statistical analysis

Differences between the means of the proportions of infected cells (over the total) at each time point between species were assessed using a two-way analysis of variance (ANOVA), where the variables included species (i.e., type of cell) and time point, with a post-hoc Tukey’s test for multiple comparisons. There were \( n = 3 \) and \( n = 4 \) replicates for each time point and cell type in the multi- and single-step growth curves, respectively. Analysis was conducted using the GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA), and significance was set at \( p < 0.05 \).

### 3.4 Results

#### 3.4.1 ABBV-1 can efficiency spread in GEF and DEF cells, but not CEF and TEF, over the same time period.

GEF, DEF, CEF, and TEF were infected with ABBV-1 at an MOI of 0.01, cells were split every other day, and the proportion of infected cells at each time point was determined with IF. Dissemination of ABBV-1 in GEF and DEF was slow initially, with less than 20% of cells positive for the N protein within the first 11 days; however, there was a significant difference in the proportion of positive GEF compared DEF by 9 dpi (\( p < 0.0001 \)). (Fig. 3-3). Exponential increase in the proportion of infected GEF and DEF was seen between 11 and 17 dpi, and 100% of cells were immunoreactive after 17 dpi for GEF, 19 dpi for DEF. The rate of growth was faster in GEF than in DEF, with the proportion of positive cells significantly higher in GEF from 9 dpi until 15 dpi (\( p < 0.0001 \) at each time point in between). Immunoreactivity in infected GEF and DEF appeared speckled in the nucleus, and finely diffuse in the cytoplasm (Figs. 3-4 and 3-5). In contrast, at no point over the 21-day time period did CEF or TEF show immunofluorescence signal (Figs. 3-3, 3-6, and 3-7). All negative controls lacked immunoreactivity.
3.4.2 GEF, DEF, CEF and TEF cells can be infected with ABBV-1, upon infection of a high quantity of virus; however, CEF and TEF cells differ in ability to maintain persistent infection and replication rate.

GEF, DEF, CEF, and TEF were infected with ABBV-1 at an MOI of 5, and virus spread was evaluated by IF over a three-week period. Virus infection was detected rapidly in GEF, DEF, and CEF, with 20% of cells immunoreactive within 1 dpi, while no infected TEF were visible within this timeframe (Fig. 3-8). Growth in DEF was significantly greater within the first 48 hours compared to CEFs and GEFs (p<0.0001), although by 4 dpi 100% of GEFs, DEFs, and CEFs were infected showing no significant differences in the proportion of infected cells between species. At 6 dpi, a lower percentage (4%) of infected cells became detectable in TEFs, which was significantly lower compared to other groups, and remained fairly consistent (<10%) until the end of the experiment (21 dpi). Starting at 6 dpi, the proportion of infected CEFs significantly decreased compared to GEF and DEF (p<0.0001), declining to 90% at 6 dpi, and dipping to <10% by the end of the experiment (21 dpi). The percentage of infected cells in GEF and DEF did not decrease or fluctuate after 100% infection was achieved at 4 dpi, and was maintained through the entire three weeks of the experiment (Fig. 3-8).

Infected GEF and DEF showed the same immunofluorescence pattern described for the multi-step growth curve (Figs. 3-9, 3-10 and 3-11, 3-12), mainly characterized by speckled nuclear and diffuse cytoplasmic immunoreactivity. Infected CEF showed a similar pattern initially, however at later time points cells gradually lost cytoplasmic reactivity, maintaining only the nuclear signal (Fig. 3-13, 3-14). Immunofluorescence in TEF, when infected cells became detectable at 6 dpi, showed both a nuclear and cytoplasmic signal, similar to what observed in GEF and DEF (Fig. 3-15, 3-16).

3.5 Discussion

In this study, we assessed the replication kinetics of ABBV-1 in primary fibroblasts derived from chicken, turkey, duck, and goose embryos through multi- and single-step viral growth curves. Using IF for the viral N protein, results showed that GEF and DEF can best support ABBV-1 replication, and establishment of persistent infection. The multi-step growth curve showed that ABBV-1 is not able to spread in CEF and TEF; while the single-step showed that CEF and TEF
can both be infected, but with differences in replication and ability of ABBV-1 to persist. Overall, these results indicate that primary embryo fibroblasts may have different levels of permissivity to ABBV-1, which possibly parallels what seen in natural infection.

Infection of GEF and DEF with a low MOI (multi-step growth curve) resulted in growth kinetics characterized by an initial slow growth, followed by exponential increase of infected cells and a plateau reached by the end of experiment, indicating infection of all cells in the population. This kinetic is similar to what is described with BoDV-1, which reached a plateau at 30 and 15 days post-infection in Vero cells and hippocampal neurons, respectively (Charlier et al. 2013; Bajramovic et al. 2003). On the other hand, infection of CEF and TEF with a low MOI did not yield any infected cells (as shown by IF) over the 21-day duration of the experiment. Studies with Estrildid finch bornavirus-1 (EsBV-1) has indicated that the time for spread and infection of cells can take multiple passages and last up to several months before a persistently infected population, where all cells are infected, is established (Rubbenstroth et al. 2014). In the present study, all cells were primary embryo fibroblasts, and experiments were not protracted for great numbers of passages due to cellular senescence. Although immunofluorescence was negative in CEF and TEF infected with low MOI, virus replication may have been present at low levels, detectable only by a more sensitive method, such as RT-qPCR. As noted in Chapter 2, ABBV-1 infected-DF-1 cells (a type of immortalized chicken fibroblasts [Schaefer-Klein et al. 1998]), despite being negative for immunofluorescence and unable to produce detectable infectious titre, yielded detectable viral RNA and protein levels, indicating a low-level or incomplete virus replication.

Single-step growth curves are not routinely performed with bornaviruses, due to difficulties in producing sufficiently large virus titres for high MOIs. Here, the yield of virus titre from persistently infected GEF (used as producer cell line) was increased by treating cells with sodium butyrate, and concentrating cell-free virus with polyethylene glycol. Sodium butyrate is a histone deacetylase inhibitor that has been shown to increase infectious virus production with BoDV-1 and other viruses, although specific mechanisms remain unknown (Pauli and Ludwig, 1985). These enhancements to virus production and further concentration allowed for high MOI infection for single-step growth curves, although relatively small amounts of cells had to be used.
GEF and DEF yielded a similar trend of ABBV-1 growth in the single-step curve as was seen in the multi-step curve, albeit at a much faster rate, and were able to maintain an infected state for three weeks (i.e., persistent infection). In contrast to what observed in the multi-step growth curve, a few infected TEF were detectable by immunofluorescence after 6 days post infection, although the proportion never increased above 10% of the total cells. These results suggest that TEFs can become infected, and that continued passaging may result in persistent infection of the whole cell population, although at a slower rate than what is seen with GEF and DEF. Similar to GEF and DEF, CEF also rapidly became infected upon inoculation with ABBV-1 at a high MOI, however the intensity of fluorescence signal and amount of positive cells decreased with the number of passages. The initial burst of replication is likely due to infection with an overwhelming amount of virus, followed by decreasing replication rates, possibly caused by poor adaptation of ABBV-1 to the chicken host. However, this pattern of growth is in obvious contrast to what is seen in TEF, where cells did not become infected initially.

Differences in the staining pattern between the CEF, and the TEF, GEF, and DEF cells were seen with immunofluorescence. Where there was both nuclear and cytoplasmic staining in the CEF initially, the staining became predominately nuclear with little to no cytoplasmic signal, in contrast to what was seen in infected GEF and DEF, where signal was in both (and in the TEF at the later time points when positive cells were visualized). The immunoreactivity pattern is a result of the localization of the N protein, which, for BoDV-1, has been shown to exist as two different isomers: p40, which is nuclear, and p38, which is cytoplasmic as it is transcribed without the N-terminal nuclear localization signal (Pyper & Gartner, 1997). In BoDV-1, both isoforms are critical for nucleo-cytoplasmic shuttling of viral RNA, and are essential for virus replication (Kobayashi et al. 2001; Honda & Tomonaga, 2013). The lack of cytoplasmic staining seen in the CEF in the later time points may indicate that replication is not efficient and decreases as a result.

