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Studies on bornaviruses infection on companion animals

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Studies on bornaviruses infection on companion animals

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STUDIES ON BORNAVIRUSES INFECTION ON COMPANION ANIMALS

Tese de Candidatura ao grau de Doutor em Ciências Veterinárias;
Instituto de Ciências Biomédicas Abel Salazar, da Universidade do Porto, Portugal.

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À minha família / To my family

Da caixa de Pandora rapidamente saíu a ganância, a inveja, o ódio, a fome, a dor, a doença, a pobreza, a guerra e a morte. Por fim, uma criatura muito pequena e frágil saiu perseguindo todos os males,

a ESPERANÇA.

From the box of Pandora came out quickly the greed, envy, hatred, hunger, pain, sickness, poverty, war and death. Finally, a creature very little and fragile went out chasing all the evils,

the HOPE.

The research presented in this Thesis was conducted in the Laboratory of Microbiology and Infectious Diseases of the Department of Veterinary Clinics of the School of Medicine and Biomedical Sciences (Instituto de Ciências Biomédicas Abel Salazar | ICBAS), University of Porto, Porto, Portugal, and at the Section of Virology, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (Sveriges lantbruksuniversitet | SLU), Uppsala, Sweden. The PhD program was supported by QREN - Operacional Potencial Humano (POPH) from European and national funds of the Fundação para a Ciência e Tecnologia (FCT), Portugal, through a PhD studentship (SFRH/BD/91436/2012) and partially by Sveland Foundation for Animal Health and Welfare.

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Communications at scientific meetings

I. **Marlene Cavaleiro Pinto**, Mikael Berg, Veronica Rondahl, Erik Ågren, Júlio Carvalheira, Gertrude Thompson, Jonas Johansson Wensman. Epidemiology of parrot bornavirus 4: genotypes clusters, geographic distribution, host species and the relationship with mammalian bornaviruses. IVX Congresso Hospital Veterinário Montenegro. Santa Maria da Feira, Portugal. 24 – 25 February 2018.

II. **Marlene Cavaleiro Pinto**, Mikael Berg, Júlio Carvalheira, Gertrude Thompson. Real-time PCR TaqMan-base detection assay of Bornavirus for challenging specimens. 27th European Congress of Microbiology and Infectious Diseases. Vienna, Austria. 22 – 25 April 2017.

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Summary

Bornaviruses are entities that have an envelope and are between 80 and 100 nanometres in diameter. These viruses have a negative, single-stranded, non-segmented, RNA genome of about 8900 nucleotides in length. Bornaviruses have the skill to replicate in the nucleus of nervous cells from several organs, and often establish persistent non-cytolytic infections, and exploit the mechanisms of cell splicing. The organization of their genome consists of six open reading frames (ORF), concerning the prototype member of bornaviruses. Along the virus genome, there are alternative transcription start and end sites as splicing events that result in the production of several messenger RNA (mRNA) molecules. The first transcription unit contains an ORF for the nucleoprotein (N), the second has an overlap of two ORFs, referring to X protein (X) and phosphoprotein (P). The third transcription unit has several transcriptions start and end signals, for the matrix protein (M), the glycoprotein (G), as well as the RNA-dependent RNA-polymerase (L), with an overlap of the ORF of the matrix protein with that of the glycoprotein.

Bornaviruses are members of the order *Mononegavirales* of the *Bornaviridae* family. To date, the genera *Carbovirus*, *Cultervirus* and *Orthobornavirus* have been identified, which include 11 species. Bornavirus infection can cause the development of severe neurological or/and gastrointestinal disease, as well as the death of its hosts. The disease was reported in humans and various species of pets, farmed and wild animals. Two species infect mammals (*Mammalian 1* and *2 orthobornavirus*), five species infect birds (*Passeriform 1* and *2 orthobornavirus*, *Psittaciform 1* and *2 orthobornavirus* and *Waterbird 1 orthobornavirus*) and three infect reptiles (*Queensland carbovirus*, *Southwest carbovirus* and *Elapid 1 orthobornavirus*). Among the species *Mammalian 1* and *2 orthobornavirus* there are some viruses with recognized zoonotic potential, such as the Borna disease virus 1 (BoDV-1) and the variegated squirrel bornavirus 1 (VSBV-1), which were the etiological agents of lethal encephalitis in humans. However, some BoDV-1 genotypes also infected ostriches (in captivity) and wild birds (such as mallards and jackdaws). Wild birds showed a wide variety of viruses belonging to the species *Waterbird 1 orthobornavirus* and *Psittaciform 1 orthobornavirus* and, also, several genotypes of aquatic bird bornavirus 1 and 2 (ABBV-1 and 2) and parrot bornavirus 2 and 4 (PaBV-2 and 4).

Members of the *Psittaciform 1* to *2 orthobornavirus* species (Parrot bornavirus 1 to 8 (PaBV-1 to 8)) cause severe effects on the well-being and health of captive psittacines. Also, infection can have severe repercussions at the level of biodiversity and economics in the context of outbreaks in zoos, in reserves, in breeding projects for rare species and

private collections of psittacines. However, asymptomatic infections in captive and wild animals have also been reported. Moreover, in captivity, some naturally infected psittacines that tested positive to bornavirus-RNA remained seronegative. The virus is released through the excretions (faeces and urine) and secretions (saliva) of the hosts, and this can occur intermittently. According to studies already published, bornavirus infection can persist for several years in flocks of captive psittacines.

Histological and immunohistochemical examination of central nervous system tissues were suggested as standard laboratory tools for detecting bornavirus infections. However, the very invasive procedures to collect samples from live animals, from wild flocks and in captivity to identify reservoirs is controversial from the point of view of feasibility and ethics. Even in clinical practice, from the point of view of feasibility, the collection of tissues from the central nervous system, from live animals, to confirm the suspicion of infection is a complex procedure, as well as invasive procedures present an inherently high risk for the animal. In the literature there are described diagnostic tools that allow the use of samples collected by non/less invasive methods (for example, faeces, urine, saliva and blood) but, the serological tests available have produced inconsistent results when used. Viral isolation methods are laborious, expensive and time-consuming, and the accurate quantification of viral load in samples is difficult using the conventional polymerase chain reaction (PCR) technique. The conventional PCR techniques/methodologies are laborious and time-consuming compared to tools based on real-time PCR. However, the viral load quantification systems used in real-time PCR tests, previously reported in the literature, are expensive, require laborious preparation and have short expiration dates. The high cost and lengthy response time, in some cases, prevent access to laboratory diagnostics, as well as reduce the likelihood of timely interventions, respectively. Therefore, the development of cost-effective diagnostic tools is an asset in the surveillance of captive parrots, in clinical diagnosis support, as in studies with wild animals.

The epidemiology of bornaviruses is a puzzle that remains unclear. Namely, the eventual role of wild birds in the epidemiology of bornaviruses is unknown. Currently, there are gaps in the knowledge about the role played by wild birds in favouring the spread and emergence of novel pathogenic strains, as well as their role in introducing the virus in captive flocks. Also, concerning psittacines in captivity, there is no consensus on the route(s) of bornavirus transmission, as well as the risk factors associated with the occurrence of outbreaks, which are not established. Besides, the effects of the geographical spread of the infection's impact on the welfare and health of pet parrots, reported in the literature, are just the top of the iceberg. According to the published literature, the psittacine trade has a much larger geographic spectrum than the spectrum of areas included in studies that address bornavirus infection in parrots. For example, in Europe, published studies on

avian bornavirus infections were mainly conducted in countries from the central region. Additionally, the consequences of the psittacine trade on the infection spread and biodiversity are unknown.

The aims of this Thesis are to contribute to the development of knowledge about the epidemiology of bornavirus infection, addressing the following specific objectives: 1) develop a cost-effective real-time PCR assay, SYBR dye-based, to detect bornavirus reservoirs; 2) develop a cost-effective real-time PCR assay, TaqMan probe-based to test animals suspected to be infected by bornavirus, to assist the clinical diagnosis; 3) screen captive flocks of psittacines housed in breeding projects and zoos, to detect virus reservoirs; 4) characterize the bornavirus detected in captive parrots in Portugal and Sweden; 5) evaluate the relationship between bornavirus worldwide detected in captive psittacines and those detected in Portugal and Sweden; 6) evaluate the relationship between bornavirus worldwide detected in captive psittacines and those detected in wild birds worldwide distributed. commercial

To accomplishing the objectives of the study, a two-step quantitative real-time PCR assay based on SYBR® Green was developed to screen flocks of animals and identify bornavirus reservoirs. Also, a quantitative real-time two-step PCR assay based on TaqMan® was developed to assist clinical diagnosis. The phosphoprotein (P) gene from bornavirus was the target in both developed assays. The absolute quantification systems for the real-time PCR assays were developed *in-house*. The performance of both assays was evaluated considering the following parameters: amplitude of the dynamic linear range, the upper limit of detection (capacity), the lower limit of detection (sensitivity), efficiency, precision and reproducibility.

Using the developed assays, healthy psittacines living in Portugal were screened using the developed assays. The psittacines belonged to flocks in captivity (for reproductive and commercial purposes) and a Zoo. From the parrots, samples of excretions (faeces and urine), secretions (saliva), as peripheral blood were collected. The samples collected were screened for bornavirus RNA. The investigation also included psittacines followed in hospitals and clinics in Portugal and Sweden, with a clinical history compatible with infection by bornavirus. The psittacines that died with a clinical history suggesting bornavirus infection underwent standardized necropsy, and samples of tissues from different organs belonging to several systems were collected. The selected samples were subjected to immunohistochemistry, PCR and histopathological tests. The genetic and protein profiles of the bornaviruses identified in the evaluated samples were established using bioinformatics. Also, bioinformatics analyses were conducted to produce epidemiological knowledge by integrating metadata obtained from information extracted from a public

database (GenBank®). The collection of GenBank® information was performed according to a previously established protocol.

The results obtained revealed a high performance of both developed real-time PCR assays. Namely, both real-time PCR tests showed a wide dynamic range of detection (with 8 orders of magnitude), high capacity (the upper limit of detection was $2X10^7$ copies of the phosphoprotein (P) gene) and high sensitivity (the lower limit of detection was 2 copies of the P gene). Additionally, both tests showed precision (correlation coefficients (R^2)> 0.99) and reproducibility (threshold cycle (Ct) ≤0.5). The real-time PCR based on SYBR® Green showed an efficiency of 99.42%, and the test based on TaqMan® showed an efficiency of 99.02%.

Additionally, the research work carried out made it possible to recover one-sixth of avian bornavirus genomes (including genes N, X, P and M). Parrot bornavirus 4 (PaBV-4) was identified as the etiologic agent associated with the death of pet psittacines residing in Portugal and Sweden. The PaBV-4 genotypes identified in this study proved to be more related to some genotypes identified in wild birds than to some identified in pet psittacines, distributed worldwide. The genetic clusters identified for PaBV-4 distributed worldwide were not linked with the geographical origin, the year of sampling and the species of infected psittacines. The phosphoprotein (P) of PaBV-4 was found to be more related to the phosphoprotein of members of the species *Mammalian 2 orthobornavirus* (genotypes of the variegated squirrel bornavirus 1 zoonotic) than to some avian bornaviruses. Namely, phosphoprotein showed a higher degree of conservation than X protein within and between different bornavirus species. Since the highest conservation degree of the phosphoprotein was observed in the region shared with X protein. Additionally, the phosphoprotein of PaBV-4 showed to be evolutionarily closer to genotypes of variegated squirrel bornavirus 1; and its nucleoprotein showed to be evolutionarily closer to avian bornaviruses. Besides, the phosphoprotein and nucleoprotein of bornavirus detected in wild birds revealed to be ancestral proteins versions of the Borna disease virus 1 genotypes, which were the cause of lethal encephalitis in humans.

In conclusion, the real-time PCR tests, based on SYBR® Green and TaqMan® developed, showed to have advantages compared to the real-time PCR tests described in the literature. Namely, both assays showed better detection limits (capacity and high sensitivity), proved to be less expensive, less laborious and less time-consuming. Consequently, the SYBR® Green-based test will be an asset in screening large populations of psittacines (such as flocks housed in zoos, in breeding projects for rare species, in private collections and wild flocks). Also, the test based on TaqMan® will be an asset as a complementary tool for clinical diagnosis.

The research also allowed the characterization of the genetic and protein profiles of avian bornaviruses that infected pet psittacines that lived in Portugal and Sweden. Molecular epidemiology has suggested that Portugal and Sweden are on the route of the world trade in psittacines; and that this occurs without biosafety measures. Besides, it showed that horizontal transmission of the infection occurred within and between different taxonomic orders of parrots in captivity. Additionally, molecular epidemiology has revealed that bornavirus infection contributed to a decrease in the number of psittacines from endangered species.

Bioinformatics analyses suggested that wild birds played a role in the emergence of new genotypes and the spread of bornavirus infection. As well as wild birds presumably participated in the infection introduction in flocks of captive psittacines.

Additionally, bioinformatics approaches suggested that among members of the *Bornaviridae* family, genome rearrangement events occurred. Besides, they allowed to conclude that the high conserved region of phosphoprotein found is a suitable candidate as the universal target in the laboratory diagnosis of bornaviruses.

Resumo

Os bornavirus são entidades que possuem um envelope, e têm entre 80 e 100 nanómetros de diâmetro. Estes vírus têm um genoma de ARN, não segmentado, de cadeia simples, de sentido negativo, com cerca de 8900 nucleótidos de comprimento. Os bornavirus têm a capacidade de se replicarem no núcleo das células nervosas infetadas de vários órgãos, e frequentemente estabelecem infeções persistentes não citolíticas e exploram os mecanismos de *splicing* celular. A organização do seu genoma consiste em seis estruturas de leitura aberta (*open reading frame* (ORF)), no que respeita ao membro protótipo dos bornavirus. Ao longo do genoma existem locais alternativos de início e término da transcrição, assim como ocorrem eventos de *splicing* resultando na produção de várias moléculas de ARN mensageiro (ARNm). A primeira unidade de transcrição contém uma ORF para a nucleoproteína (N), a segunda contém a sobreposição de duas ORF, referentes à proteína X (X) e à fosfoproteína (P). A terceira unidade de transcrição possui diversos sinais de início e término da transcrição, permitindo a expressão da proteína da matriz (M), da glicoproteína (G), bem como do ARN-dependente ARN - polimerase (L), existindo sobreposição da ORF da proteína da matriz com a da glicoproteína.