The differential growth of ABBV-1 in the cell lines of the present work are likely to be explained by species-specific differences in the innate immune response or permissivity to infection and replication. Future research will need to address the differences in expression of antiviral genes that are activated upon ABBV-1 infection, especially considering that bornaviruses have multiple methods of antagonizing the innate immune signaling and establish persistent
infection (Lin et al. 2013). Additionally, factors that control virus entry and cell-to-cell spread could explain the difference in replication kinetics between CEF, TEF, GEF, and DEF, including receptor and host factor availability. Tissue tropism could also be investigated using different cell types. As ABBV-1 is highly neurotropic in vivo, the use of neural cell lines might provide a more realistic understanding of replication kinetics, however the use of the fibroblast model to address the question of host range is appropriate, considering that goose and duck fibroblasts were able to be infected and maintain infection. Overall, the results here show that chicken and turkey fibroblasts are semi-permissive to infection, although live animal experimental infections will need to be carried out to fully understand the impact that ABBV-1 infection may have on commercial poultry.
3.6 Figures and tables

Figure 3-1: Experimental outline for the multi-step growth curve.

0.1x10^6 goose, duck, chicken, and turkey embryo fibroblasts (GEF, DEF, CEF, TEF, respectively) were each seeded into 5 separate wells of a 12-well plate and let adhere overnight. The following day, 1 well from each cell type was dislodged and cells were counted using a hemocytometer. Three (n = 3) of the remaining 4 wells were infected with ABBV-1 at an MOI of 0.01, based on the number of cells counted from the 1 well, and the final well was inoculated with an equal volume of PBS. Virus was let to adsorb to cells for 1 hour before infectious inoculum was removed, rinsed 3 times with PBS and replaced with fresh media. Cells were incubated for 2 days before being split in thirds, with 1/3 being passaged into a separate well of a 12-well plate, and 2/3 passaged into separate wells of a 6-well plate. Cells were let adhere overnight and virus infected cells were visualized in the 12-well plates using immunofluorescence. The cells in the 6-well plate were maintained and split every other day in the same manner, for a total of 21 days. The time points when the virus was visualized by IF were: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 days post infection (dpi).
Figure 3-2: Experimental outline for the single-step growth curve.

- **n = 21** Overnight
  - Plate 0.05 x $10^6$ cells in 5 wells for each host

- **n = 21**
  - Count cells from one well for each host using a hemocytometer

- **n = 20** 1 hour
  - Infect 15 wells at an MOI of 5 (negative with PBS)

  **n = 20**
  - Remove infectious inoculum

  **n = 21** 0 dpi
  - Add fresh media

  **n = 4** 1 dpi

  **n = 4** 2 dpi

  **n = 4** 4 dpi

  **n = 4** 5 dpi

  **n = 4** 6 dpi

  **n = 4** 21 dpi

  **n = 4** 14 dpi

  **n = 4** 10 dpi

  **n = 4** 2/3

  **n = 4** 1/3

  **n = 4**
(Figure 3-2 cont’d). 0.05x10^6 goose, duck, chicken, and turkey embryo fibroblasts (GEF, DEF, CEF, TEF, respectively) were each seeded into 21 separate wells of a 24-well plate and let adhere overnight. The following day, 1 well from each cell type was dislodged and cells were counted using a hemocytometer. Fifteen \( (n = 15) \) of the remaining 20 wells were infected with ABBV-1 at an MOI of 0.01, based on the number of cells counted from the 1 well, and the final well was inoculated with an equal volume of PBS. Virus was let to adsorb to cells for 1 hour before infectious inoculum was removed, rinsed 3 times with PBS and replaced with fresh media. Virus was detected using immunofluorescence (IF) in 4 of the wells per species (3 infected and 1 PBS control) at each of the following time points: 0, 1, 2, and 4 days post infection (dpi) without splitting the cells in between. At 5 dpi the remaining 4 wells per each species were split in a 1:3 ratio with 2/3 being maintained and 1/3 being split into a 24-well plate for detection of virus by IF on 6 dpi. This continued for timepoints of 10, 14, and 21 dpi.
Figure 3-3: Multi-step growth curve of ABBV-1 in primary embryonic fibroblasts.

Multi-step growth curve of ABBV-1 in primary embryonic fibroblasts from goose (GEF), duck (DEF), chicken (CEF), and turkey (TEF). Cells were infected at an MOI of 1 and at 3, 5, 9, 11, 13, 15, 17, 19, and 21, days post infection a portion of infected cells was fixed and virus was detected using immunofluorescence. Cells were counterstained with DAPI to determine percentage of infected cells over the total. For each cell line at each time point, n = 3.
Figure 3-4: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in GEF from multi-step growth curve at 3, 11, and 21 days post infection. For each picture, 10x magnification.
Figure 3-5: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in DEF from multi-step growth curve at 3, 11, and 21 days post infection. For each picture, 10x magnification.
Figure 3-6: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in CEF from multi-step growth curve at 3, 11, and 21 days post infection. For each picture, 10x magnification.
Figure 3-7: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in TEF from multi-step growth curve at 3, 11, and 21 days post infection. For each picture, 10x magnification.
Single-step growth curve of ABBV-1 in primary embryonic fibroblasts from goose (GEF), duck (DEF), chicken (CEF), and turkey (TEF). Cells were infected at an MOI of 5 and at 1, 2, 4, 6, 10, 14, 21 days post infection and a population of cells were fixed and virus was detected using immunofluorescence. Cells were counterstained to determine percentage of infected cells over time. For each cell line at each time point, n = 3.

Figure 3-8: Single-step growth curve in primary embryonic fibroblasts
Figure 3-9: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in GEF from single-step growth curve at 1, 4, and 21 days post infection. For each picture, 10x magnification.
Figure 3-10: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in GEF at 6 dpi. For each picture, 63x magnification.
Figure 3-11: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in DEF from single-step growth curve at 1, 4, and 21 days post infection. For each picture, 10x magnification.
Figure 3-12: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in DEF at 6 dpi. For each picture, 63x magnification.
Figure 3-13: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in CEF from single-step growth curve at 1, 4, and 21 days post infection. For each picture, 10x magnification.
Figure 3-14: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in CEF at 6 dpi. For each picture, 63x magnification.
Figure 3-15: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in TEF from single-step growth curve at 1, 4, and 21 days post infection. For each picture, 10x magnification.
Figure 3-16: Immunofluorescence for ABBV-1 N antigen (left), counterstained with DAPI (middle) and merged (right) in TEF at 6 dpi. For each picture, 63x magnification.
Chapter 4. Embryonated eggs as a model to characterize the host restriction and pathogenesis of aquatic bird bornavirus 1 in chickens, turkeys, and ducks

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Running head: Avian bornavirus infection in ovo
4.1 Abstract

Aquatic bird bornavirus 1 (ABBV-1) is the causative agent of a chronic and debilitating ganglioneuritis and meningoencephalitis. ABBV-1 is highly prevalent in migratory waterfowl but has been sporadically isolated from other birds including gulls, bald eagles, and emus, suggesting a broad host range. Given the ability to infect multiple species, ABBV-1 may have the potential to infect commercial poultry (i.e., chickens and turkeys). In this study, we aimed to evaluate the host restriction of ABBV-1 through growth and lesion development in avian embryos (in ovo). Groups of duck, chicken and turkey embryonated eggs were inoculated with ABBV-1 into the yolk sac (day 5 of embryonation for chickens; day 8 for turkeys and ducks) or into the chorio-allantoic cavity (day 9 of embryonation for chickens; day 12 for turkeys and ducks). Embryonic tissue was harvested at two time points during the experiment—early (day 12 of embryonation for chickens; day 15 for turkeys and ducks) and late (day 19 of embryonation for chickens; day 24 for turkeys and ducks). Virus growth was evaluated through RT-qPCR of embryonic tissues and immunohistochemistry (IHC) on sections of central nervous tissue. Lesion development in embryos was assessed through histopathology. Results showed that growth of ABBV-1 and lesion development in embryonic tissues was absent in all tested species, suggesting that either embryos are resistant to infection, or ABBV-1 spread from the inoculation site is inefficient or not possible. This research is the first to evaluate an in ovo model to assess ABBV-1 host restriction and pathogenesis, and suggests that avian embryos cannot be successfully employed to reproduce ABBV-1-induced disease.

Keywords: Aquatic bird bornavirus, embryo infection, in ovo pathogenesis, host restriction, experimental infection, poultry
4.2 Introduction

Proventricular dilatation disease (PDD) is a chronic and fatal wasting disease affecting psittacines (Tizard et al., 2016). In PDD, inflammation and neuronal degeneration in the myenteric and gastric ganglia causes impaired intestinal motility and atrophy of the smooth muscle lining the gastrointestinal (GI) tract, leading to impaired digestion, starvation, and ultimately death (Tizard et al., 2016). The peripheral nervous system of the GI tract is the most common location of inflammation, although other organs can be affected, including the central nervous system, the retinal ganglion, and the cardiac plexus (Tizard et al., 2016). Common microscopic lesions are characterized by lymphoplasmacytic infiltrates arranged into perivascular cuffs in peripheral and central nervous system (Tizard et al., 2016).

A viral etiology for PDD was discovered in 2008, when the full genome of a novel virus was sequenced from the brains of psittacines that were presumptively diagnosed with PDD based on clinical and pathological findings (Honkavuori et al., 2008; Kistler et al., 2008). The agent belonged to the family Bornaviridae, which encompasses a group of highly neurotropic enveloped viruses with a round morphology and a negative sense, single stranded RNA genome. Since this initial discovery, multiple bornaviruses have been isolated from a variety of avian species (i.e., avian bornaviruses; ABVs). The most recent organization of the family Bornaviridae by the International Committee on Taxonomy of Viruses (ICTV) has identified five species of ABV within the genus Orthobornavirus: Passeriform 1 orthobornavirus, Passeriform 2 orthobornavirus, Psittaciform 1 orthobornavirus, Psittaciform 2 orthobornavirus, and Waterbird 1 orthobornavirus (Maes et al., 2019).

Symptoms and lesions similar to PDD in psittacine birds have been observed in other avian species, including finches and various species of waterfowl infected with ABVs. PDD-like lesions have been noted in Canada geese as far back as 1991 (Daoust et al., 1991); however, a possible etiological agent was not identified until 2011, with the successful detection of an ABV from healthy Canada geese, which was designated aquatic bird bornavirus 1 (ABBV) (Payne et al., 2011; Kuhn et al., 2015). ABBV-1 is highly prevalent throughout Southern Ontario in multiple species of migratory waterfowl, including Canada geese (Branta canadensis), trumpeter swans.
(Cygnus buccinator), and mute swans (Cygnus olor). As the majority of seropositive birds show no signs of disease, populations of healthy carriers must exist, which can maintain the virus in the wild (Delnatte et al., 2013). Pathological findings in ABBV-1-infected waterfowl are mostly consistent to what is observed with PDD in psittacines, although with some notable differences. Namely, in a retrospective review of postmortem waterfowl species that were diagnosed with ABBV-1 by RT-PCR, non-purulent perivascular cuffs in the central nervous system appeared to be much more common compared to psittacines with PDD (Delnatte et al., 2011). In the same study, there was a notable lack of obvious lesions in the ganglia of the proventriculus and ventriculus (Delnatte et al., 2011).

While the typical host range of ABBV-1 includes geese and swans, ABBV-1 has been isolated from and/or found to cause disease in birds taxonomically distinct from waterfowl, including eagles (Payne et al., 2012), gulls (Guo et al., 2015), and an emu (Nielsen et al., 2017). Histological lesions in these birds are consistent to what is seen in waterfowl. Similarly, other ABVs have been shown to have a broad host range, outside the common avian species reservoirs. For instance, a Himalayan monal (Lophophorus impejanus; order Galliformes) that had clinical signs relating to PDD-like illness was found to have lesions indicative of a bornavirus infection at necropsy (Bourque et al., 2015). Further RNA isolation and sequencing was able to isolate parrot bornavirus-4 (PaBV-4; Psittaciform 1 orthobornavirus) from tissue (Bourque et al. 2015).

Despite the high prevalence in waterfowl, and propensity to cause disease in multiple avian species, the ability of ABBV-1 to infect and cause disease in agriculturally significant poultry species (i.e., chickens, turkeys, and domestic ducks) has not been evaluated. Infection and establishment of ABBV-1 in poultry species is a hazard that could lead to potential economic losses. In this study, the ability of ABBV-1 to infect and cause disease in chickens, turkeys and domestic ducks was evaluated using an in ovo model, by means of infecting embryos. In ovo models are attractive systems to study pathogenesis because of reduced cost, ease of handling and no requirements for specially designed containment areas (Jacobson et al., 2010). Fertilized eggs of each species were inoculated into either the yolk or chorio-allantoic cavity, and embryos were harvested at mid or late embryonation. Lesion development in tissues was assessed by
histopathology, and viral replication was evaluated by immunohistochemistry (IHC) and RT-qPCR for viral RNA. We hypothesized that embryonated eggs represent a model for the study of ABBV-1 pathogenesis and host restriction. This research will help to develop an understanding regarding the pathogenic potential of ABBV-1 in commercial poultry.

4.3 Materials and methods

4.3.1 Eggs

Fertilized White Leghorn chicken, Hybrid turkey, and Pekin duck eggs were obtained respectively from the Canadian Food Inspection Agency (CFIA; Ottawa, ON, Canada), Hendrix Genetics (Kitchener, ON, Canada), and King Cole Ducks Ltd. (Whitchurch-Stouffville, ON, Canada). Chicken and turkey eggs were specific-pathogen free (SPF). All eggs were incubated at 37.5°C with 65% humidity and rocked every two hours in an egg incubator (GQF manufacturing, Savannah, Georgia, United States).

4.3.2 Virus

ABBV-1 was harvested from persistently infected finite goose embryo fibroblasts (ABBV-GEFs). Details of ABBV-1 stock production and titration are reported in detail in Chapters 2 and 3. Briefly, infected ABBV-GEFs were grown in maintenance media (Dulbecco’s modified Eagle’s medium [DMEM; Wisent, St. Bruno, Quebec, Canada] with 10% fetal bovine serum [FBS; Wisent, St. Bruno, Quebec, Canada] and 1% penicillin/streptomycin/amphotericin B [PSA; Hyclone, ThermoFisher, Mississauga, Ontario]) at 37°C with 10% CO₂. Forty eight hours before planned virus harvesting, media was removed from the cells and was replaced with maintenance media supplemented with 10 mM sodium butyrate (Alfa Aesar, ThermoFisher). After 48 hours of incubation, the media was removed and replaced with a hypertonic buffer (maintenance media supplemented with 250mM MgCl₂, and 10mM HEPES) and incubated with cells for 2 hours at 37°C and 10% CO₂. The virus-containing media was then clarified through a 0.45 µm filter, and incubated overnight at 4°C in a 2:1 ratio of polyethylene glycol 8000 (PEG8000; Fisher BioReagents, ThermoFisher) supplemented with 0.4M sodium chloride (Fisher BioReagents, ThermoFisher), with constant shaking. After incubation, the mixture was centrifuged at 4000 rpm for 15 minutes at 4°C, and the virus pellet was re-suspended in PBS with 1% FBS, with a final
ratio of 1:600 of resuspension volume to originally harvested virus supernatant. Virus stocks were
titrated using a 50% tissue culture infectious dose (TCID$_{50}$) assay (Chapter 2) and titre was
calculated using the Spearman-Karber method. Stock was aliquoted and stored at -80°C.