Os bornavirus são membros da ordem *Mononegavirales*, da família *Bornaviridae*. Até ao presente, estão identificados os géneros *Carbovirus*, *Cultervirus* e *Orthobornavirus*, que incluem 11 espécies. A infeção por bornavirus pode causar o desenvolvimento de doença neurológica e/ou gastrointestinal grave, assim como a morte dos seus hospedeiros. A doença foi reportada em humanos e em várias espécies de animais de estimação, de produção e em animais selvagens. Duas espécies infetam mamíferos (*Mammalian 1* e *2 orthobornavirus*), cinco espécies infetam pássaros (*Passeriforme 1* e *2 orthobornavirus*, *Psittaciform 1* e *2 orthobornavirus* e *Waterbird 1 orthobornavirus*) e três infetam répteis (*Queensland carbovirus*, *Southwest carbovirus* e *Elapid 1 orthobornavirus*). Dentre as espécies *Mammalian 1* e *2 orthobornavirus*, existem alguns vírus com potencial zoótico reconhecido, tais como o Borna disease virus 1 (BoDV-1) e o variegated squirrel bornavirus 1 (VSBV-1). Nomeadamente, o Borna disease virus 1 (BoDV-1) e o variegated squirrel bornavirus 1 (VSBV-1) foram os agentes etiológicos de encefalite letal em humanos. Porém, alguns genótipos de BoDV-1 também mostraram ter a capacidade de infetar avestruzes (em cativeiro) e aves selvagens (como o pato-real e o corvo). As aves selvagens, revelaram ser hospedeiras de uma grande variedade de vírus pertencentes às espécies *Waterbird 1 orthobornavirus* e *Psittaciform 1 orthobornavirus* e, adicionalmente,

vários genótipos de *aquatic bird* bornavirus 1 e 2 (ABBV-1 e 2) e de *parrot* bornavirus 2 e 4 (PaBV-2 e 4).

Os membros das espécies *Psittaciform 1* to *2 orthobornavirus* (Parrot bornavirus 1 to 8 (PaBV-1 to 8)) causam efeitos severos no bem-estar e na saúde dos psitacídeos em cativeiro, e assim como a infeção pode ter repercussões graves a nível de biodiversidade e economia no âmbito dos surtos em jardins zoológicos, em reservas, em projetos de criação de espécies raras e em coleções privadas de psitacídeos. No entanto, foram também relatadas infeções assintomáticas em animais de cativeiro e selvagens. Assim como, psitacídeos de cativeiro infetados naturalmente, e que testaram positivo para RNA de bornavirus permaneceram seronegativos. O vírus é libertado através das excreções (fezes e urina) e das secreções (saliva) dos hospedeiros, sendo que a libertação pode ocorrer intermitentemente. De acordo com estudos já publicados, a infeção por bornavirus pode persistir por vários anos em bandos de psitacídeos em cativeiro.

O exame histológico e imunohistoquímico de tecidos do sistema nervoso central foram sugeridos como ferramentas laboratoriais padrão para detetar infeções por bornavirus. No entanto, o uso de procedimentos muito invasivos para a colheita de amostras em animais vivos, de bandos selvagens e em cativeiro, para identificar reservatórios é controverso do ponto de vista da exequibilidade e ético. Mesmo na prática clínica, do ponto de vista da exequibilidade, a recolha de tecidos do sistema nervoso central, de animais vivos, para confirmar a suspeita de infeção é um procedimento complexo, assim como os procedimentos invasivos apresentam um risco inerentemente elevado para o animal. Na literatura, estão descritas ferramentas de diagnóstico que permitem a utilização de amostras recolhidas por métodos não/menos invasivos (por exemplo, fezes, urina, saliva e sangue), no entanto, os testes sorológicos disponíveis produziram resultados inconsistentes quando utilizados. Os métodos de isolamento viral são trabalhosos, caros e demorados e a quantificação precisa da carga viral, em amostras, é difícil utilizando a técnica convencional da reação em cadeia da polimerase (PCR). As técnicas/metodologias convencionais de PCR são trabalhosas e demoradas em comparação com as ferramentas baseadas em PCR em tempo real. No entanto, os sistemas de quantificação da carga viral utilizados nos testes de PCR em tempo real, previamente relatados na literatura, são dispendiosos, requerem preparação trabalhosa e têm prazos de validade curtos. O custo elevado e o tempo de resposta alongado impedem, em alguns casos, o acesso aos diagnósticos laboratoriais, bem como reduzem a probabilidade de intervenções em tempo útil, respetivamente. Pelo que, o desenvolvimento de ferramentas de diagnóstico com uma boa relação custo-benefício é uma mais-valia na vigilância de psitacídeos em cativeiro, no apoio ao diagnóstico clínico, bem como em estudos com animais selvagens.

A epidemiologia das bornaviroses é um *puzzle* que permanece inacabado nomeadamente, o eventual papel das aves selvagens na epidemiologia das bornaviroses é desconhecido. Atualmente existem lacunas no conhecimento sobre a eventual relevância das aves selvagens no favorecimento da disseminação e emergência de novas estirpes patogénicas, assim como é desconhecido o seu papel na introdução do vírus nos bandos em cativeiro. Adicionalmente, em relação aos psitacídeos em cativeiro, não há consenso sobre a(s) via(s) de transmissão dos bornavirus bem como, não estão estabelecidos os fatores de risco associados à ocorrência de surtos. Além disso, os efeitos da dispersão geográfica do impacto da infeção no bem-estar e na saúde dos papagaios de estimação, relatada na literatura, é, muito provavelmente, apenas a parte do *iceberg* acima do nível da água. De acordo com a literatura publicada, o comércio de aves tem um espectro geográfico muito maior do que o espectro das áreas incluídas nos estudos que abordam a infeção por bornavirus em psitacídeos. Por exemplo, na Europa, os estudos publicados sobre as bornaviroses aviárias, maioritariamente, foram conduzidos em países da região central. Além disso, as consequências do comércio de psitacídeos na disseminação da infeção e na biodiversidade são desconhecidas.

O propósito da presente dissertação é o de contribuir para o desenvolvimento do conhecimento sobre a epidemiologia da infeção por bornavirus, atendendo aos seguintes objetivos específicos: 1) desenvolver um teste de PCR em tempo real baseado no corante SYBR, com boa relação custo/beneficio, para detetar reservatórios de bornavirus; 2) desenvolver um teste de PCR em tempo real baseado numa sonda TaqMan, com boa relação custo/beneficio, para testar animais com suspeita de infeção por bornavirus com o propósito de auxiliar o diagnóstico clínico; 3) rastrear bandos de psitacídeos em cativeiro de projetos de criação e de jardins zoológicos, para detetar reservatórios; 4) caracterizar os bornavirus detetados em psitacídeos de cativeiro em Portugal e na Suécia; 5) avaliar a relação entre os bornavirus detetados mundialmente em psitacídeos de cativeiro e os detetados em psitacídeos de cativeiro em Portugal e na Suécia; 6) avaliar a relação entre os bornavirus detetados mundialmente em psitacídeos de estimação e os detetados mundialmente em aves selvagens.

Neste estudo, um teste de PCR em tempo real quantitativo, de duas etapas, baseado em SYBR® Green, foi desenvolvido para rastrear bandos de animais e para identificação de reservatórios de bornavirus. Além disso, um teste de PCR quantitativo em tempo real, de duas etapas, baseado em TaqMan® foi desenvolvido para auxiliar o diagnóstico clínico. O gene da fosfoproteína (P) de bornavirus foi o alvo em ambos os testes desenvolvidos. Os sistemas de quantificação absoluta, de ambos os testes de PCR em tempo real, foi desenvolvido *in-house*. O desempenho, de ambos os testes, foi avaliado considerando os seguintes parâmetros: amplitude da faixa linear dinâmica, limite superior de deteção

(capacidade), limite inferior de deteção (sensibilidade), eficiência, precisão e reprodutibilidade.

Na investigação realizada foram rastreados psitacídeos, aparentemente saudáveis, residentes em Portugal. Os psitacídeos pertenciam a bandos em cativeiro (com fins reprodutivos e comerciais) e a um Zoo. Dos psitacídeos foram recolhidas amostras de excreções (fezes e urina), secreções (saliva), como sangue periférico. Nas amostras recolhidas foi pesquisada a presença de ARN de bornavirus. A investigação incluiu também psitacídeos, seguidos em hospitais e clínicas em Portugal e na Suécia, com história clínica compatível com infeção por bornavirus. Os psitacídeos, que morreram com histórico clínico compatível com infeção por bornavirus, foram submetidos a necropsia padronizada e foram recolhidas amostras de tecidos, de diversos órgãos, pertencentes a diversos sistemas. As amostras selecionadas foram submetidas a testes de imunohistoquímica, de PCR e exame histopatológico. Os perfis genéticos e proteicos dos bornavirus identificados, nas amostras avaliadas, foram estabelecidos com recurso à bioinformática. Adicionalmente, análises de bioinformática foram realizadas para produção de conhecimento epidemiológico através da integração de meta-dados obtidos a partir de informação extraída de um banco de dados públicos (GenBank®). A recolha da informação do GenBank® foi realizada de acordo com um protocolo previamente estabelecido.

Os resultados obtidos revelaram que ambos os testes desenvolvidos de PCR em tempo real tiveram um desempenho elevado. Nomeadamente, ambos os testes de PCR em tempo real apresentaram uma ampla faixa dinâmica de deteção (com 8 ordens de magnitude), uma capacidade elevada (o limite superior de deteção foi de $2X10^7$ cópias do gene da fosfoproteína (P)) e elevada sensibilidade (limite inferior de deteção foi de 2 cópias do gene P). Adicionalmente, ambos os testes mostraram precisão (coeficientes de correlação (R^2)> 0,99) e reprodutibilidade (*threshold cycle* (Ct) ≤0,5). O PCR em tempo-real, baseado em SYBR® Green mostrou uma eficiência de 99,42% e o teste baseado em TaqMan®, apresentou uma eficiência de 99,02%.

Adicionalmente, o trabalho de investigação conduzido permitiu recuperar um sexto de genomas de bornavirus aviários (abrangendo os genes N, X, P e M). Nomeadamente, foi identificado parrot bornavirus 4 (PaBV-4) como o agente etiológico associado à morte de psitacídeos de estimação residentes em Portugal e na Suécia. Os genótipos de PaBV-4 identificados, neste estudo, mostraram ser mais aparentados com alguns genótipos identificados em aves selvagens do que com alguns identificados em papagaios de estimação, mundialmente distribuídos. Os *clusters* genéticos identificados para os PaBV-4, mundialmente distribuídos, não estavam relacionados com a origem geográfica, o ano da amostragem e as espécies dos psitacídeos infetados. A fosfoproteína (P) dos PaBV-4 revelou ser mais aparentada com a fosfoproteína de membros da espécie *Mammalian 2*

orthobornavirus (genótipos do zoótico variegated squirrel bornavirus 1) do que com alguns bornavirus aviários. Nomeadamente, a fosfoproteína revelou maior grau de conservação do que a proteína X, dentro e entre diferentes espécies de bornavirus. Sendo que, o maior grau de conservação da fosfoproteína foi observado na região partilhada com a proteína X. Adicionalmente, a fosfoproteína dos PaBV-4 revelou ser evolutivamente mais próxima de genótipos de variegated squirrel bornavirus 1; e a sua nucleoproteína revelou ser evolutivamente mais próxima de bornavirus aviários. Ademais, a fosfoproteína e a nucleoproteína de bornavirus que infetaram aves selvagens revelaram ser versões ancestrais das proteínas de genótipos de Borna disease virus 1, os quais foram a causa da encefalite letal em humanos.

Em conclusão, os testes de PCR em tempo real, baseados em SYBR® Green e TaqMan® desenvolvidos possuem vantagens comparativamente com os testes de PCR em tempo real descritos na literatura. Nomeadamente, ambos os testes apresentaram melhores limites de deteção (capacidade e sensibilidade elevada), revelaram-se menos dispendiosos, menos laboriosos e menos demorados. Consequentemente, o teste baseado em SYBR® Green será uma mais-valia no rastreio de populações numerosas de psitacídeos (como bandos alojados em jardins zoológicos, em projetos de criação de espécies raras, em coleções privadas e em bandos selvagens). Adicionalmente, o teste baseado em TaqMan® será uma mais-valia como ferramenta complementar do diagnóstico clínico.

A investigação realizada permitiu ainda a caracterização do perfil genético e proteico de bornavirus aviários que infetaram psitacídeos residentes em Portugal e na Suécia. A epidemiologia molecular sugeriu que Portugal e a Suécia estão na rota do comércio mundial de psitacídeos; e que este ocorre sem medidas de biossegurança. Assim como, mostrou que ocorreu transmissão horizontal da infeção dentro e entre diferentes ordens taxonómicas de psitacídeos em cativeiro. Adicionalmente, a epidemiologia molecular revelou que a infeção por bornavirus contribuiu para a diminuição do número de psitacídeos de espécies ameaçadas de extinção.

As análises bioinformáticas sugeriram que as aves selvagens tiveram um papel no surgimento de novos genótipos e na disseminação da infeção por bornavirus. Assim como as aves selvagens presumivelmente participaram na introdução da infeção em bandos de psitacídeos em cativeiro.

Adicionalmente, as abordagens bioinformáticas sugeriram que entre os membros da família *Bornaviridae* ocorreram eventos de rearranjo do genoma. Além disso, elas permitiram concluir que a região altamente conservada da fosfoproteína é uma candidata adequada como alvo universal no diagnóstico laboratorial das bornaviroses.