4.3.3 Experimental outline

A schematic representation of the experimental plan is reported in Figure 4-1. Eighty ($n = 80$) eggs per species were obtained and incubated as described above. Half ($n = 40$) were inoculated in the yolk sac and the other half in the chorio-allantoic cavity. For each route, half of the embryos ($n = 20$) were inoculated either with ABBV-1 or PBS (control). On account of different developmental rates and times at hatch (i.e., 21 days for chickens and 28 days for turkeys and ducks), inoculation and embryo harvest were carried out at different days of embryonation (DOE). Yolk inoculation was conducted at 5 DOE in chickens, and 8 DOE in turkeys and ducks. Allantoic inoculation was conducted 9 DOE in chickens, and 12 DOE in turkeys and ducks. Therefore, considering inoculation route and inoculum type, for each species there were four groups, each of $n = 20$ embryos (yolk/ABBV-1, yolk/PBS, chorio-allantoic/ABBV-1, chorio-allantoic/PBS). From each group, $n = 10$ embryos were harvested at two time points: early and late embryonation. Early harvest was 11 DOE for chickens, 15 DOE for turkeys and ducks; late harvest was 19 DOE for chickens, 24 DOE for turkeys and ducks. Half of the embryos ($n = 5$) sampled at each time point were used for histology/immunohistochemistry (IHC), and half for RNA extraction. Based on the results of RT-qPCR (see below), IHC was only done on brain and spinal cord tissue only from $n = 2$ PBS inoculated and $n = 3$ ABBV-1 inoculated embryos per harvest time point.

4.3.4 Egg inoculation

Inoculation into the yolk and chorio-allantoic sacs was conducted according to standard procedures (Labzofsky 1974), with minor modifications. For yolk inoculation, eggs were candled to identify a site corresponding to the top of the air sac. This area was sterilized using Septisol (50% iodine, 50% ethanol) and pierced using scissors to make a small hole directly into the egg shell that entered the air sac. Using a syringe, 100 µL of ABBV inoculum (1.0x10$^5$ FFU) was delivered into the yolk sac by directing the needle (22-gauge, 1.5-inch) perpendicular to air sac
(Figs. 2A/C). The needle was then removed, and the hole was covered with tape. An equivalent volume of PBS was inoculated into control eggs.

For chorio-allantoic inoculation, the eggs were candled to identify a site of air sac just above a site of the chorio-allantoic membrane with little to no vasculature, in order to avoid traumatic bleeding upon inoculation. The site was sterilized and pierced using scissors as described above. A syringe (22-gauge, 1-inch needle) was used to deliver 100 µL of ABBV inoculum (1.0x10^5 FFU) in the chorio-allantoic cavity, making sure to follow the mark that identified non-vascular areas (Figs. 2B/D). The needle was then removed slowly and the hole was covered with clear tape. An equivalent volume of PBS was inoculated into control eggs. After inoculation, eggs were candled every day to assess embryo viability. Embryos that died the day after inoculation were discarded (death interpreted as a consequence of inoculation); embryos that died before harvesting time were regularly sampled as described below.

4.3.5 Harvest of embryonic tissue

At the set time points described above, embryos were euthanized by refrigeration 6 hours to overnight prior to harvest; sterile scissors were used to open up the shell along the air sac and release the embryo into a sterile petri dish for dissection. Harvested tissues were: allantoic fluid (RNA extraction only), chorio-allantoic membrane (histology only), yolk and vitelline membrane (yolk with vitelline membrane were harvested together due to the thickness of yolk making it difficult to pipette; RNA extraction and histology), brain and spinal cord (RNA extraction and histology), liver (RNA extraction and histology), and proventriculus and ventriculus (proventriculus and ventriculus [PV] were harvested together; RNA extraction and histology). For RNA extraction, fluids and tissues were stored in screw cap tubes and frozen at -80°C. For histology, tissues were fixed in 10% neutral buffered formalin for 48 hours and then preserved in 70% ethanol until being embedded in paraffin wax and routinely processed for hematoxylin and eosin (H&E) staining and IHC (see below).
4.3.6 Immunohistochemistry

Immunohistochemistry was performed in brain tissue only, representing the mostly likely area of virus replication based on literature sources (Delnatte et al. 2011). The primary antibody was an affinity purified rabbit monospecific directed against a 22-amino acid synthetic-peptide representing a stretch of conserved amino acid residues at the C terminus of the ABBV nucleoprotein (N) (Chapter 2; Pacific Immunology, Ramona, CA, USA). Briefly, after deparaffinization, sections were unmasked using Proteinase K (Dako, Agilent Technologies, Mississauga, ON) for 12 minutes at room temperature, and blocked with non-serum block (Dako, Agilent Technologies, Mississauga, ON) for 12 minutes at room temperature. The primary antibody, diluted 1:6000, was applied onto the slides for 30 minutes at room temperature, followed by a polymer (Envision, Agilent Technologies, Mississauga, ON) directed against rabbit and mouse antibodies and conjugated with peroxidase. Positive reaction was visualized using Nova-Red chromogen for 10 minutes. Positive control sections derived from the brain of Canada geese (Branta canadensis) naturally infected with ABBV-1.

4.3.7 RNA extraction and reverse transcriptase quantitative PCR (RT-qPCR)

For RNA extraction, a total of 300 µL of allantoic fluid, 300 µL of yolk (or 300 mg of yolk with vitelline membrane at late harvest time points due to the yolk fluid being too thick to pipette), or 300 mg of solid tissue (brain, liver, PV) were homogenized in 1 mL of TRizol LS (Invitrogen, ThermoFisher, Mississauga, Ontario) reagent using a Precellys 24 homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France) and 1.0 mm sterile glass beads (BioSpec products, Bartlesville, Oklahoma, United States). After homogenization, samples were centrifuged at 15,000 x g for 3 minutes to pellet large debris, and supernatant was mixed with 200 µL chloroform (Fisher Chemical, ThermoFisher, Mississauga, Ontario), inverted to mix and centrifuged at 11,000 x g for 10 minutes. The upper phase (500 µL) was mixed with 350 µL (0.7 volumes) of isopropanol (Fisher Chemical, ThermoFisher, Mississauga, Ontario) and incubated at -20°C overnight to allow precipitation of nuclei acids. RNA was purified using RNeasy Mini Kit (QIAGEN Inc. Toronto, Ontario, Canada).
Extracted RNA was used in a one-step reverse-transcriptase (RT) quantitative PCR (qPCR) (New England BioLabs, Whitby, Ontario, Canada) using a TaqMan protocol, with forward (5’-ATGCACCTGGCAGCTCTTAGAC-3’) and reverse (5’-TCCCCATAAAACCTCCCAAC-3’) primers and probe (5’-6 FAM-CCCTGCCGCAGGAAATCCAT-BHQ-3’) designed to target the ABBV-1 N gene. Reactions were prepared in a 20 µL volume according to manufacturer instructions, with 0.8 mM primers, 0.4 mM probe, and 100 ng of RNA template. The cycling conditions were as follows: 55°C reverse transcription for 10 minutes, 95°C initial denaturation for 1 minutes, and 40 cycles of 95°C for 10 seconds for denaturation and 60°C for 30 seconds for both annealing and extension.