Contents

List of figures

Page

Chapter 6

List of tables

Page

Chapter 5

Chapter 6

List of abbreviations and units

List of abbreviations

2´-FdC	2´-fluoro-2´- deoxycytidine
ABBV-1 to 2	Aquatic bird bornavirus 1 to 2
Ara-C	1-beta-D-arabinofuranosylcytosine
BiP	Immunoglobulin heavy chain-binding protein
BLAST®	Basic local alignment search tool
BoDV-1	Borna disease virus 1
cDNA	Complementary deoxyribonucleic acid
CICs	Circulating immunocomplexes
CITES	Conservation on International Trade in Endangered Species of Wild Fauna and Flora
CKII	Casein kinase II
CnBV-1 to 3	Canary bornavirus 1 to 3
CNS	Central nervous system
DAB	3,3´-Diaminobenzidine
DEF	Duck embryonic fibroblasts
DNA	Deoxyribonucleic acid
EBLG	Endogenous bornavirus-like glycoprotein
EBLL	Endogenous bornavirus-like RNA-dependent RNA-polymerase
EBLM	Endogenous bornavirus-like matrix protein
EBLN	Endogenous bornavirus-like nucleoprotein
EBLS	Endogenous bornavirus-like
ECL	Electrochemiluminescence
EDTA	Ethylenediamine tetraacetic acid
eEBLL	Endogenous bornavirus-like RNA-dependent RNA-polymerase in bats of the genus *Eptesicus*
ELISA	Enzyme-linked immunosorbent assay
EVES	Endogenous viral elements
MDCK	Madin-Darby Canine kidney
R^2	Correlation coefficient
ε	Efficiency

List of abbreviations (continuation)

EsBV-1	Estrildid finch bornavirus 1
E-value	Expected value
G	Glycoprotein
GAPDH	Glyceraldehyde-3’-phosphate dehydrogenase
GP-1	Glycoprotein 1
GP-2	Glycoprotein 2
HE	Haematoxylin
HMGB-1	High mobility group protein 1
IAP	Intracisternal A-type particle
IB	Immunoblotting
ICTV	International Committee on Taxonomy of Virus
Id	Identity
IFA	Indirect immunofluorescence assay
IFN	Type I interferon
Ig	Immunoglobulins
IHC	Immunohistochemistry
IP	Immune precipitation
IVCN	International Union for Conservation of Nature
JCPV	Jungle carpet python virus
LDL	Lower detection limit
LGSV	Loveridge’s garter snake virus 1
LINE-1	Long interspersed nuclear element
Log	Logarithm
LTR	Long terminal repeat
M	Matrix protein
MEGA	Molecular Evolutionary Genetics Analysis
MEK	Extracellular signal-regulated kinase
mRNA	Messenger ribonucleic acid
mRNAcap	Messenger RNA with 5’-terminal cap-structure
MTase	Methyltransferase
NK	Natural Killer
T-705	Favipiravir
ΔRn	Maximum magnitude of the signal

List of abbreviations (continuation)

(TNF)-1β	Tumour necrosis factor β
(TNF)-α	Tumour necrosis factor α
Ct	Threshold cycle
L	Large protein or RNA-dependent RNA-polymerase
N	Nucleoprotein
NES	Nuclear export signal
NLS	Nuclear localization signal
Non-LTR	Non-long terminal repeat
NTC	No-template negative control
ORF	Open reading frame
P	Phosphoprotein
PaBV-1 to 8	Parrot bornavirus 1 to 8
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDD	Proventricular dilatation disease
PKCε	Protein kinase Cε
Raf/MEK/ERK	Rapidly accelerated fibrosarcoma/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signalling pathway
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RNPs	Ribonucleoproteins
RT	Reverse transcription
RT $^{-}$	Reserve transcriptase minus
SD	Standard deviation
Spp.	Species
SWCPV	Southwest carpet python virus
TSDs	Target site duplication
UV	Ultraviolet
VSBV-1	Variegated squirrel bornavirus 1
WSV	Wuhan sharpbelly bornavirus
X	X protein
α	Slope

List of units

%	Percent
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microliter
µm	Micrometre
µM	Micromolar
bp	Base pair
cm^2	Centimetre cubic
Kb	Kilobase
KDa	Kilodalton
mg	Milligram
min	minute
ml	Millilitre
mM	Millimolar
ng/µl	Nanograms per microliter
nm	Nanometres
nM	Nanomolar
ºC	Degrees Celsius
s	Second
U	Units
U/ml	Units per millilitre
W/V	Weight per volume
X g	Times gravity

Chapter 1 – General introduction | Literature review

1.1. Bornavirus: history and disease

In the 18th century, in Germany, a neurological disease in horses named "Kopfkrankheit der Pferde" (head disease of horses) was described (Dürrwald R *et al*., 1997). At the time, the "head disease of horses" was occasionally reported in the southern and the south-eastern of Germany (Dürrwald R *et al*., 1997). However, in 1895 occurred an outbreak around the Germany city Borna, in Western Saxony and a high number of horses died from a cavalry regiment (Dürrwald R *et al*., 1997). Following the outbreak, the disease became known as "Bornasche Krankheit", that means Borna disease (Lipkin WI *et al*., 2011). In 1907, the Ministry of Home Affairs of the Kingdom of Saxony ordered a thorough investigation to characterize the clinical signs, pathology and aetiology of Borna disease (Wensman JJ, 2011). Therefore, the clinical signs and the pathology of Borna disease were then well-characterized by Joest and Degen in 1911 and by Schmidt in 1912 (Dürrwald R *et al*., 1997). However, at the time, the etiologic agent of Borna disease remained unknown after several efforts (Dürrwald R *et al*., 1997). Initially, bacterial causes were proposed as etiologic agent and later was suggested to be a bacterial toxin, because of the absence of purulent inflammation in the diseased animals (Dürrwald R *et al*., 1997). At the beginning of the 20th century, Zwick and his co-workers, in Germany, as Nicolau and Galloway, working in France and England, respectively, established the viral aetiology of this disease and the virus nature (Dürrwald R *et al*., 1997). In 1928, it was proposed, for the first time, that the Borna disease virus (BoDV) was the etiological agent of Borna disease (Dürrwald R *et al*., 1997). Borna disease was characterized as a non-purulent meningoencephalomyelitis, often fatal in horses and sheep, farmed in Germany and the upper Rhine valley (between Switzerland, Austria and the Principality of Liechtenstein) (Weissenböck H *et al*., 1998; Staeheli P *et al*., 2000). Horses were the first recognized host species of Borna disease virus, but later studies reported the presence of Borna disease virus in sheep and cattle (Dürrwald R *et al*., 1997). As knowledge about Borna disease virus infection progressed during many years of research, it was revealed that it was distributed across the five continents, infecting a wide range of mammalian species (Kinnunen PM *et al*., 2013). This also Include humans as antibodies against Borna disease virus were detected in humans with psychiatric disorders (Arias I *et al*., 2012). As Borna disease virus is known to induce persistent emotional, cognitive and behavioural alterations in experimentally infected animals (Lancaster K *et al*., 2007), the question has arisen of whether the virus could also be a human pathogen and the cause of neuropsychiatric disorders. However, the issue remained controversial until the first decade of the 21st

century. Since then, within the scientific community, it is accepted that the virus can infect humans and to cause lethal encephalitis (Korn K *et al*., 2018; Schlottau K *et al*., 2018; Petzold J *et al*., 2019). However, the relationship between Borna disease virus infection and human psychiatric disease remains to be clarified (Lipkin WI *et al*., 2011).

In 2008, as an etiologic agent of Proventricular dilatation disease (PDD) in parrots, a novel bornavirus was proposed (Honkavuori KS *et al*., 2008; Kistler AL *et al*., 2008). The novel virus showed to be closely related to the Borna disease virus (Honkavuori KS *et al*., 2008; Kistler AL *et al*., 2008). The name given to the novel virus was "Avian bornavirus" (ABV) (Honkavuori KS *et al*., 2008; Kistler AL *et al*., 2008). From then until nowadays, there is evidence of infection by avian bornaviruses in several species of birds (e.g., ostriches, geese, trumpeter swans, mallards, gulls and canaries) (Malkinson M *et al.*, 1993; Berg M *et al*., 2001; Guo J *et al*., 2012; Guo J *et al*., 2014 (a); Guo J *et al*., 2015; Murray M *et al*., 2017; Nielsen AMW *et al*., 2018; Philadelpho NA *et al*., 2019). Since the first publication several species of avian bornaviruses have been identified (Malkinson M *et al.*, 1993; Berg M *et al*., 2001; Guo J *et al*., 2012; Guo J *et al*., 2014 (a); Guo J *et al*., 2015; Murray M *et al*., 2017; Nielsen AMW *et al*., 2018; Philadelpho NA *et al*., 2019).

In 2014, for the first time, the infection by bornaviruses in reptiles, a novel virus species was isolated from a wild *Elapsoidea loveridgei* and later named as Loveridge's garter snake virus 1 (LGSV-1) was reported (Stenglein MD *et al*., 2014), and two more novel species of reptiles bornaviruses were published in 2018 (Hyndman TH *et al*., 2018).

In addition, for the first time, in 2015, a new species of mammalian bornaviruses was associated with the occurrence of lethal encephalitis in squirrel breeders (Hoffmann B *et al*., 2015). At the time, the study alluded to the zoonotic potential of this novel virus, as it was both detected in breeding squirrels, and their dead breeders (Hoffmann B *et al*., 2015). The new virus was named variegated squirrel bornavirus 1 (VSBV-1) (Hoffmann B *et al*., 2015). In the last years, new cases of lethal encephalitis caused by variegated squirrel bornavirus 1 have been published (Tappe D *et al*., 2018; Tappe D *et al*., 2019 (a); Tappe D *et al*., 2019 (b)).

1.2. Taxonomy: the history of bornaviruses classification and nomenclature

The etiologic agent of Borna disease was established in the 1920s (Kuhn JH *et al*., 2015) and from then on, the virus had several nominations, such as: "Bornavirus," "virus of Borna disease" or "Borna virus" (Kuhn JH *et al*., 2015). From the mid-1970s, the etiologic agent became frequently named "Borna disease virus", as well as "BoDV", the abbreviation became the most used (Kuhn JH *et al*., 2015). For the first time the virus was mentioned in

the third report (1979) of the International Committee on Taxonomy of Viruses (ICTV) and was designated as an unclassified virus or virus-like agent (Kuhn JH *et al*., 2015). The virus also was listed as an unclassified virus or virus-like agent in the fourth (1982), fifth (1991), and sixth ICTV reports (1995) (Kuhn JH *et al*., 2015). In 1996, the ICTV established a new mononegavirus family, designating it as *Bornaviridae*, which included only the genus *Bornavirus*, containing a single species, Borna disease virus (at the time not italicized) (Kuhn JH *et al*., 2015). The virus classification and its nomenclature remained unchanged in reports of ICTV Seventh (in 2000), Eighth (in 2005), and Ninth (in 2011), however, being italicized the species Borna disease virus (Kuhn JH *et al*., 2015).

In 2015, the family *Bornaviridae* was reorganized by establishing five species in the genus *Bornavirus* (Kuhn JH *et al*., 2015) following a non-Latinized binomial species name format (Kuhn JH *et al*., 2015). Therefore, the genus *Bornavirus* was reorganized into the following species: 1) *Mammalian 1 bornavirus* (the classical Borna disease virus, as well as the divergent Borna disease virus, isolate No/98), 2) *Psittaciform 1 bornavirus* (avian/psittacine bornaviruses 1, 2, 3, 4, 7), 3) *Passeriform 1 bornavirus* (avian/canary bornaviruses C1, C2, C3, LS), 4) *Passeriform 2 bornavirus* (estrildid finch bornavirus EF), and 5) *Waterbird 1 bornavirus* (avian bornavirus 062) (Kuhn JH *et al*., 2015). A snake bornavirus, proposed to be named Loveridge's garter snake virus 1, was classified as a member of an additional species (Elapid 1 bornavirus) (Kuhn JH *et al*., 2015). Avian bornaviruses 5, 6, MALL, and another "reptile bornavirus" ("Gaboon viper virus") stayed as unclassified until further information becomes available (Kuhn JH *et al*., 2015).

In 2016, two new species were added to the genus *Bornavirus Elapid 1 bornavirus* (for Loveridge's garter snake virus 1) and *Psittaciform 2 bornavirus* (for parrot bornavirus 5) (Afonso CL *et al*., 2016); also in 2017, the new species *Mammalian 2 orthobornavirus* was added, for the newly discovered variegated squirrel bornavirus 1, according to the International Committee on Taxonomy of Viruses (ICTV) (Amarasinghe GK *et al*., 2017).

The family *Bornaviridae* expanded again, as in 2018 a second genus (genus *Carbovirus*) was added, as well as two new species for the newly discovered jungle carpet python virus (JCPV) and southwest carpet python virus (SWCPV) found in carpet pythons (*Pythonidae: Morelia spilota*) were included (Amarasinghe GK *et al*., 2018). Moreover, in 2018 the genus *Bornavirus* was renamed as *Orthobornavirus,* aiming to remove the ambiguity of the terms "bornavirus"/"bornaviral" that resulted due to the establishment of the second genus (Amarasinghe GK *et al*., 2018). Furthermore, in 2018 all binomial species names of the genus *Bornavirus* were adjusted by replacing the genus epithet "bornavirus" with "orthobornavirus" (Amarasinghe GK *et al*., 2018).

In 2019, the family *Bornaviridae* incorporated the species *Sharpbelly cultervirus* belonged to the new genus *Cultervirus* (Amarasinghe GK *et al*., 2019) (Figure 1).

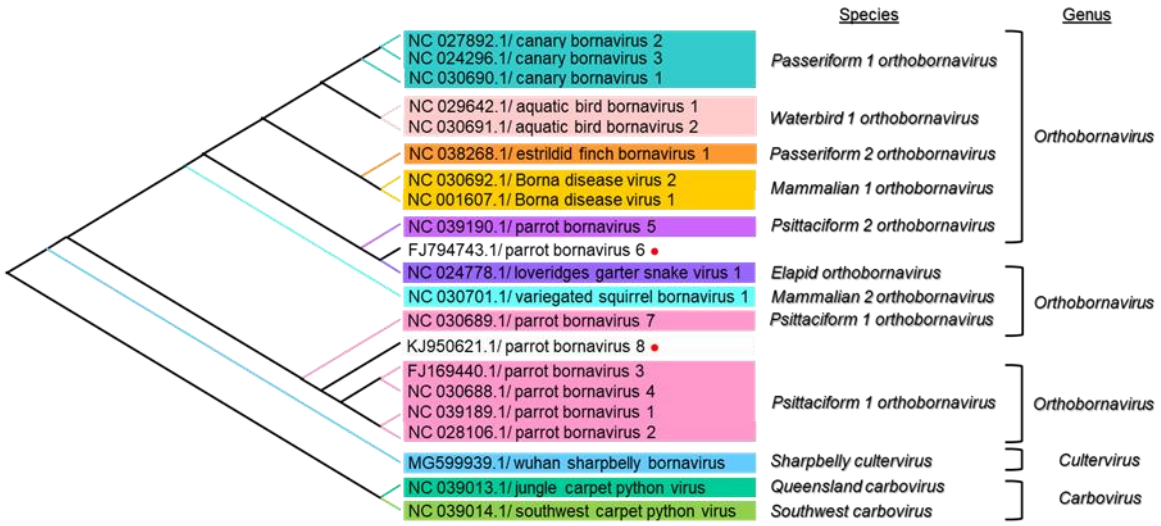

Figure 1. The phylogeny shows the virus names, species and genus belonging to the *Bornaviridae* family identified and described so far (by Marlene Cavaleiro Pinto). The phylogenetic relationship is based on a segment of the M gene. The genetic sequences used were from the GenBank® database and are identified with the accession numbers. Bornaviruses marked with a red circle are not classified following the currently accepted taxonomy (Amarasinghe GK *et al*., 2019).

1.3. Virion properties, genome, antigens and life cycle

When it was proposed that a virus could be the etiologic agent of Borna disease, at that time, the characteristics of bornaviruses and the structures of viral particles (virions), were unknown. The first published studies describing the etiologic agent of Borna disease, emerged in the twenties and thirties of the last century (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Namely, studies addressed the physical properties (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). At that time, it was proposed that the virus were spherical enveloped particles ranging in diameter from 85 to 125 nm for the virion (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). In the late 1960s, for the first time, viral antigens were observed by immunofluorescence (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017), and in the early seventies the cultivation of Borna disease virus in tissue culture was performed successfully (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). In 1997, it was proposed the Borna disease virus as an

RNA virus (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017), which frequently persisted inside of the infected cells and was only sporadically released (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). In the same year, the first electron micrographs of the virus revealed spherical particles (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017), corroborating the theory published in the late 1930s, but the virion morphology was confirmed only in the 1990s (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The composition of the virus genome remained unknown until about the 1990s. Namely, the establishment the full sequence of the virus genome was in 1994 (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The next paragraphs address the genome, proteins and life cycle of bornaviruses.

1.3.1. Virion properties

The observation, by electron microscopy, of extracts from cells culture infected with bornaviruses revealed the presence of spherical, enveloped particles ranging in diameter from 40 to 190 nm (Lipkin WI *et al*., 2011). Particles ranging in diameter equally or higher than 90 nm showed an electron-dense core (50–60 nm) suggesting being the infectious virions (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). However, the smaller particles, with a diameter less than 90 nm, were proposed to be defective (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Additionally, spikes of 7 nm were observed on the larger particles (≥90 nm), suggesting representing the viral glycoprotein; however, this has not been confirmed by immune-electron microscopy (Lipkin WI *et al*., 2011). Nevertheless, details about the ultrastructure of bornavirus remain vague because of the low amounts of complete virions produced during an infection, making it difficult to advance this knowledge (Payne S, 2017).

1.3.2. Genome organization

Bornaviruses have monopartite, linear, compact, non-segmented, negative-strand RNA (non-polyadenylated) genomes of around 9000 nucleotides in length, whose prototype member is Borna disease virus 1 (BoDV-1) (Lipkin WI *et al*., 2011). Bornaviruses are singular among the non-segmented, negative-sense RNA viruses, as they replicate in the nucleus of host, and they use splicing mechanisms to generate some mRNAs (Payne S, 2017). In the genome of bornaviruses was mapped a single promoter, (positioned near the 3'-end of the genome, which drives initiation of transcription), four transcription termination sites and three reinitiating sites (Payne S, 2017).

Bornaviruses uses the cellular splicing mechanisms to efficiently use their genome that contain three transcription units that encode six open reading frames (ORFs) (Figure 2) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). In the prototype member (which is Borna disease virus 1 (BoDV-1)), the first transcription unit (1.2 kb) is monocistronic and contain one ORF for the nucleoprotein gene (N) (Figure 2). The second transcription unit (0.8 kb) is bicistronic and comprises two overlapping ORFs for the phosphoprotein gene (P), and the X protein gene (X) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017), being the X gene a small non-structural gene (Figure 2) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The third transcription unit (2.8 or 7.1 kb RNA) is tricistronic, splice differently, and also has alternative transcription start and stop sites (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017); resulting in the production of three proteins, such as: the matrix protein (M), the glycoprotein (G), and the large protein or RNA-dependent RNA-polymerase (L) (Figure 2) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The M and G ORFs overlap each other (Figure 2) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

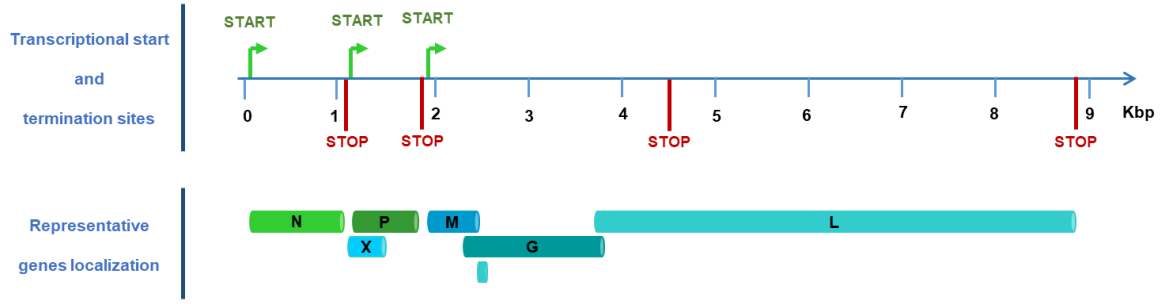

Figure 2. The genomic map of Borna disease virus 1 (BoDV-1) prototype member of bornaviruses (by Marlene Cavaleiro Pinto). BoDV uses alternative transcription strategies, like over-lapping open reading frames (ORFs) and the host cellular splicing mechanisms. Abbreviations: (N) nucleoprotein gene; (X) X protein gene; (P) phosphoprotein gene; (M) matrix protein gene; (G) glycoprotein gene; (L) Large protein gene or RNA-dependent RNA-polymerase gene.

From the viral genome about 0.6% of the nucleotides are not transcribed, they are not present into sub-genomic RNAs produced (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). In the primary viral transcripts, the trailer region (55 nucleotides) at the 5'-end of the genome is not transcribed (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Additionally, the viral genome has a 42-nucleotide sequence rich in adenosine/uridine, located between the 3'-end and the first base belonging to the first transcriptional unit (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

Moreover, complementary extracistronic sequences at the 3' (leader) and 5' (trailer) termini of the virus genome were found, whereby these structures have potential to align forming a terminal panhandle (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

The genome of the Loveridge's garter snake virus 1 (LGSV-1), has a similar organization to mammalian and avian bornaviruses (Stenglein MD *et al*., 2014). However, the genome order of the jungle carpet python virus (JCPV) and the southwest carpet python virus (SWCPV) are different from other bornaviruses (Hyndman TH *et al*., 2018). In these two viruses, the order of genes is 3'-N-X-P-G-M-L-5', representing a transposition of the G and M genes compared to other bornaviruses (Hyndman TH *et al*., 2018). The genome of JCPV and SWCPV contain 93 and 71 nucleotides of predicted noncoding region at their 3' ends and 50 and 77 nucleotides at their 5' ends, respectively (Hyndman TH *et al*., 2018). The JCPV and SWCPV share less than 45% pairwise amino acid identity with other bornavirus (Hyndman TH *et al*., 2018).

1.3.3. Viral proteins and immunogenicity

In the following paragraph, the six polypeptides encoded by the genome of bornaviruses are described.

The six open reading frames (ORFs) in the genome of the BoDV-1 prototype member of bornaviruses encode for the following polypeptides: nucleoprotein (N, p40), phosphoprotein (P, p23), X protein (X, p10) matrix protein (M, p16), glycoprotein (G, p57) and L-polymerase (L, p190) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The denomination of p40, p23 and so forth indicates their respectively molecular weights (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

The nucleoprotein (N) was the most abundant in infected cells, and predominantly located inside the nucleus (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Additionally, nucleoprotein was the predominantly component found in the nucleocapsid or ribonucleoprotein (RNP) complex, with the genomic RNA. The nucleoprotein and the RNA forming the backbone of the ribonucleoprotein (RNP) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Furthermore, the nucleoprotein interacts with phosphoprotein during the intracellular transport of ribonucleoprotein complexes (RNPs) into and from the nucleus (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The N protein is present in Borna disease virus 1 (BoDV-1) in two isoforms (p40 and p38), which differ in 13 amino acids (aa) at the N-terminus produced by use of alternative start codons (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Moreover, the N protein contains a nuclear localization signal (NLS) (located at the N-terminus of the p40 isoform) and a

nuclear export signal (NES) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

The phosphoprotein (P) down-regulates the polymerase activity upon phosphorylation (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The P protein phosphorylation is essential for the efficient viral dissemination in infected cells, suggesting that it is a protein with crucial functions in viral transmission (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The P protein is phosphorylated at serine residues principally by protein kinase Cε (PKCε) and to a lesser extent by casein kinase II (CKII) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The phosphoprotein forms homomers (as tri- or tetramers), which can interact with RNA-dependent RNA-Polymerase (large protein (L)) and serving as a cofactor for large protein (L) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). However, this interaction is blocked when the nucleoprotein is not bound to the ribonucleoprotein complex (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Additionally, the P protein and the X protein can interact, preferably as a monomer, indicating that X protein has a role in multimerization of P protein (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The P protein is acidic (predicted isoelectric point of 4.8) and has a high serine-threonine content (16%) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The P protein forms a central structural unit in the assembly of the active polymerase complex (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). P protein binds to N, X and L proteins through protein-protein interactions, suggesting that it may contribute to the nuclear position of X and N protein, once P protein contains two nuclear localization signals (NLS) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S *et al*., 2017).

The X protein is a small non-structural protein found in the nucleus of infected cells and interacts with P protein (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The X protein has a role in RNA replication, it participates in the regulation of polymerase complex activity (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Additionally, it has been proposed that, X protein, has an inhibitory role in the apoptosis process of infected cells, leading to a persistent infection (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Nevertheless, the green monkey kidney (Vero) cell line infected with BoDV-1 showed apoptosis progression (Wensman JJ, 2011). Moreover, it was proposed that the X protein participates in the mediation of nuclear shuttling of viral gene products such as unspliced RNAs and the ribonucleoprotein complexes (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

The matrix protein (M) constitutes a covering (an outer layer) of the ribonucleoprotein (RNP) complex in the complete enveloped virions, suggesting having a protective function (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Additionally, the matrix

protein can form tetra- or octamers, which by the interaction with phosphoprotein becomes a part of the ribonucleoprotein (RNP) complex, without inhibitory effects of the polymerase activity (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Besides, M protein binds to single-stranded RNA and interacts with lipid membranes, suggesting that has a crucial role in the production of ribonucleoprotein (RNPs) complexes, as well as of complete enveloped virions (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Antibodies against matrix protein neutralize the virus infectivity, suggesting that the protein is present on the surface of complete enveloped virions (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). However, virus also spreads as ribonucleoprotein (RNPs) complexes, from cell to cell inside the central nervous system (CNS) as well as in cell culture (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Therefore, it was suggested that the neutralizing effect of antibodies towards matrix protein probably is due to neutralization of infectious ribonucleoprotein (RNPs) complexes (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

Glycoproteins (G) can bind to the membrane receptors in target cells, allowing the virus entry. Glycoprotein is posttranslationally modified by N-glycosylation to yields a 93-94-KDa primary product (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Some of the glycosylated proteins are cleaved by the cellular protease furin into two biologically active proteins (G-1 and G-2) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Namely, the cleavage produces a molecule of 51 KDa (p51) and 43 KDa (p43), corresponding to the amino-terminal (G-1) and carboxyl-terminal (G-2) products, respectively (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The G-1 protein is responsible for the attachment to the host cell surface (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). One potential cellular receptor was the immunoglobulin heavy chain-binding protein (BiP), an endoplasmic chaperone expressed on the cell surface, which is also called glucose-regulated protein 78 (Honda T *et al*., 2009). However, further studies are required for a full understanding of the association processes between bornavirus and the host cells (Honda T *et al*., 2009). After the biding of G-1 protein to the cellular receptor, virus enters to the host cell through endocytosis (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011). The virus, once inside of the endosome, at the cytoplasm, the G-2 mediates pH-dependent fusion of the viral and endosomal membranes, to release the ribonucleoprotein (RNP) complex (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Neutralizing antibodies against the viral glycoproteins are produced by the host in the chronic phase of the disease (Furrer E *et al*., 2001). The BDV-specific monoclonal antibodies against the glycosylated glycoprotein, produced by hybridomas, showed to neutralize the infectious virus and to prevent Borna disease in experimentally infected rats when given prophylactically (Furrer E *et al*., 2001). Also, monoclonal antibodies

against the glycosylated glycoprotein, produced by chronically infected rats, showed specificity for the 43-KDa C-terminal furin cleavage product, providing evidence of conformational epitope recognition (Furrer E *et al*., 2004).

The large protein (L) is an RNA-dependent RNA-polymerase of about 190 kDa, the catalytic subunit of the transcription/replication complex (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). It was also denominated as RNA-Dependent RNA-Polymerase that, can translocate into the nucleus by itself (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). However, there are viral proteins within the RNP that potentiated this nuclear translocation (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The host cellular kinases phosphorylate the viral L protein, and it is therefore involved in the polymerase activity regulation (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). However, several viral proteins (N, P, and L) are required to constitute a polymerase complex, for successful transcription and replication (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The polymerase complex activity is negatively regulated, through the phosphorylated P protein, which previously bound to X protein (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

1.3.4. Life cycle

The virion binds to the host cell and release into the cytoplasm the viral ribonucleoprotein complexes (RNPs) aggregated with the viral RNA genome (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The envelope glycoprotein (or G protein) is responsible for the virus attachment to the host cells. Namely, the amino-terminal 244 aa of the glycosylated G protein (94-kDa primary product) and/or G-1 protein (51-KDa product) participate in host receptor binding (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011). However, the binding process between glycoproteins of the virus and the cellular receptors of the host is not completely known (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). After the virion entrance by endocytosis, the RNP complexes are released into the cytosol through the fusion between G-2 protein (43-KDa product) and the membrane of intracellular vesicles (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011). The hydrophobic amino-terminus of G-2 protein initiates the pH-dependent fusion process however, the molecular details of the fusion process are unknown (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011). Once inside the host cell, the nucleocapsid or ribonucleoprotein complex (single strand of RNA packaged by nucleoprotein, linked to the phosphoprotein and the large protein (L) migrates from cytoplasm to the nucleus, where genome replication occurs (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The duplication of the

viral genome requires three proteins: the nucleoprotein, phosphoprotein, and large protein (L) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). There is the synthesis of anti-genomes, which are full-length positive-strand copies of the negative-strand RNA viral genome (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The anti-genome serves as a template for new negative-strand progeny genomes (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The viral genome strand reveals truncations at the ends, resulting in the elimination of promoters (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017), suggesting that this event downregulates the virus replication and transcription process. Moreover, it suggests to has a role in the persistent nature of bornaviruses infection (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The copy of the genomic termini results in non-triphosphorylated 5'-termini, (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The four-terminal bases of genome and anti-genome appear to be copied from internal template motifs through a realignment mechanism, allowing later cleavage of 5-triphosphorylated terminal bases from progeny strands without loss of genetic information (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The transcription process occurs in the nucleus of host cells, and it requires the N, P and L proteins (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Also, L caps and polyadenylates viral mRNAs, while the cellular splicing machinery, are likely to process some transcripts (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Transcription of the viral genome results in the synthesis of four essential primary, 5-capped and 3-polyadenylated RNAs with chain lengths about 0.8 kb, 1.2 kb, 2.8 kb and 7.1 kb (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). A sequential and polar transcription results in a transcriptional gradient (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017) therefore, the most abundant transcript is the mRNA encoding the nucleoprotein and the least encoding the L protein (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). Namely, the first two transcripts are found at similar levels, whereas the third transcript was found at lower levels (Briese T *et al*., 1994). Then, the mRNAs migrate to the cytoplasm, where their translation produce six proteins (nucleoprotein, phosphoprotein, X protein, matrix protein, Glycoprotein and Large protein (L)) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The matured viral proteins migrate to the nucleus, where they will participate in the assembling of new nucleocapsids (at present the stages of the nucleocapsid formation process are unknown) (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017). The exportation of the new nucleocapsids is from the nucleus to the cytoplasm; where they can follow one of the two pathways: 1) they can participate in the assembling of new complete enveloped virions or 2) they proceed as infectious particles. Therefore, in addition to transmission by

extracellular virions, the spread between cells occurs in the form of the RNP complexes (Tomonaga K *et al*., 2002; Lipkin WI *et al*., 2011; Payne S, 2017).