Viral RNA in tissues was quantitated using a standard curve, which was developed as follows. Virus was harvested from 4.0 x 10^6 ABBV-GEFs using freezing and thawing, and the virus yield reported in focus-forming units (FFU), as outlined in Chapter 2 and 3. RNA was extracted from an equivalent amount of cells (i.e., 4.0 x 10^6) using a High Pure RNA Isolation Kit (Roche, Mississauga, ON, Canada), and 10-fold serial dilutions of 100 ng of RNA were used as template for RT-qPCR, using the same conditions specified above. The threshold value (i.e., Ct value) of each dilution was then plotted against the relative amount of FFU/100 ng of RNA, to create a standard curve. Additionally, and since total RNA was extracted from the whole amount of harvested samples (either 300 mg or 300 µL), the standard curve was further modified to plot Ct value versus FFU/mg or µL of sample (Fig. 4-3). Positive samples were determined using a Ct value of 35 and as the cut-off (i.e., any Ct value above 35 was considered to be negative).

4.3.8 Statistical analysis

Differences between the proportion of positive/negative samples in the ABBV-1 inoculated eggs and PBS inoculated eggs, as well as difference in proportion of positive samples between each host were determined using a Chi-squared test using GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA).
4.4 Results

4.4.1 Egg incubation and harvesting

Four \((n = 4)\) chicken and duck, and 8 turkey eggs were either not fertilized or contained dead embryos prior to inoculation and were discarded, leaving a total of 76 chicken and duck eggs and 72 turkey eggs for inoculation. Within 24-hours after inoculation, a total of 26 embryos across all three species died; this was assumed to be the consequence of inoculation, and not ABBV-1 infection (Table 4-1). As a consequence, the following were discarded: 10 turkey embryos (2 yolk/ABBV-1, 5 chorio-allantoic/PBS, 3 chorio-allantoic/ABBV-1); 9 chicken embryos (3 yolk/ABBV-1, 3 yolk/PBS, 2 chorio-allantoic/PBS, 1 chorio-allantoic/ABBV-1); and 7 duck embryos (3 yolk/PBS, 2 chorio-allantoic/PBS, 3 chorio-allantoic virus) (Table 4-1). The final number of chicken, turkey and duck embryos that reached the end of the experiment were respectively 67, 62, and 69, for a total of 198 embryos.

Due to the decreased volume of the chorio-allantoic sac at the later stages of embryo development, 24 allantoic fluid samples could not be harvested from eggs for RNA extraction across the three species including 7 turkey and 15 duck embryos (Table 4-2). A total of 261 and 251 tissues from ABBV-1- and PBS-inoculated embryos, respectively, were available for RNA extraction. A total of 270 and 282 tissues from ABBV-1- and PBS-inoculated embryos, respectively, were available for histopathology (Table 4-3). A total of 60 embryos were tested by IHC (Figure 4-1).

4.4.2 ABBV-1 does not cause lesions in embryonic tissues

Fertilized chicken, turkey, and duck eggs were inoculated into the yolk sac or the allantoic cavity with \(10^5\) FFU of ABBV-1 or an equivalent volume of PBS. Embryos were harvested at an early time point, and a late time of embryonation, and brain, spinal cord, liver, PV, and chorioallantoic and vitelline membranes were assessed for presence of microscopic lesions. No inflammatory lesions consistent with ABBV infection, such as lymphoplasmacytic infiltration and presence of mononuclear perivascular cuffs in the central (brain and spinal cord) or peripheral nervous tissues were observed in tissues from any ABBV- or PBS-inoculated embryo (Figure 4-4).
4.4.3 ABBV-1 does not replicate in embryonic tissues, as assessed by RT-qPCR and IHC

Immunohistochemistry on brain tissues and spinal cords from 60 embryos were negative, regardless of inoculum, route, or harvest time (Figs. 4-5A/B). Only one late-harvest duck embryo inoculated with ABBV into the yolk sac showed rare, but weak, immunoreactive nuclei (Fig 4-5C); however other embryos in the same grouping were confirmed negative by RT-qPCR (see below). Therefore, this weak staining was interpreted as artifact. The positive control for IHC (brain of a naturally infected Canada goose [Branta canadensis]) showed numerous immunoreactive nuclei within the neuroparenchyma (Figure 4-5D). IHC on chorio-allantoic and vitelline membrane from select tissues that were RT-qPCR positive were negative (data not shown).

In addition to IHC (described above), replication in the embryos was assessed by detection of the ABBV-1 N gene by RT-qPCR in brain, PV, liver, yolk and allantoic fluid. Table 4-4 summarizes the results of the RT-qPCR, and the estimated amount of FFU of ABBV per mg or uL of tissue from positive samples is provided in Table 4-5 and Figure 4-5. For infected embryos, the majority of positive cases derived from the yolk and allantoic fluid samples, with 15/53 (28.3%) and 13/46 (28.3%) samples, respectively, testing positive from virus inoculated eggs. A total of 4/162 (2.5%) solid tissue samples (i.e. brain, PV and liver) from inoculated embryos tested positive, with all 4 positive samples derived from chicken brains. The 32 positive samples had high Ct values (just below the cut-off of 35), ranging from the lowest of 29.09 to the highest of 34.42. For control embryos, there were a total of 10/251 samples that tested positive by RT-qPCR (Table 4). Specifically, two derived from chicken and one from duck brain tissues; three from turkey yolk tissue; and four from duck from the allantoic fluid. The 10 positive samples had Ct values ranging from the lowest of 29.80 (chicken brain inoculated via the chorio-allantoic route) to the highest of 33.75 (duck allantoic fluid inoculated via the chorio-allantoic route). Statistical analysis showed that there was a significant difference (p < 0.05) between the proportions of positive to negative samples from ABBV-inoculated and PBS-inoculated embryos (p < 0.0001, chi-squared test). Additionally, there was no significant difference in the proportions of positive to negative samples between host species in ABBV-1 inoculated eggs (p = 0.0981, chi-squared test).
Taking into account the very high Ct value of the samples derived from PBS-inoculated birds, these positive results were interpreted as experimental contamination during embryo harvesting. Therefore, a new Ct value threshold was established, which considered negative any sample that had a Ct value between 29.80 (the highest Ct value from a control [PBS] sample) and 35 (the original cut off, as determined from the standard curve). This gating leaves two positive samples from ABBV-1 inoculated embryos, one chicken brain inoculated via the yolk sac harvested at an early time point (Ct = 29.74) and one duck yolk inoculated via the yolk sac harvested at a late time point (Ct = 29.09) (green data points, Figure 5). These two samples correspond to an inferred virus titer of < 0.18 FFU/µL or mg of tissue.

4.5 Discussion

In this study, we used an in ovo experimental infection model to determine the ability of ABBV-1 to replicate, and cause lesions in chickens, turkeys, and ducks. Virus inoculum or PBS was inoculated into either the yolk sac or the chorio-allantoic cavity and embryonic tissues were harvested for histology or RNA extractions mid-way or late embryonation. We observed that regardless of host, inoculation route, or harvest time, ABBV fails to cause lesions, or replicate at high levels in embryonic tissues.

Inoculation of embryonated eggs into the allantoic cavity is a common method for growing influenza and Newcastle disease virus (NDV), where virus infects the chorioallantoic membrane (CAM), replicates, and is released back into the allantoic fluid (Lee et al 2018; Yunis and Donnelly, 1969). In cases with mesogenic and velogenic NDV, virus is capable of infiltrating through the outermost epithelial layer of CAM to access the blood, and become distributed throughout embryonic tissue (Oldoni et al., 2005). In the present study, a quarter of allantoic fluid samples from ABBV-1 inoculated eggs tested positive for presence of viral RNA by RT-qPCR, when using a Ct value of 35 as the cut off. Much like with influenza and NDV, ABBV-1 may have infected the CAM, however due to the non-cytolytic nature of bornaviruses (Tizard et al., 2016) ABBV-1 was not released into the allantoic fluid. It is also possible that ABBV-1 may not capable of infecting the CAM, and that the low level ABBV RNA detected by RT-qPCR could reflect remaining inoculum. This is in agreement with the fact that all positive samples had high Ct values.
narrowly above the threshold, indicating less than one FFU of ABBV per µL of fluid in each sample. One of the four chicken brains from ABBV-1-infected embryo that tested positive by RT-qPCR, was inoculated via the chorio-allantoic cavity, although the estimated virus titre was less than one FFU/mg. This single positive sample, along with the low estimated virus titre, suggests that ABBV-1 does not spread from the cavity to the embryo efficiently, if at all, and that the low-level detection is likely representative of contamination.

Yolk sac inoculation is less common compared to the chorio-allantoic route, but has been utilized as a route for Marek’s disease virus (Gallid alphaherpesvirus 2; GaHV-2) and Coxsackie virus (Peers, 1952; Katz and Kohn, 1971). In these cases, the virus infiltrates the yolk sac membrane before distributing throughout embryonic tissues, much like with highly pathogenic NDV as described above. In this study, a quarter of total yolk samples from ABBV-1 inoculated embryos tested positive for presence of viral RNA by RT-qPCR, when using a Ct value of 35 as the cut off. Three of the 4 chicken brains from ABBV-1-inoculated embryos that tested positive via RT-qPCR, were inoculated via the yolk sac with low estimated FFU/mg in all cases. Similarly to what observed with the chorio-allantoic route of inoculation, the low amount of positive samples from embryos inoculated through the vitelline sac, along with high Ct values, suggests that virus spread is inefficient, and positive tissue samples likely represent the initial inoculum or contamination.

Overall, 10 tissues from PBS-inoculated eggs tested positive. While the proportion of total positive samples from ABBV-1 inoculated embryos is significantly higher than the proportion of those inoculated with PBS, it is expected that all negative controls would be negative. When positive samples are gated by disqualifying any positive that was higher in Ct value than the highest PBS positive only two samples remained positive: one chicken brain and one duck yolk. Rather than true positives, these samples more likely represent errors due to contamination of tissue during sample collection, or errors during RNA extraction or RT-qPCR.

In addition to RT-qPCR, virus replication was evaluated by IHC using a monospecific antibody against the ABBV N protein, in both the brain and spinal cord. In naturally infected Canada geese, brains show scattered immunoreactivity for ABBV in the nuclei of both neurons
and glial cells (Delnatte et al., 2013). In the present study, immunoreactivity was only observed in one brain from a ABBV-1 inoculated duck egg. However, the general weakness of the staining, along with the proximity of the nuclei to the edge of the tissue strongly suggests that this represented an artifact, commonly known as edge effect—a situation of overexposure due to sections of tissue near the edge lifting up, with the result that both sides of the tissue open to the peroxidase reaction (True, 2008).

Microscopic lesion development was assessed in multiple tissues. The two most common lesions documented with avian bornavirus infections in multiple avian species (including ABBV-1 infection in waterfowl) include lymphoplasmacytic infiltration of the peripheral ganglia and perivascular cuffs of mononuclear cells in the CNS (Delnatte et al., 2013). No lesions were observed in the central and peripheral nervous tissue, as well as other organs, in any of the tissues from ABBV-1- or PBS-inoculated embryos. As bornaviruses do not cause direct cell lysis, lesion development is believed to be a consequence of inflammation mediated by the cell-mediated immune response of the host (Tizard et al., 2016). The lack of a fully-functioning immune system in the developing embryo (Jankovic et al., 1975) is in agreement with the absence of lesions observed in the embryonic tissues of this study. Overall, lack of lesions associated with the negative IHC and RT-qPCR results, suggest that ABBV did not spread or replicate in embryonic tissue and did not elicit lesion development.

For a cell-associated virus, the nervous tissue (i.e., the site of ABBV replication) might have been inaccessible from a distant site of inoculation (i.e., chorio-allantoic and vitelline sac). Additionally, the short incubation time before hatch might have been too short for ABBV to fully replicate in tissues. In fact, experimental inoculation of cockatiels with brain homogenate from an African gray parrot infected with PaBV-4 failed to show clinical signs until 21 days after infection, and, in another study, cockatiels inoculated with PaBV-4 infected duck embryo fibroblasts did not develop clinical signs for 110 days (Gancz et al., 2009; Payne et al., 2011). Ultimately, the results reported here suggest that an in ovo model is inadequate to assess replication and pathogenesis of ABBV in the three species tested. Infection of birds in an in vivo trial is necessary to fully understand the pathogenesis and host restriction of ABBV.
4.6 Figures and tables

Figure 4-1: Schematic showing the experimental outline for the *in ovo* experimental infection.
(Figure 4-1 cont’d) 80 fertilized eggs were purchased per host. Half of the eggs were inoculated into the yolk sac (n = 40) at 5 days of embryonation (DOE) for chickens, 8 DOE for turkeys and ducks. The other half was inoculated into the chorio-allantoic cavity at 9 DOE for chickens and 12 DOE for turkeys and ducks. n = 40 embryos were harvested at two time points: early, 11 DOE for chickens and 15 DOE for turkeys and ducks; and late, 19 DOE for chickens and 24 DOE for turkeys and ducks. n = 10 embryos at each time point represented one of the two inoculum routes and types (i.e., n = 10 yolk/ABBV-1, n = 10 yolk/PBS, n = 10 chorio-allantoic/ABBV-1; n = 10 chorio-allantoic/PBS). Five (brain, proventriculus/ventriculus (PV), liver, yolk, allantoic fluid) and six (brain, spinal cord, PV, liver, chorio-allantoic membrane (CAM), and vitelline membrane) organs were taken for RNA extraction and histology, respectively. For each group, n = 3 brain and spinal cord samples from ABBV-1 inoculated embryos and n = 2 brains and spinal cord from PBS inoculated embryos were also subjected to IHC.
Inoculation routes used in the *in ovo* experiment. (A) shows a drawing of yolk-sac inoculation and (C) shows an actual yolk sac inoculation into a chicken egg. A 1.5 inch needle is plunged directly through the air sac into the yolk sac perpendicular to the egg shell. (B) shows a drawing of a chorio-allantoic inoculation and (D) shows an actual chorio-allantoic inoculation into a chicken egg. A mark is drawn on the shell to avoid any large blood vessels to prevent trauma that may kill the embryo if accidentally punctured.
Figure 4-3: Standard curve for quantification of ABBV-1 viral RNA.

Standard curve produced from 10-fold serial dilutions of RNA extracted from a known amount of persistently infected goose embryo fibroblasts. ABBV-1 was titrated by TCID50 assay from the same amount of cells, producing a curve of FFU/100 ng of RNA, which could be transformed to FFU/mg or uL of tissue or liquid sample, given that a known amount of sample was subjected to RNA extraction. Crossing point indicate Ct value, and Log concentration indicates the Log transformation of the ABBV-1 titer.
Table 4-1: Total number of embryos harvested after infertile eggs and dead-within-24-hours embryos were discarded

<table>
<thead>
<tr>
<th></th>
<th>Chicken $n = 80^*$</th>
<th>Turkey $n = 80$</th>
<th>Duck $n = 80$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not inoculated**</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Died after inoculation***</td>
<td>Y/V</td>
<td>A/V</td>
<td>Y/P</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Subtotal</td>
<td>9</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>TOTAL REMAINING</td>
<td>67</td>
<td>62</td>
<td>69</td>
</tr>
</tbody>
</table>

* $n = 80$ indicates the initial eggs that were incubated.