1.4. Endogenous bornavirus-like elements

Endogenous viral elements (EVEs) are sequences derived from a virus naturally inserted in the eukaryotic genome, namely in the germline (Horie M *et al*., 2019). The integration of EVEs in the eukaryotic host genomes occurred millions of years ago, and these viral sequences are considered molecular fossils of viruses (Horie M *et al*., 2019). EVEs are considered information sources of ancient viruses, such as: 1) their time scale, 2) their geographical distribution, 3) their genetic information, and 4) their hosts range (Horie M *et al*., 2019). The following paragraphs are about the biological significance of Endogenous viral elements (EVEs) and the paleovirology of bornaviruses (Horie M *et al*., 2019).

Endogenous bornavirus-like elements (EBLs) have been predominantly identified in vertebrate genomes such as: EBLN (endogenous bornavirus-like nucleoprotein), EBLM (endogenous bornavirus-like matrix protein), EBLG (endogenous bornavirus-like glycoprotein), and EBLL (endogenous bornavirus-like RNA-dependent RNA-polymerase) (Belyi VA *et al*., 2010; Horie M *et al*., 2010; Katzourakis A *et al*., 2010; Horie M *et al*., 2013; Cui J *et al*., 2014, Gilbert C *et al*., 2014; Horie M *et al*., 2019). Also, EBLLs were reported to be present in invertebrates genomes (Belyi VA *et al*., 2010; Horie M *et al*., 2010; Katzourakis A *et al*., 2010; Horie M *et al*., 2013; Cui J *et al*., 2014; Gilbert C *et al*., 2014; Horie M *et al*., 2017; Horie M *et al*., 2019). The endogenous bornavirus-like elements (EBLs) above mentioned, derived from N, M, G, and L genes, of bornaviruses, respectively. Non-bornaviral factors maybe play a role in the reverse transcription and integrations events, since bornaviruses do not encode reverse transcriptase either integrase (Horie M *et al*., 2019). Therefore, the long interspersed nuclear element (LINE-1) clade of non-long terminal repeat (non-LTR retrotransposon), was suggested to be involved in some of the viral genes integration events that occurred into the host genomes (Horie M *et al*., 2019). Moreover, the insertion of an EBLN element in the genome of squirrels (*Ictidomys tridecemlineatus*) occurred after the extinction of LINE-1 whereby, another reverse transcriptase played a role in the endogenization process (Suzuki Y *et al*., 2014). Since the EBLN, in question, is flanked by several LTR retrotransposons, it is most likely that one of them participated in the endogenization process (Suzuki Y *et al*., 2014). Also, was proposed that EBLs can be endogenized through co-infection of exogenous retroviruses (Horie M *et al*., 2019).

A distribution tendency of integrated ancient bornavirus, in the host genomes, was reported (Horie M *et al.,* 2019). The EBLNs and EBLLs are present in a wide range of animals but, EBLMs and EBLGs were found in a few lineages of vertebrates while, X or P-like EBLs have not, up to date, been reported (Belyi VA *et al*., 2010; Horie M *et al*., 2010; Katzourakis A *et al*., 2010; Horie M *et al*., 2013; Cui J *et al*., 2014, Gilbert C *et al*., 2014; Horie M *et al*., 2019). There are quite a few factors proposed to explain the integration of bornavirus genes and their distribution tendency (Horie M *et al*., 2019). Firstly, the amount of specific viral transcripts and the preference of reverse-transcriptase, were proposed as biological factors that probably affect the distribution tendency. Once, in the genome of mononegavirus, there is an increased number of transcripts from the 3' to 5'–end (Whelan SP *et al*., 2004), it is expected that N gene transcripts are the most abundant in bornavirus-infected cells (Horie M *et al*., 2019). Therefore, the chance to be inserted into the host chromosomes is increased compared with the other genes (Horie M *et al*., 2019). Additionally, the preference of reverse-transcriptase by N and L mRNA, may also play a role in the distribution tendency (Horie M *et al*., 2019). Secondly, the EBLNs and EBLLs endogenization may be likely to confer selective advantages to the hosts, whereas the other genes may be lethal for their hosts (Horie M *et al*., 2019). Namely, the phosphoprotein (P protein) showed to be a pathogenic element of Borna disease virus (Horie M *et al*., 2019). Furthermore, phosphoprotein (P protein) interacts with the high-mobility group protein 1 (HMGB-1), one of the most important chromatin proteins, inhibiting its physiological function (Kamitani W *et al*., 2001; Kamitani W *et al*., 2003). In contrast, the N protein expression inhibits infections of Borna disease virus without noticeable neurological disease or behavioural abnormalities (Schneider U *et al*., 2003; Rauer M *et al*., 2004; Horie M *et al*., 2017). Therefore, the endogenization of viral N gene confers resistance to the host against bornaviruses infections or leads to the occurrence of disease without deleterious effects (Horie M *et al*., 2019). Thirdly, the methods used to search for endogenous bornavirus-like elements (EBLs) throughout genomes could explain the distribution tendency (Horie M *et al*., 2019). The Basic Local Alignment Search Tool (BLAST®) finds regions of local similarity between sequences (Horie M *et al*., 2019). The bioinformatics tools compare nucleotide or protein sequences to sequence databases and calculates the statistical significance (E-value) of matches, based on the query sequence sizes (Horie M *et al*., 2019). Thus, the length of query genes affects the detection sensitivity (Horie M *et al*., 2019). The L gene is the longest of genes encoded by bornaviruses, while X, P and M genes are the shortest (Horie M *et al*., 2019). Consequently, it is expected that the detection of EBLLs possess a higher probability to occur by BLAST® than the other genes of bornaviruses (Horie M *et al*., 2019). Fourthly, the evolution rate of viral genes can explain their distribution trend throughout the host genome (Horie M *et al*., 2019). It is likely that genes, of bornaviruses,

with high evolutionary rate, are more difficult to be detected by BLAST® (Horie M *et al*., 2019), therefore leads to a bias introduction which resulted in an under-representation of EBLs prevalence, in host genome (Horie M *et al*., 2019). Therefore, endogenous bornavirus-like elements (EBLs) derived from the X and P genes of bornaviruses might be present, in host genomes, however not detectable by BLAST® (Horie M *et al*., 2019). Nevertheless, the expression of phosphoprotein (P protein) can be deleterious therefore, the endogenized X/P genes may not be an advantage for the host populations, from the evolutionary point of view (Horie M *et al*., 2019). An alternative proposal was that ancient bornaviruses might not have encoded X/P genes (Horie M *et al*., 2019).

The gene orthology allows the estimation dates of the insertion of the endogenous virus elements (EVEs) into the host genome (Aiewsakun P *et al*., 2015). Published studies reported that integration of some anthropoid EBLNs occurred in the host genomes before the divergence of *Platyrrhini* and *Catarrhini*, which was estimated to be 43 million years ago (Belyi VA *et al*., 2010; Horie M *et al*., 2010; Katzourakis A *et al*., 2010). Additionally, it was estimated that the integration, into the host genomes, of an afrotherian EBLN and a bat EBLL was, at least, between 65 and 11.8 million years ago, respectively (Katzourakis A *et al*., 2010; Horie M *et al*., 2016; Kobayashi Y *et al*., 2016;). However, in 2018, new facts showed that some EBLNs became endogenous before the divergence of anthropoids (43 million years ago) as previously described (Belyi VA *et al*., 2010; Horie M *et al*., 2010; Katzourakis A *et al*., 2010); as well as the insertion of some EBLNs occurred before 21.9 million years ago; and the incorporation of an EBLN and EBLM arose before 55 million years ago (Horie M *et al*., 2019). Published studies suggest that some EBLs can play a role as anti-bornaviral genes, because hosts having EBLs in their genome showed to be less susceptible to infections and diseases caused by bornaviruses (Belyi VA *et al*., 2010). However, as reported above, several identified EBLNs became endogenous at different times (Gilbert C *et al*., 2014; Horie M *et al*., 2019), which suggests that previously integrated EBLNs did not inhibit the subsequent infections by bornaviruses (Horie M *et al*., 2019). Therefore, suggesting that no expression of inserted EBLNs occurred, at the time of the subsequent infections (Horie M *et al*., 2019). Or else, that the endogenous bornavirus-like nucleoprotein (EBLNs) expression happened but was not able to stop the subsequent infections of bornaviruses (Horie M *et al*., 2019).

Endogenous viral elements (EVEs) can provide useful genetic information about ancient bornaviruses (Horie M *et al*., 2019). An EBLL in bats of the genus *Eptesicus*, (termed as eEBLL-1), preserves a long open reading frame (ORF) containing 1718 codons, which has similar length to the L genes of contemporary bornaviruses, about 1710 codons (Horie M *et al*., 2016). Moreover, the eEBLL-1 has in its proximity a target site duplication (TSD) and a poly-A stretch, suggesting that the ORF of eEBLL-1 reflects the full-length ORF

of an ancient bornavirus (Horie M *et al*., 2016), and thus eEBLL-1 would provide precious information regarding an ancient bornaviral L gene (Horie M *et al*., 2019). The length of the ORF of eEBLL-1 showed to be more like the current bornaviruses than with the ORF from L genes belonging to other mononegaviruses (Horie M *et al*., 2016). Additionally, eEBLL-1 showed absence of block VI, the one conserved region among mononegaviruses (Briese T *et al*., 1994; Poch O *et al*., 1990; Ogino T *et al*., 2011). Therefore, the transcription and replication of an ancient bornavirus, may have been performed in the cell nucleus of bats at least 11.8 million years ago (Horie M *et al*., 2019). And the truncation of the C-terminal region of L protein occurred at least 11.8 million years ago (Horie M *et al*., 2019).

Analyses of gene orthology also showed that the genome organization of some ancient bornaviruses was different from the contemporary, once, in the genome of the grey mouse lemur (*Microcebus murinus*), an EBLN and an EBLM were found close located (Horie M *et al*., 2019). The EBLs likely became endogenous at 55 million years ago (Hedges SB *et al*., 2006). The length between the EBLN and EBLM is 306 nucleotides (nt), which is much shorter than that of the corresponding region in the BoDV genome (933 nt) (Horie M *et al*., 2019).

In conclusion, the organization of the ancient genome of bornavirus is significantly different from that of modern bornaviruses, considering the above facts reported. There are proposals such as: 1) the order of genes differ between ancient and contemporary bornaviruses; 2) the X/P genes were absent, or 3) the X/P genes were very short.

Additional studies are needed to fill gaps in the knowledge.

1.5. Epidemiology of infection and Public Health

The prediction of viral outbreaks has become a public health and a welfare animal issue. A profound understanding of the viral population in key animal species acting as reservoirs and the viral shedding pathways represents a significant step towards this goal.

1.5.1. Risk factors and transmission of bornaviruses

The infection sources and the transmission routes of bornaviruses remain as subjects of current discussions, due to the heterogeneous results reported in experimental (Kerski A *et al*., 2012; Rubbenstroth D *et al*., 2013; Rubbenstroth D *et al*., 2014; Heckmann J *et al*., 2017) and observational studies (Ludwig H *et al*., 2000; Kistler AL *et al*., 2010; Lierz M *et al*., 2011; Monaco E *et al*., 2012; Rubbenstroth D *et al*., 2016; Pinto MC *et al*., 2018; Nielsen AMW *et al*., 2018; Sa-Ardta P *et al*., 2019).

Throughout the next two items, the risk factors and the transmission routes of bornaviruses will be addressed, regarding the experimental and the natural infection.

1.5.1.1. Natural infection by bornavirus

The first published epidemiological studies, on the subject, reported that Borna disease was more frequently observed in horses from traditional farms than in horses housed in modern facilities with higher hygiene and management standards (Staeheli P *et al*., 2000). However, it is unlikely that the infection chain could be maintained exclusively by virus transmission by diseased animals, since their life expectancy is low, as well as bornaviruses infection occurs mostly sporadically (Staeheli P *et al*., 2000). Also, it was suggested that the asymptomatic farmed animals (e.g., horses, sheep) play a role in the transmission of bornaviruses, once in this case the infection could have taken a non-symptomatic but highly productive persistent course (Staeheli P *et al*., 2000). An argument supporting this chance was the RNA of bornavirus detected in nasal secretions, conjunctival fluid and saliva, of infected horses (Staeheli P *et al*., 2000). However, transmission between animals by nasal and oral/faecal routes has caused debate among the scientific community, because of the heterogeneous evidence produced by epidemiological studies (observational and experimental) (Staeheli P *et al*., 2000). Namely, there are published studies showing no evidence for animal-to-animal transmission of bornavirus infection, by cohabitation, between horses, sheep and cattle (Staeheli P *et al*., 2000). The evidence absence of infection transmission between diseased animals and healthy animals in flocks of mammals (Staeheli P *et al*., 2000), as in flocks of birds was reported (Rubbenstroth D *et al*., 2014; Sa-Ardta P *et al*., 2019). Wild animals also can play a role, once a horse with Borna Disease and antibody-positive had previously eaten oats from the field where a BoDV antibody-positive root vole (*Microtus oeconomus*) was captured, suggesting a possible transmission chain (Kinnunen PM *et al*., 2007).