** indicates eggs that died or were infertile before inoculation.

*** indicates eggs that died 24 hours post inoculation and were not sampled.

A = chorio-allantoic cavity; Y = yolk; P = PBS inoculation (control); V = virus inoculation (ABBV-1).
Table 4-2: Total number of tissues harvested for RNA extraction and RT-qPCR from ABBV-1 and PBS inoculated embryos

<table>
<thead>
<tr>
<th></th>
<th>Chicken</th>
<th>Turkey</th>
<th>Duck</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>PV</td>
<td>Liver</td>
</tr>
<tr>
<td>Number of organs harvested</td>
<td>19 18 18 17 19 17 17 17 17 15 19 19 18 19 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subtotals</td>
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<td>Total</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>261</td>
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</table>

<table>
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<th>Turkey</th>
<th>Duck</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>PV</td>
<td>Liver</td>
</tr>
<tr>
<td>Number of organs harvested</td>
<td>18 17 17 17 17 17 17 17 17 10 19 19 19 19 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subtotals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PV = proventriculus/ventriculus; AF = allantoic fluid

96
### Table 4-3: Total number of tissue samples harvested for histology from ABBV-1 and PBS inoculated eggs

<table>
<thead>
<tr>
<th></th>
<th>ABBV-1-inoculated embryos</th>
<th>PBS-inoculated embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chicken</td>
<td>Turkey</td>
</tr>
<tr>
<td>Number of organs harvested</td>
<td>Brain</td>
<td>SC</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Subtotals</td>
<td>96</td>
<td>78</td>
</tr>
<tr>
<td>Total</td>
<td>270</td>
<td>282</td>
</tr>
</tbody>
</table>
Figure 4-4: Hematoxylin and eosin (H&E) stained tissue sections from ABBV-1 infected embryos.
(Figure 4-4 cont’d) Hematoxylin and eosin (H&E) stained tissue sections at 40x magnification from ABBV-infected embryos, showing normal tissue and no lesion development. A. Proventriculus and intramural ganglion of a late harvest turkey embryo inoculated with ABBV-1 via the chorio-allantoic cavity. B. Late harvest duck brain inoculated with ABBV-1 via the yolk sac. C. Spinal cord of turkey inoculated with ABBV-1 via the chorio-allantoic cavity. D. Chorio-allantoic membrane (CAM) of duck inoculated with ABBV-1 via the chorio-allantoic cavity. E. Vitelline membrane of late harvest chicken inoculated with ABBV-1 via the yolk sac. F. Liver of late harvest duck infected with ABBV-1 via the chorio-allantoic cavity.
Figure 4-5: Immunohistochemistry of brain tissue sections.

Immunohistochemistry (IHC) of brain tissue sections at 40x magnification for detection of ABBV-1 using anti-ABBV-N antibody. (A) is duck brain inoculated with ABBV-1 via the yolk sac showing two weakly reactive nuclei that most likely represent artefact; (B) is chicken brain inoculated with ABBV-1 via the yolk sac showing no reactivity; (C) is positive control from an adult Canada goose brain showing multiple scattered reactive nuclei.
Table 4-4: Summary of RT-qPCR results from infected and PBS inoculated eggs.

<table>
<thead>
<tr>
<th>Inoculation route / harvest time</th>
<th>ABBV-1-inoculated embryos</th>
<th>PBS-inoculated embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chicken</td>
<td>Turkey</td>
</tr>
<tr>
<td></td>
<td>Brain PV Liver Yolk AF</td>
<td>Brain PV Liver Yolk AF</td>
</tr>
<tr>
<td>Yolk / Early</td>
<td>2/5 0/4 0/4 0/4 0/5 0/5 0/5 2/5 0/5 0/4 0/4 3/5 0/5</td>
<td>0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 3/17 0/10</td>
</tr>
<tr>
<td>Allantoic / Early</td>
<td>1/5 0/5 0/5 0/5 2/5 0/4 0/4 0/4 1/4 2/4 0/5 0/5 1/5 4/5</td>
<td>0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>Yolk / Late</td>
<td>1/4 0/4 0/4 0/4 0/4 0/4 0/4 0/4 1/4 0/4 0/4 0/4 4/4 2/2</td>
<td>0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>Allantoic / Late</td>
<td>0/5 0/5 0/5 0/4 3/5 0/4 0/4 0/4 1/4 0/4 0/4 0/4 2/5 N/A</td>
<td>0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>Subtotals</td>
<td>4/19 0/18 0/17 5/19 0/17 0/17 0/17 5/17 2/15 0/19 0/19 0/18 10/19 6/12</td>
<td>0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17 0/17</td>
</tr>
<tr>
<td></td>
<td>Total ABBV-1 chicken positive: 9/91</td>
<td>Total ABBV-1 turkey positive: 7/83</td>
</tr>
<tr>
<td></td>
<td>Total ABBV-1 positive: 32/261</td>
<td>Total PBS chicken positive: 2/86</td>
</tr>
<tr>
<td></td>
<td>Total PBS positive: 10/251</td>
<td></td>
</tr>
</tbody>
</table>
(Table 4-4 cont’d) Summary of one-step RT-qPCR results detecting viral RNA in the brain, proventriculus/ventriculus (PV), liver, yolk, and allantoic fluid (AF) of infected chicken, turkey and duck embryos. Rows represent experimental groups as identified by inoculation route (yolk, allantoic) and harvest time (early, late). Positive samples were determined based on a standard curve (data not shown) of serial dilutions of RNA from a known number of persistently infected goose embryo fibroblasts with Ct values ranging from 18-34. An FFU assay determined the number of FFU per cell to correlate Ct values to estimate FFU/ng of RNA. Fractions indicate the number of positive results out of total number of biological replicates per experimental group. Each biological replicate received three technical replicates for the one-step qRT-PCR.
Table 4-5: Estimates of viral titre from positive tissue samples

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Host</th>
<th>Inoculation route</th>
<th>Harvest time</th>
<th>Ct value</th>
<th>FFU/mg or µL of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABBV-1 inoculated positives</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Chicken</td>
<td>Allantoic</td>
<td>Early</td>
<td>32.50</td>
<td>9.17 x 10^3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34.35</td>
<td>2.11 x 10^2</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>29.74*</td>
<td>1.6 x 10^-1*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Late</td>
<td>32.36</td>
<td>5.3 x 10^-3</td>
</tr>
<tr>
<td>Allantoic fluid</td>
<td>Chicken</td>
<td>Allantoic</td>
<td>Early</td>
<td>32.58</td>
<td>3.5 x 10^-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>33.43</td>
<td>1.5 x 10^-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Late</td>
<td>32.20</td>
<td>3.7 x 10^-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34.03</td>
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<td>33.36</td>
<td>2.2 x 10^-5</td>
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<tr>
<td></td>
<td>Duck</td>
<td>Allantoic</td>
<td>Early</td>
<td>32.41</td>
<td>5.97 x 10^-5</td>
</tr>
<tr>
<td></td>
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<td>31.92</td>
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<td>33.64</td>
<td>1.67 x 10^-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Late</td>
<td>32.72</td>
<td>3.17 x 10^-5</td>
</tr>
<tr>
<td></td>
<td>Yolk</td>
<td>Late</td>
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<td>32.74</td>
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<td>31.90</td>
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<td>Yolk</td>
<td>Early</td>
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<td>31.29</td>
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<td>5.69 x 10^-3</td>
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(Table 4-5 cont’d) Numbers with an asterisk (*) are the two positive samples when the cut-off range is decreased to 29.80, to account for the positive samples in the PBS-inoculated group.
Figure 4-6: Graphic summary of data presented in Table 5.