1.5.1.2. Experimental infection with bornavirus

Experimental studies showed successfully achieved intranasal or oral infection in mammals and birds (Staeheli P *et al*., 2000; Kinnunen PM *et al*., 2007). Infection could be induced by intranasal route on rats, mice, sheep and horses (Staeheli P *et al*., 2000, Kinnunen PM *et al*., 2007; Tizard I *et al*., 2016 (a)), and by oral route on parrots and canaries (Gancz AY *et al*., 2009; Rubbenstroth D *et al*., 2014). Therefore, the evidence produced by the experimental studies suggest that natural infection can occur by intranasal and/or by

oral route (Staeheli P *et al*., 2000, Kinnunen PM *et al*., 2007; Gancz AY *et al*., 2009; Rubbenstroth D *et al*., 2014; Tizard I *et al*., 2016 (a)).

Moreover, naturally and experimentally infected animals release the virus through saliva, nasal secretions, connective fluid and faeces (Staeheli P *et al*., 2000, Kinnunen PM *et al*., 2007; Gancz AY *et al*., 2009; Rubbenstroth D *et al*., 2014; Tizard I *et al*., 2016 (a)). Consequently, findings suggest that transmission between farmed/production animals (such as sheep, goats, cattle and ostriches) (Staeheli P *et al*., 2000, Kinnunen PM *et al*., 2007; Tizard I *et al*., 2016 (a)) and between captive flocks (parrots and canaries) (Gancz AY *et al*., 2009; Rubbenstroth D *et al*., 2014) may occur through inhalation of viruses present in the excretions and secretions produced by the diseased animals.

Experimental studies also showed that vertical and horizontal transmission occurred in rodents (Okamoto M *et al*., 2003; Sauder C *et al*., 2003), and that in captive birds occurred vertical spread, of avian bornaviruses (Gancz AY *et al*., 2009; Rubbenstroth D *et al*., 2014).

1.5.2. Reservoirs of bornaviruses

The accumulated evidence strengthened the ubiquity of bornaviruses (Kinnunen PM *et al*., 2013). In the next paragraphs, the advances achieved and the questions that remain unanswered regarding reservoirs of bornaviruses, are addressed.

In the early 21st century, it was reported the first evidence of bornaviruses reservoirs. However, before this finding, the epidemiological datasets, on the subject, suggested that small wild mammals could play a role in the epidemiology of bornaviruses, as reservoirs (Kinnunen PM *et al*., 2013). For instance, cats showed a sevenfold increased risk of infection by bornaviruses when roaming free in a rural environment or hunting rodents. (Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014). Dogs that lived in the neighbourhood of stables housing horses that tested antibody-positive for bornaviruses were at risk to also become antibody-positive; suggesting that there was a shared source of infection between neighbouring farms (Kinnunen PM *et al*., 2011). Besides, in farms lacking proper rodent control and hygiene, the occurrence of Borna disease virus 1 (BoDV-1) was reported to be higher (Kinnunen PM *et al*., 2013). Published studies revealed that bornaviruses epidemics were independent of the region and the host species and reported at intervals of 2 to 5 years (Kinnunen PM *et al*., 2013). A link between the observed facts and the fluctuation of wild rodent numbers was proposed (Kinnunen PM *et al*., 2013). Thus, the territorial factor, the enhanced occurrence of Borna disease or markers of infection in stables with poor hygiene/rodent control and the seasonal periodicity of the disease showed to be compatible with the existence of a rodent reservoir (Kinnunen PM *et al*., 2013).

The first unequivocal detection of a reservoir species of bornaviruses was in 2006 (Hilbe M *et al*., 2006). For the first time, the bicoloured white-toothed shrew (*Crocidura leucodon*) was identified as reservoir, in an endemic area of Borna disease, at Switzerland (Hilbe M *et al*., 2006). Since then, several published studies, conducted in diverse geographical regions, have reported the evidence of several species of small wild rodents as reservoirs of Borna disease virus 1 (Kinnunen PM *et al*., 2007; Bourg M *et al*., 2013; Dürrwald R *et al*., 2014; Nobach D *et al*., 2015; Weissenböck H *et al*., 2017). Namely, in 2007, in Finland, it was reported the direct evidence of bornaviruses infection in wild bank vole (*Myodes glareolus*) and root vole (*Microtus oeconomus*) (Kinnunen PM *et al*., 2007). In 2013, in disease-endemic areas of Bavaria (Germany), BoDV-1 infection in wild shrews *(Crocidura leucodon)* was detected (Bourg M *et al*., 2013). Additionally, in 2014, a published study, conducted in Germany at the district of Borna, reported Borna disease virus 1 (BoDV-1) infection in wild bicoloured white-toothed shrew (*Crocidura leucodon*) (Dürrwald R *et al*., 2014). Also, in Germany, at two sites in the district of Swabia, a known endemic area, wild bicoloured white-toothed shrews (*Crocidura leucodon*) infected with Borna disease virus 1 (BoDV-1) were found (Nobach D *et al*., 2015). In 2017, Upper Austria was stated as a novel endemic area, in which shrews (*Crocidura leucodon* and *Sorex araneus*) infected with BoDV-1 were found (Weissenböck H *et al*., 2017).

In addition to the facts reported above there are evidences that argues for a general role of small wild rodents as reservoirs, such as: 1) the absolute homology found between the BoDV-1 genome detected in several species of wild rodents and the farmed animals, 2) the widespread distribution of BoDV-1 antigen in the nervous system, in epithelial and in mesenchymal tissues without pathological alterations and the asymptomatic course of infection, 3) the presence of large amounts of viral RNA in saliva, urine, skin swabs, lacrimal fluid and faeces are sources of infection for natural and spill-over hosts, 4) the close genetic relationship between regional BoDV-1 genotype clusters detected in domestic animals and the genotypes obtained from wild rodents, 5) the consecutive years of BoDV-1 infection in rodents, points towards a self-sustaining infection cycle in these animals and 6) analyses of behavioural and population features of rodents species revealed that they may indeed play a role as an indigenous host of BoDV-1 (Dürrwald R *et al*., 2014; Nobach D *et al*., 2015).

Also, arthropods were suggested as reservoirs of bornaviruses, but the idea was refuted, since bornaviruses does not cause the high viremia, needed for the transmission of arboviruses (Kinnunen PM *et al*., 2013).

Over the years, several wild bird species have also been suggested as reservoirs of bornaviruses (Berg M *et al*., 2001; Payne S *et al*., 2011; Delnatte P *et al*., 2014; Guo J *et al*., 2014 (a); Guo J *et al*., 2015). In 2001, for the first time, BoDV-1 RNA detection was

reported in wild mallards (*Anas platyrhyncos*) and jackdaws (*Corvus monedula*). These findings stood up for the fact that wild birds could be asymptomatic carriers of mammalian bornaviruses, as they could play a role in transmission (Berg M *et al*., 2001).

In 2011, for the first time, a published study reported the presence of avian RNA in oropharyngeal secretions and cloaca from healthy wild Canada geese (*Branta canadensis*) (Payne S, 2011). Four years later, another study showed that several species of free-ranging and healthy waterfowl had antibodies against avian bornaviruses and was shedding virus by cloaca (Delnatte P *et al*., 2014). Besides, the infection by bornaviruses in the trumpeter swans (*Cygnus buccinator*), mute swans (*Cygnus olor*), and mallards (*Anas platyrhynchos*) was investigated (Delnatte P *et al*., 2014). Also in 2014, a study that reported the absence of inflammation in cerebrums of wild mallards (*Anas platyrhynchos*), ducks (*Aix sponsa*) and green-winged teal (*Anas crecca* and *Anas acuta*) naturally infected with avian bornaviruses, which not showed to be suffering from overt disease was published (Guo J *et al*., 2014 (b)). Additionally, for the first time, in 2015 it was reported the presence of RNA of avian bornaviruses in brains of apparently healthy gulls (Herring Gulls (*Larus argentatus*), Ring-billed Gulls (*Larus delawarensis*), and Laughing Gulls (*Leucophaeus atricilla*) (Guo J *et al*., 2015).

In 2014, for the first time, four species of insectivorous bats (*Pipistrellus pipistrellus*, *Hypsugo savii*, *Myotis nattereri* and *Eptesicus serotinus*), living geographically close to humans, were proposed as reservoirs of bornaviruses (Dacheux L *et al*., 2014). The published study, conducted in France, reported the detection of new bornaviruses in the brain, lungs and liver of these mammalian species (Dacheux L *et al*., 2014). The new bornaviruses were termed as bat bornavirus, however until present, they remained as unclassified *Bornaviridae*, resulting from the absence of enough data (Dacheux L *et al*., 2014).

In addition to wild animals, natural BoDV-1 infection in domesticated animals can be asymptomatic (Zhang L *et al*., 2014 (a)), with the release of bornaviruses in saliva and faeces (Zhang L *et al*., 2014 (a)). Infection in several healthy companion and production animals (e.g., cats, horses, dogs, birds, squirrels, donkeys, pigs, rabbits, cattle, goats and sheep) was also detected (Zhang L *et al*., 2014 (a)). In infected pet squirrels were found high viral loads in their central nervous system, as well as, in organs capable of secretion and excretion (kidneys, urinary bladder, skin and oral cavity); being them probably a natural reservoir (Hoffmann B *et al*., 2015; Schlottau K *et al*., 2017 (a); Schlottau K *et al*., 2017 (b)). Therefore, humans probably are cohabiting with reservoirs of bornaviruses.

1.5.3. Geographic distribution of bornaviruses

Bornavirus infection and its diseases, for years, have been considered endemic to Central Europe (Staeheli P *et al*., 2000). Therefore, for years, studies on bornaviruses were exclusively conducted in some central European countries (Staeheli P *et al*., 2000; Kinnunen PM *et al*., 2013). When the research on bornaviruses was extended to other regions of Europe, the accumulated evidence showed that bornavirus infection and its diseases were detected and reported across Eastern, Northern, Southern and Western of Europe (Reeves NA *et al*., 1998; Kinnunen PM *et al*., 2007; Weissenböck H *et al*., 2009; Kinnunen PM *et al*., 2011; Kinnunen PM *et al*., 2013; Dacheux L *et al*., 2014; Thomsen AF *et al*., 2015; Pinto MC *et al*., 2019). Also, it has been reported, outside of Europe, with studies of the infection conducted in Africa, America, Asia and Oceania (Salvatore M *et al*., 1997; Nunes SO *et al*., 2008; Last RD *et al*., 2012; Encinas-Nagel N *et al*., 2014, Guo J *et al*., 2014 (b); Guo J *et al*., 2015; Philadelpho NA *et al*., 2019; Sa-Ardta P *et al*., 2019). Despite the advances on bornaviruses epidemiology, there are remaining gaps regarding the geographical distribution of infection and its diseases. Therefore, to better understand the extent of infectious on animals and humans, more studies are needed, namely in geographical areas where bornaviruses have not been studied.

1.5.4. Bornavirus infection and Public Health

The zoonotic potential of Borna disease virus 1 (BoDV-1) was a controversial issue until the middle of the first decade of the 21st century. During the last century, studies reported BoDV-1 infection markers in humans (Kinnunen PM *et al*., 2013; Azami M *et al*., 2018). However, at the time, some scientific community members argued that the infection markers detected in humans were likely to be a result of laboratory contamination (Staeheli P *et al*., 2000), as the validity and reproducibility of the serological tests used were questionable (Staeheli P *et al*., 2000). However, in 2015 a study undoubtedly showed that some bornaviruses are zoonotic (Hoffmann B *et al*., 2015). The study was conducted in the state of Saxony-Anhalt (Germany) between 2011 and 2013, having as a case study three breeders of variegated squirrels (*Sciurus variegatoides*), who developed encephalitis and died 2 to 4 months after onset of the clinical symptoms (Hoffmann B *et al*., 2015). The brain samples analysed revealed infection by a novel bornavirus (Hoffmann B *et al*., 2015). The novel virus was identified as the etiologic agent of encephalitis (Hoffmann B *et al*., 2015) and called variegated squirrel 1 bornavirus (VSBV-1), belonging to the species *Mammalian*

2 orthobornavirus (Hoffmann B *et al*., 2015). The variegated squirrel bornavirus 1 (VSBV-1) was identified as the causative agent, once: 1) the similarity of the complete coding sequences obtained from samples of squirrels and humans, 2) the detection of viral RNA in the brain tissue of all three patients, 3) the antigen immunostaining results, 4) the resemblance of the clinical picture amongst the three patients, 5) the anti-bornavirus IgG titres in the serum and the cerebrospinal fluid of one of the patients and 6) the epidemiologic link among all three cases (Hoffmann B *et al*., 2015). Since then, the infection by VSBV-1 started to be considered by physicians as a possible etiologic agent of encephalitis, which were of unclear origin. In the subsequent years, cases of lethal encephalitis caused by VSBV-1 were reported (Tappe D *et al*., 2018; Tappe D *et al*., 2019 (a)). Namely, in 2018 in the north of Germany, the VSBV-1 infection was the cause of death of a Zoo animal caretaker (Tappe D *et al*., 2018). Molecular assays and immunohistochemistry detected a limbic distribution of the virus in brain tissue of the animal caretaker (Tappe D *et al*., 2018). Antibodies against bornaviruses were detected in the animal caretaker cerebrospinal fluid by Enzyme-linked Immunosorbent Assay (ELISA) and immunoblot (Tappe D *et al*., 2018). Phylogenetic analyses established that infection spreading from the Prevost's squirrel to the animal caretaker (Tappe D *et al*., 2018). In 2019, a published retrospective epidemiological investigation reported human cases of past infections by variegated squirrel bornavirus 1 (VSBV-1), among exotic squirrel breeders in Germany, regarding the period 2005 to 2018 (Tappe D *et al*., 2019 (a)). However, human cases of VSBV-1 infection among breeders and caretaker of squirrels, maybe underreported due to the complexity of exotic squirrels trade networks and the difficulties in conducting epidemiological investigations in these communities (Tappe D *et al*., 2019 (a)). For instance, some private breeders disagreed in participating in the seroprevalence surveys, and the absence of registry of squirrel breeders/holders was also an issue (Tappe D *et al*., 2019 (a)). Besides, tracing back the squirrels' trade showed to be difficult once the provided information on squirrel exchange by breeders was limited (Tappe D *et al*., 2019 (a)). Therefore, a human case of past VSBV-1 infection, a private holder and the zoo holdings, could not be tied to other holdings with positive squirrels; as it was not possible to establish an eventual connection between zoo animal and private squirrel trade (Tappe D *et al*., 2019 (a)).

In conclusion, VSBV-1, a zoonotic bornavirus from squirrels, was associated with fatal central nervous systems infections in humans (Tappe D *et al*., 2018; Tappe D *et al*., 2019 (a)). The import of exotic rodents as pets into Europe, and the trade of live squirrels within Europe is recurrent (Tappe D *et al*., 2019 (a)). Therefore, there is an expectation of a widespread of the VSBV-1. Further studies, including seroepidemiologic and molecular studies in putative animal reservoirs and human patients, specifically those with unexplained encephalitis or meningoencephalitis, are needed.

Once established a link between variegated squirrel bornavirus 1 (VSBV-1) infection and the occurrence of lethal encephalitis, the Borna disease virus 1 (BoDV-1) started to become also searched in human cases of encephalitis, by informed clinicians (Korn K *et al*., 2018; Schlottau K *et al*., 2018; Coras R *et al*., 2019; Niller HH *et al*., 2020). In 2018, for the first time a publication confirmed the association between BoDV-1 infection and the development of lethal encephalitis in a cluster of solid organ transplant recipients, of a donor from the Bavaria region of southern of Germany (Schlottau K *et al*., 2018). Moreover, the BoDV-1 genome detected in human brain samples shared 99.3 to 99.7% of identity with field isolates of BoDV-1 from shrews and horses, from Bavaria region (Schlottau K *et al*., 2018). In the same year, there was another human case of lethal encephalitis caused by BoDV-1 infection (Korn K *et al*., 2018). In 2019, a retrospective study revealed that BoDV-1 was the causative agent of acute encephalitis and Guillain Barré polyradiculitis in humans (Coras R *et al*., 2019). In 2020, a published retrospective study reported eight humans with BoDV-1 infections, in Bavaria (Germany) (Niller HH *et al*., 2020). The study analysed brain samples collected between 1999 and 2019, from fatal encephalitis cases (Niller HH *et al*., 2020). Phylogenetic analysis suggested multiple independent infections from a local reservoir (Niller HH *et al*., 2020).

The available evidence show 1) that the lethal BoDV-1 infection in humans is not a newly emerging entity in the known endemic regions but appears to have occurred unnoticed for decades, 2) that BoDV-1 can be transmitted by solid organ transplantation, 3) the BoDV-1 is a relevant zoonotic pathogen, 4) bornaviruses infection in encephalitis cases of potential direct or indirect contact with reservoirs should be considered (Schlottau K *et al*., 2018; Korn K *et al*., 2018; Coras R *et al*., 2019; Niller HH *et al*., 2020). Therefore, searching for mammalian bornaviruses infection, in samples collected from human cases diagnosed with encephalitic and central nervous system disease will be a suitable approach.

The produced findings and remaining gaps reinforce the necessity to consider the "One Health" approach, for designing and implementing programmes, policies, legislation and research, in which multiple sectors communicate and work together to achieve better public health outcomes.

1.6. Diagnostics

1.6.1. Clinical diagnosis in animals

The clinical picture associated with Borna disease, in mammalian species, is similar between companion and production animals regardless of the affected host species (Ludwig H *et al*., 2000; Kinnunen PM *et al*., 2013; Tizard I *et al*., 2016 (a)). The variability of clinical signs is related to the brain areas affected, and they can range from minor to severe, according to the intensity of the inflammatory response (Vahlenkamp TW *et al*., 2002). Horses can develop the following clinical signs: depression (apathy, somnolence and stupor) or excitability, narcolepsy, fever, swallowing difficulties, anorexia that precede ataxia (abnormalities in eye movements), colic, paresis, disordered-movements (spread their legs or cross them), repetitive behaviours (such as circling), vacuous slow-motion chewing, neurogenic torticollis, blindness, severe tooth grinding, aggressiveness and decreased sensory, proprioceptive and reflex functions (Ludwig H *et al*., 2000; Lipkin WI *et al*., 2011). The clinical picture evolution leads to coma and death (Ludwig H *et al*., 2000; Lipkin WI *et al*., 2011; Kinnunen PM *et al*., 2013). Death usually occurs between the first and the fourth week after the onset of clinical signs, and the mortality exceeds 80% of the cases (Ludwig H *et al*., 2000; Lipkin WI *et al*., 2011). Sheep with Borna disease developed behavioural changes, depression (such as somnolence and multiple deficits), progressive ataxia, swallowing difficulties and dyskinesia (seizures) (Ludwig H *et al*., 2000; Kinnunen PM *et al*., 2013, Tizard I *et al*., 2016 (a)). The death as an outcome has been reported and estimated to 50% of Borna disease cases (Tizard I *et al*., 2016 (a)). In cattle, Borna disease causes the development of anorexia, repetitive behaviours (such as circling) ataxia, paresis and death (Ludwig H *et al*., 2000, Kinnunen PM *et al*., 2013, Okamoto M *et al*., 2002 (a)). In cats, the reported clinical signs, at an initial stage, were the fever, apathy and a reduced appetite (Wensman JJ *et al*., 2014; Lutz H *et al*., 2015). The clinical signs can progress for anorexia, ataxia, gait disturbances, blindness, lower back pain, behavioural changes, loss of postural reactions, hind-leg paralysis and hyperaesthesia (seizures) (Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014). According to published literature, there were affected cats incapacitated to retract their claws (Lutz H *et al*., 2015). Borna disease in dogs causes the development of neurologic symptoms (severe and acute progressive disorder) behavioural changes (aggressiveness), anorexia and depression (lethargy) (Ludwig H *et al*., 2000; Okamoto M *et al*., 2002 (b)).

In birds, the clinical picture can be quite different. In parrots, for example, virus belonging to the taxonomic group *Psittaciform 1 to 2 orthobornavirus* (parrot bornavirus 1 to 8) are the etiologic agents of Proventricular dilatation disease, a clinical condition that can produce gastrointestinal and/or neurologic disturbs. The tissue regions where lesions occur define the clinical signs presented by the bird (Berhane Y *et al*., 2001; Tizard I *et al*., 2016 (b)). A severe proventricular dilatation usually is linked to the disease (Tizard I *et al*., 2016 (b)) however, few cases developed neurologic signs without the occurrence of proventricular dilatation, according to published studies (Raghav R *et al*., 2010; Lierz M *et al*., 2009). Besides, in other studies there were additional clinical signs and outcomes reported, such as: weight loss, intestinal dilatation, regurgitation, mal-digestion (the presence of undigested food in faeces), malnourishment, tremors, ataxia, seizures, blindness, depression, ataxia, convulsions and death (Berhane Y *et al*., 2001, Weissenböck H *et al*., 2009; Hoppes SM *et al*., 2013). However, a few infected parrots died suddenly without developing clinical signs, (because of the severe myocarditis) (Tizard I *et al*., 2016 (b)). Regarding waterfowl, gastrointestinal and/or neurological signs were reported in geese (*Branta canadensis*), mute swans (*Cygnus olor*) and Emu (*Dromaius novaehollandiae*). Specifically, the occurrence of proventricular impaction, weakness/poor body condition, somnolence, and lethargy, blindness, lameness, torticollis, ataxia, inability to stand or fly, hypermetry (voluntary muscular movements), head tremors, stargazing and opisthotonos (abnormal posturing caused by muscle spasms) were described (Tizard I *et al*., 2016 (b)).

In conclusion, the naturally infected mammalian that developed Borna disease exhibit clinical signs like those described in cases of infections caused by other microbiological agents. Namely, Borna disease share a similar clinical picture with the rabies, equine Herpesviruses, tick-borne encephalitis, botulism, listeriosis, West Nile virus encephalitis, parasitic encephalomyelitis, Bovine Spongiform Encephalopathy and ovine scrapie (Dauphin G *et al*., 2002). Also, the clinical signs developed by birds infected with avian bornaviruses are like those found in birds infected by *Baylisascaris* spp., psittacine beak and feather disease virus, West Nile virus, *Chlamydophila psittaci* (Thompson AB *et al*., 2008; Bakonyi T *et al*., 2016; Razmyar J *et al*., 2016). Therefore, the clinical picture cannot be sufficient to diagnose the disease whereby, complementary diagnostic methods are required.

1.6.2. Clinical diagnosis in humans

In humans, the reported clinical symptoms linked to the infection by Borna disease virus 1 (BoDV-1) and variegated squirrel bornavirus 1 (VSBV-1) started with fever and

cognitive dysfunction (confusion), psychomotor slowing that progressed to ocular paresis, facial paraesthesia, dysarthria, dysphagia, tetraparesis, and focal seizures, myoclonus, opisthotonos, coma and death (Hoffmann B *et al*., 2015; Korn K *et al*., 2018; Schlottau K *et al*., 2018; Tappe D *et al*., 2018; Tappe D *et al*., 2019 (a)).

1.6.3. Laboratorial diagnosis

The laboratory diagnosis can be based on the genome, antigens and antibodies detection of bornaviruses, and searching to pathologic alterations in infected tissues (macroscopic and microscopic evaluation) (Bode L *et al*., 2001; Wolff T *et al*., 2006; Bode L, 2008; Honkavuori KS *et al*., 2008; Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013; Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014; Zhang L *et al*., 2014 (a); Zhang L *et al*., 2014 (b); Hoffmann B *et al*., 2015; McHugh JM *et al*., 2015; Ando T *et al*., 2016; Tizard I *et al*., 2016 (a); Tizard I *et al*., 2016 (b)). The currently used approaches include: 1) protocols for molecular techniques for the detection of infection in a live or dead host, 2) serologic protocols and 3) protocols for bornaviruses isolation from infected tissues by culture in cell lines (Bode L *et al*., 2001; Wolff T *et al*., 2006; Bode L, 2008; Honkavuori KS *et al*., 2008; Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013; Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014; Zhang L *et al*., 2014 (a); Zhang L *et al*., 2014 (b); Hoffmann B *et al*., 2015; McHugh JM *et al*., 2015; Ando T *et al*., 2016; Tizard I *et al*., 2016 (a); Tizard I *et al*., 2016 (b)). As part of a diagnostic workup, the genotyping of detected bornaviruses is imperative. However, none of the laboratory diagnosis, above-identified, is the gold-standard established to search for bornavirus infection in mammalian and reptiles samples. However, the histological examination, of the serosal surface of the proventriculus, is the gold-standard method for detection of bornaviruses infection in birds (Gancz AY *et al*., 2010). Consequently, it requires the collection of one appropriately sized biopsy in live birds. However, it is a technically challenging procedure to conduct with live birds, highly invasive and requiring anaesthesia (Gancz AY *et al*., 2010).

In the next paragraphs the diagnostic tools available and used to detect infection, as well as, to genotype and sequencing bornaviruses are addressed.

1.6.3.1. Serology

Bornavirus antigens present in the peripheral blood bind to *Bornavirus*-specific antibodies forming circulating immunocomplexes (CICs) (Bode L *et al*., 2001). Circulating immunocomplexes (CICs), antigen and antibodies have been detected using a reverse-type

sandwich triple ELISA (Bode L, 2008). However, the results were not reproducible in studies conducted and published posteriorly (Wolff T *et al*., 2006). Also, the indirect immunofluorescence assay (IFA) immunoblotting (IB), immune precipitation (IP) and the electrochemiluminescence (ECL) immunoassay to detect bornavirus antibodies from serum and cerebrospinal fluid samples were used (Kinnunen PM *et al*., 2013). The available serological tests target the most immunogenic bornavirus proteins (such as the nucleoprotein and the phosphoprotein), as well as the detection of antibodies directed against these immunogenic proteins (Zhang L *et al*., 2014 (a); Zhang L *et al*., 2014 (b); McHugh JM *et al*., 2015; Ando T *et al*., 2016). The indirect immunofluorescence assay (IFA) was considered the most consistent method for bornavirus antibody detection (Staeheli P *et al*., 2000). However, the IFA tools showed less sensitivity than ELISA tools (Johansson M *et al*., 2002). On the other hand, IFA showed to have higher specificity (Johansson M *et al*., 2002). However, no published studies used agreed methods to conduct assays validation. Moreover, animals infected by members of bornavirus taxon usually produced very low antibody titres, which are commonly detectable during the acute phase of the disease but, are challenging to detect in a subacute or chronic phase of the disease (Katz JB *et al*., 1998; Staeheli P *et al*., 2000). Therefore, to avoid false negatives occurrence, additional methods or repeated sampling are recommended (Ludwig H *et al*., 2000; Kinnunen PM *et al*., 2013).

In conclusion, the available serological tools can detect circulating immunocomplexes, antigens or antibodies in samples of live humans and animals, however, the use of serological tests alone may not be effective in detecting infection.

1.6.3.2. Histopathology

When suspected of bornaviruses infection as a cause of death, the central nervous system is the favourite tissue to conduct histopathologic examination (Eisenman LM *et al*., 1999). In cerebellum, interruptions in the Purkinje cell layer are the lesions usually observed in mammalian and birds (Ouyang N *et al*., 2009).

In the peripheral nerves of psittacines, including the sciatic, brachial, vagus and dorsal root ganglia, the occurrence of lymphoplasmacytic neuritis are the lesions observed (Berhane Y *et al*., 2001). The histological lesions include mild, diffuse or focal mononuclear cell infiltration; presence of focal clusters of lymphoplasmacytic infiltrates; axonal swelling and myelin degeneration; and the existence of perivascular cuffs. In rats, as in birds, the lesions showed perivascular cuffing with lymphocytes and monocytes (but rarely plasma cells or B cells). In rats, the predominant infiltrating cells are CD8+ T cells with some CD4+

T cells and natural killer (NK) cells (Hatalski CG *et al*., 1998). Additionally, the activation of microglia released pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1ß further disrupting brain functions (Tizard I *et al*., 2016 (a)).

The blindness, in mammalian and birds, is a consequence of retina inflammation, resulting from numerous infected cells and lymphocytes infiltrates. The cellular infiltrates are also present within the optic nerve (Steinmetz A *et al*., 2008). Namely, the T-cells infiltrated in the retina of Lewis rats consisted of alfa-beta TCR+, CD4+ and CD8+ cells (Stahl T *et al*., 2003). In addition to the retina inflammation, there were reported cases of chorioretinitis in the canaries (Rubbenstroth D *et al*., 2014). In typical cases of proventricular dilatation disease (PDD), severe muscle atrophy of the proventricular wall is usually present (Tizard I *et al*., 2016 (b)).