The two green data points represent the two positive samples above the 29.80 threshold set by the negative control.
Chapter 5. General discussion and summary

Aquatic bird bornavirus 1 (ABBV-1) is a RNA virus in the genus *Orthobornavirus* and family *Bornaviridae*, associated with chronic and fatal infections of waterfowl causing non-suppurative inflammation throughout the central and peripheral nervous system (Guo et al. 2013). While waterfowl act as the primary hosts, ABBV-1 infection is not limited to these species. Since the identification of ABBV-1 in 2009, case reports have sporadically isolated ABBV-1 from birds taxonomically distinct from waterfowl including gulls (Guo et al. 2015), bald eagles (Payne et al., 2012), and emus (Nielsen et al., 2017). Even though ABBV-1 has been shown to cause infection in multiple species of birds, the ability of ABBV-1 to become established and cause disease in agriculturally important species of poultry (i.e., chickens and turkeys) is currently unknown. Thus, the overall goal of this thesis was to characterize the ability of ABBV-1 to replicate and/or cause lesions in agriculturally important species of birds through *in vitro* and *in ovo* experiments to determine a relative risk of ABBV-1 becoming established in commercial poultry, which could have potential damaging economic losses.

The first objective was to evaluate ABBV-1 propagation, including replication and production of infectious virus in continuous cell lines from ducks (duck embryo fibroblasts; CCL-141), chickens (chicken embryo fibroblasts; DF-1), and quail (quail fibrosarcoma; QT-35). Continuous cell lines were chosen because of obvious advantages, such as lack of senescence, increased consistency between batches and laboratories, and the ease of access of these cell lines over primary cell lines (no need to acquire producer eggs or animals). Replication was best supported in CCL-141 over the other two immortalized cell lines, with similar production of infectious virions in comparison to primary duck embryo fibroblast (DEF), which is the commonly used cell line for avian bornavirus (ABV) propagation (see Table 1-1). ABBV-1 viral RNA and N protein were present in QT-35 cells at levels similar to CCL-141, however a significantly lower amount of infectious virus was produced. In DF-1, viral RNA and N protein were detectable at very low levels compared to CCL-141 and QT-35, however no amount of infectious virions could be measured by TCID$_{50}$. The proposed explanation for the differences observed here is host specificity. Ducks are known to be susceptible to infection with avian bornaviruses. Indeed, a different genotype within the same species *Waterbird 1 orthobornavirus*, ABBV-2, has been found
to infect ducks in North America and exposure of ducks to ABVs has also been described (Guo et al. 2014; Gray et al. 2009). To date, there has been no experimental infection of chickens or quail with ABVs, and no reports document natural infection of chickens or quails with ABVs. The ability of ABBV-1 to at least infect and replicate in QT-35 may largely be due in part to ABVs ability to replicate in cell lines from different hosts. Long-term passaging of Estrildid finch bornavirus-1 (EsBV-1) has shown the virus to be capable of establishing a persistent infection in Vero cells, an immortalized African green monkey kidney cell line. It is within the realm of possibility then that continued passaging of ABBV-1 through DF-1 and QT-35 could produce infectious titres of ABBV-1 comparable to those seen in CCL-141. The consequence of this is that the use of immortalized cell lines in this manner may not be reflective of the true host range of ABBV-1.

While continuous cell lines are easily accessible and easy to propagate, they generally accrue multiple mutations that cause divergence from the normal cell biology. To gather a better understanding of host restriction in a more physiologically relevant system, the second objective was to evaluate the replication kinetics of ABBV-1 in primary embryonic fibroblasts of chicken (CEF), turkey (TEF), duck (DEF), and goose (GEF), with two kinds of growth curves: multi-step (low multiplicity of infection; MOI) and single-step (high MOI). Infection with a low MOI showed efficient infection and spread in GEF and DEF, however no infected cells were seen throughout the 21-day experiment in CEF and TEF. High MOI infection in the same cell lines showed a similar curve pattern for GEF, and DEF. Infection in CEF exhibited a similar curve initially as seen in the GEF and DEF however, the percentage of infected cells started to decrease by 6 dpi. Infection with a high MOI in TEF indicated few positive cells (10%) by 6 dpi although this number did not increase by the end of the experiment. Persistent infections are a hallmark of bornavirus infections, and we propose that the decrease in the percentage of infected cells seen in CEF is due to decreased viral replication. Initial peak in replication may be due to an overwhelming amount of virus and as the experiment continued, the lack of adaptation of ABBV-1 to the CEF may have resulted in a subsequent decrease in replication. Immunoreactivity in later time points for CEF showed a lack of cytoplasmic staining and only nuclear staining. The staining pattern is indicative of the location of two isoforms of the N gene, p40 which is nuclear and p38 which is cytoplasmic (Pyper &
The two isoforms are necessary for efficient nucleocytoplasmic shuttling of viral RNA (Pyper & Gartner, 1997; Kobayashi et al. 2001; Honda & Tomonaga et al. 2013). Thus, the lack of cytoplasmic staining, and decrease in number of infected cells may reflect decreased replication efficiency. The differences in susceptibility of TEF and CEF to ABBV-1 infection may be due to intrinsic host factors, either innate immune response or decreased permissivity to infection due to lack of receptor in TEF.

As bornaviruses are highly neurotropic in vivo, growth kinetics in fibroblasts may not be fully representative of virus-host interactions. The third objective of this study was to evaluate the use of an in ovo model to study the host restriction and pathogenesis of ABBV-1 in chickens, turkeys, and ducks. Regardless of the species, the route of inoculation, or the harvest time, there was no increase in virus replication, as assessed by RT-qPCR or immunohistochemistry and no lesion development in any tissue. As avian bornavirus infections cause chronic disease (e.g., proventricular dilatation disease in parrots), infection of developing embryo might have not provided sufficient time, due to the short period of incubation. Additionally, common microscopic lesions associated with ABV infections are perivascular cuffing in the central nervous system and lymphoplasmacytic infiltration in the peripheral ganglia (Delnatte et al. 2013). Both of these lesions are due to activation of a cell-mediated immune response, which is not fully developed in an embryo (Jankovic et al. 1975), and this might have accounted for lack of lesions in the tissues of this study. Therefore, lack of virus replication in the embryo, the chronic nature of ABV infections, as well as the poorly developed immune system, are all reasons that suggest the in ovo system is not suitable for the study of ABBV-1 pathogenesis. Necessitate the need for in vivo trials to study pathogenesis and host restriction.

The results highlight the differences of ABBV-1 replication in different avian species. The next steps towards understanding the reasons for these differences involve multiple different aspects. Identification of host factors that allow for ABBV-1 replication could be elucidated with knock-out studies. Discovering intrinsic host factor differences would explain the difference in permissivity between cell lines. Furthermore, understanding the host response to infection is an important aspect of determining how well ABBV-1 replicates in each host. This could be done
through a cytokine array for common genes or more high-throughput screens like next generation sequencing to identify changes in the transcriptome that bear specific signatures in ABBV-1 permissive and non-permissive cells. If propagation of ABBV-1 in different cell lines results in production of infectious titre similar to what is seen in cells from natural hosts (i.e., ducks), then viral RNA can be extracted and sequenced to identify mutations that might provide an advantage in replicating in specific cell lines and/or hosts. The limitations of the in ovo model to understand pathogenesis were broken down into the time constrains of incubation time of an embryo and the absence of preferred cell types at the site of inoculation. Due to the chronic nature of bornavirus infections and the neurotropism future studies in pathogenesis would be better explained using an in vivo model. The overall conclusion of the work presented here suggests that poultry species in vitro are semi-permissive to infection; however, more research, particularly in vivo pathogenesis experiments – is needed to fully evaluate the capacity for ABBV-1 infection in these species to understand the true threat of infection to commercial flocks.
REFERENCES


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APPENDICES

Appendix 1: Primers used for sequencing ABBV-1

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<tr>
<th>Name</th>
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<th>Genome Position</th>
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