Additionally, injuries are present within the gastric plexus, the duodenal myenteric plexus and the celiac ganglion (Tizard I *et al*., 2016 (b)). The neurons in these ganglia seems to be damaged and replaced by a lymphoplasmacytic infiltration with some monocytes (Tizard I *et al*., 2016 (b)). Likewise, branches of the myenteric plexus that innervate the muscular layer revealed many lymphocytes (Tizard I *et al*., 2016 (b)). It can also be found mononuclear cell infiltrates in the connective tissue, between the muscle fibers. Moreover, the viral antigen detection in neurons that survive can be through immunohistochemistry (Tizard I *et al*., 2016 (b)). The gold standard for diagnosing Proventricular dilatation disease (PDD) has been and will likely remain histologic examination (Gancz AY *et al*., 2010). Consequently, in live birds, it implies to collect a biopsy with appropriate size, from a relevant anatomic place (Gancz AY *et al*., 2010). Namely, ideally, a biopsy of the serosal surface of the proventriculus and/or ventriculus should be collected because these sites are the most affected by PDD (Gancz AY *et al*., 2010).

1.6.3.3. The virus isolation

The detection of bornaviruses occurred in several tissues from infected hosts (Guo J *et al*., 2014 (b); Thomsen AF *et al*., 2015; Schlottau K *et al*., 2017 (a); Schlottau K *et al*., 2017 (b); Tappe D *et al*., 2018). However, the brain revealed to be the organ most regularly positive (Guo J *et al*., 2014 (b); Thomsen AF *et al*., 2015; Schlottau K *et al*., 2017 (a); Schlottau K *et al*., 2017 (b); Tappe D *et al*., 2018). Whereby, it is the organ of selection for attempts at virus isolation. The mammalian bornaviruses are cultivable on Vero cells (monkey kidney cells) and MDCK (dog kidney cells) (Kamitani W *et al*., 2001; Bajramovic JJ *et al*., 2002; Bajramovic JJ *et al*., 2004; Bonnaud EM *et al*., 2015). For the cultivation of avian bornaviruses, the cell line of choice is the primary duck embryonic fibroblasts (DEF)

(Guo J *et al*., 2014 (a)). The mammalian and avian bornaviruses persistently infects cells lines usually without cytopathic effects (Guo J *et al*., 2014 (a)). Therefore, it is required to search for the presence of viral nucleic acids or proteins to determine if the virus is in the cultured samples (Guo J *et al*., 2014 (a)). Nevertheless, some members of mammalian bornaviruses revealed to have the capacity to induce lysis of Vero cells (Wensman JJ, 2011). However, viral isolation methods from tissues are time-consuming and expensive.

1.6.3.4. Molecular biology approaches: conventional polymerase chain reaction and real-time polymerase chain (PCR)

Molecular biological assays to detect viral nucleic acids, in samples from screenings and clinical practice were used (Hoppes SM *et al*., 2013; Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014). Specifically, the conventional PCR and real-time PCR (Hoppes SM *et al*., 2013; Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014). For *post-mortem* detection of mammalian bornaviruses, the most consistent results of molecular biological assays, were obtained when conducted detection in samples of specific regions of the central nervous system (CNS), such as: olfactory bulb, grey matter of the brain stem, basal ganglia and hippocampus (Hoppes SM *et al*., 2013; Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014). On the other hand, for *post-mortem* detection of avian bornaviruses, the most consistent results were obtained testing tissues samples of brain, crop, proventriculus, *ventriculus* and adrenal glands (Hoppes SM *et al*., 2013; Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014).

The *ante-mortem* detection by searching viral RNA using molecular biological assays has been reported to be difficult, due to the nature of persistent infection within the central nervous system (CNS), in mammalians (Hoppes SM *et al*., 2013; Kinnunen PM *et al*., 2013; Wensman JJ *et al*., 2014).

In a live bird, three types of samples are readily available—blood, feathers, crop tissue, secretions (e.g., saliva) and excretions (e.g., urine/faeces) (Guo J *et al*., 2014 (a)). However, the use of blood samples for viral RNA detection has been discouraged for reasons described in the next paragraphs (Guo J *et al*., 2014 (a)). The collecting blood samples from large birds is practicable (e.g., macaws, cockatoos, and African Grey parrots) (Guo J *et al*., 2014 (a)); however, it is often impractical to collect blood samples from little birds (e.g., finches) (Guo J *et al*., 2014 (a)). Moreover, usually the infection does not induce viremia and therefore, the viral RNA may not be detectable in blood until the late stage of the disease (Guo J *et al*., 2014(a)). The use of freshly plucked feathers as samples for laboratory diagnostic is a controversial issue (Guo J *et al*., 2014 (a)). Published studies

showed the existence of viral RNA in the air of aviaries, and therefore, contamination of the outer feathers can occur (Guo J *et al*., 2014(a)). Consequently, the detection of viral RNA on the feathers indicated the virus presence within an aviary but did not identify the bird(s) infected (Guo J *et al*., 2014(a)). On the other hand, cloaca swabs were suggested as the most convenient samples to obtain from live birds. Since, cloaca swabs can be collected in large quantities, repeatedly and without subjecting the animal to very invasive procedures (Guo J *et al*., 2014(a)).

1.6.3.5. Metagenomics-based approaches

Viral metagenomics is an emerging tool in veterinary medicine that has contributed to expand the knowledge of bornaviruses (Honkavuori KS *et al*., 2008; Dacheux L *et al*., 2014; Hoffmann B *et al*., 2015). The metagenomics assays for the detection of bornaviruses are based mainly on the sequence-independent amplification of nucleic acids from samples, in combination with next-generation sequencing platforms and bioinformatics tools for sequence analysis (Honkavuori KS *et al*., 2008; Dacheux L *et al*., 2014; Hoffmann B *et al*., 2015). Once viral metagenomics is a culture-independent method that allows sequencing the whole viral genetic populations from samples, it becomes a powerful tool for identifying and characterizing novel viruses, which might be highly divergent from those that are already described (Honkavuori KS *et al*., 2008; Dacheux L *et al*., 2014; Hoffmann B *et al*., 2015).

For more than 40 years, the etiologic agent of Proventricular dilatation disease (PDD) remained unknown (Honkavuori KS *et al*., 2008). For the first time, in 2008 it was possible to identify the etiologic agent of Proventricular dilatation disease (PDD), by metagenomics approaches (Honkavuori KS *et al*., 2008). The metagenomics-based methodologies detected a virus with a bornavirus-like genome organization, in brain samples of captive parrots with PDD (Honkavuori KS *et al*., 2008). This virus showed a high degree of genetic divergence from all prior bornaviruses identified, and the name proposed at the time was avian bornavirus (Honkavuori KS *et al*., 2008). After six years, in 2014, a new mammalian bornaviruses was discovered during a screening of insectivorous bats, using metagenomics-based methodologies (Dacheux L *et al*., 2014). In the next year, in 2015, the detection of a novel species of mammalian bornaviruses occurred using a metagenomics approach (Hoffmann B *et al*., 2015). Namely, it was detected a previously unknown bornavirus in brain samples of breeders of variegated squirrels (*Sciurus variegatoides*), who died from encephalitis (Hoffmann B *et al*., 2015). The virus was named variegated squirrel 1 bornavirus (VSBV-1) (Hoffmann B *et al*., 2015). Additionally, in 2018, the complete

genomes of novel bornavirus were identified in samples from Australian carpet pythons (*Morelia spilota*) with neurological disorders by metagenomics-based methodology (Hoffmann B *et al*., 2015).

1.7. Prevention and management of the disease

1.7.1. Prevention of the disease: biosafety measures

At present, there is no medical prophylaxis being used to prevent the occurrence of Borna disease in breeding and production animals, as in companion animals. However biosafety measures such as the quarantine, isolation of sick animals and the implementation of hygienic procedures can reduce the spread of disease, at breeding projects, as well as in farmed animals (Ludwig H *et al*., 2000; Staeheli P *et al*., 2000, Kinnunen PM *et al*., 2013). Published studies reported that animals that did not go outdoor were at lower risk to acquire the infection than animals with unlimited access (Wensman JJ *et al*., 2014; Lutz H *et al*., 2015). However, there are species of companion animals that for those the outdoor access is an essential component of their wellbeing, such as cats (Wensman JJ *et al*., 2014; Lutz H *et al*., 2015).

The control of avian bornaviruses infection become relevant in the context of psittacines housed in reserves, in breeding projects of rare species, in private collections and zoos, because of the severe effects it may cause for bird welfare, economy, and biodiversity levels. Published studies on molecular epidemiology, of avian bornaviruses, suggested that a world trade of psittacines without biosafety measures has been carried out (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). Since vaccine development is in the experimental phase (Olbert M *et al*., 2016; Runge S *et al*., 2017; Hameed SS *et al*., 2018), the establishment and implementation of biosafety measures are essential to reduce the risk of infection (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013). Therefore, the control of avian bornaviruses in captive birds requires a multimodal approach, such as: 1) the isolation of sick birds, 2) the screening of bornaviruses in apparently healthy birds, 2) the control of the birds trade, 3) have adequate infrastructures of sanitation, 4) implement of good cleaning/disinfection practises and 5) diagnostic necropsies and histopathology should be performed on all birds that die of unknown causes (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013). Regarding the asymptomatic birds, their screening is advisable, once they can be persistent shedders of bornaviruses (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013). Given the limitations of the available diagnostic tools, the published literature recommended combining serologic and molecular diagnostic tests to conduct screenings of populations,

aiming to reduce the occurrence of false negatives (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013). As well as, the literature recommended to conduct, over time, multiple molecular tests to samples of faeces or swabs from cloaca, due to the intermittent shedding of the virus (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013).

The literature proposes the separation of offspring from infected progenitors and the manual feeding of offspring to prevent vertical transmission (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013). Also, it suggests measures to prevent horizontal transmission of infection. Namely, the asymptomatic baby birds should be separated from infected or sick birds and monitored, aiming a suitable detection of, an eventual, disease development (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013).

1.7.2. Prevention of the disease: vaccines

The first immunization studies, against bornaviruses infection, were conducted in horses. Published studies showed that immunized horses did not develop clinical signs after induced infection (Wensman JJ, 2011). In Germany, at the endemic regions, the immunization was carried out from the 1920s until the late 1970s in the former Federal Republic of Germany (West Germany) (Wensman JJ, 2011), as in the former German Democratic Republic (East Germany) until the reunification of Germany (Wensman JJ, 2011). The conducted immunization was with a live vaccine, which was suspected to be linked with the occurrence of a limited spread of Borna disease virus, based on molecular epidemiological data (Ludwig H *et al*., 2000).

During the second decade of the 21st century, the first published studies reporting the development of vaccines against avian bornavirus were published (Olbert M *et al*., 2016; Runge S *et al*., 2017; Hameed SS *et al*., 2018). Specifically, the vaccines aimed to target bornaviruses that infect species of psittacines (Olbert M *et al*., 2016; Runge S *et al*., 2017; Hameed SS *et al*., 2018). In 2016, an experimental study showed that it was possible to repress avian bornaviruses infections by vaccine-induced immunity, in cockatiels (*Nymphicus hollandicus*) and common canaries (*Serinus canaria forma domestica*) (Olbert M *et al*., 2016). The study showed that viral vector vaccines expressing the phosphoprotein and nucleoprotein genes of parrot bornavirus 4 (PaBV-4) and canary bornavirus 2 (CnBV-2) could delay the course of homologous bornavirus experimental infections (Olbert M *et al*., 2016). However, cockatiels still developed signs of proventricular dilatation disease, when vaccine failed to prevent viral persistence (Olbert M *et al*., 2016). The following studies refined the vaccine systems, achieving a full protection against a heterologous experiment infection and consequently against proventricular dilatation disease (Runge S *et al*., 2017).

In 2018, a vaccine produced with killed parrot bornavirus plus recombinant PaBV-4 nucleoprotein, also showed to prevent the occurrence of proventricular dilatation disease in cockatiels (*Nymphicus hollandicus*) experimentally infected with a virulent parrot bornavirus 2 (PaBV-2) (Hameed SS *et al*., 2018). However, despite cockatiels vaccinated remained free of the proventricular dilatation disease, the virus persisted in many organs (Hameed SS *et al*., 2018). Moreover, combining the developed vaccine systems with the cyclosporine A, when administered one day before induced infection with PaBV-2, prevented the manifestation of clinical signs in cockatiels (*Nymphicus hollandicus*) (Hameed SS *et al*., 2018). However, the study reported that the addition of cyclosporine A did not confer protection to cockatiels, against infection-induced with PaBV-2 (Hameed SS *et al*., 2018).

1.7.3. Management of the disease: *in vitro* and *in vivo* studies

The first therapeutic approaches date back to the 19th century and early 20th century, which consisted of the administration of various herbal and medical treatments, to horses with Borna Disease (BD) diagnose (Wensman JJ, 2011). However, these therapeutic approaches were unsuccessful (Wensman JJ, 2011).

In the late 20th and early 21st century, the first studies, which evaluated the antiviral effect of drugs in cell lines and experimentally infected mammalian were published (Cubitt B *et al*., 1997; Hallensleben W *et al*., 1997; Mizutani T *et al*., 1998; Jordan I *et al*., 1999; Planz O *et al*., 2001; Solbrig MV *et al*., 2002; Lee BJ *et al*., 2008).

In 1997, the performance of the antiviral effect of amantadine, on cells culture infected with Borna disease virus, was questioned (Cubitt B *et al*., 1997; Hallensleben W *et al*., 1997). Namely, antiviral activity of amantadine against Borna disease virus showed to be ineffective to reduce the number of infected cells and to decrease levels of Borna disease virus RNA or proteins (Cubitt B *et al*., 1997; Hallensleben W *et al*., 1997). Also, the treatment of cells culture with amantadine did not affect the virus multiplication (Cubitt B *et al*., 1997). Additionally, it was reported that the application of the amantadine to persistently infected mice had no effect on the concentration of the Borna disease virus in their brains (Hallensleben W *et al*., 1997). In the years 1998 and 1999, in cell cultures experiments, the ribavirin, an antiviral nucleoside analogue with antiviral activity against a range of RNA and DNA viruses, showed promising results against mammalian bornaviruses infection (Borna disease virus 1 (BoDV-1)) (Mizutani T *et al*., 1998; Jordan I *et al*., 1999). After two years, the immunomodulatory U0126 showed to avoid the spread of BoDV-1 to the neighbourhood cells, inhibiting the MEK, in the cellular Raf/MEK/ERK signalling pathway (Planz O *et al*., 2001). However, the immunomodulatory U0126 was ineffective in eliminating the infection

from cells (Planz O *et al*., 2001). The findings demonstrated that the nucleoside analogue 1-beta-D-arabinofuranosylcytosine (Ara-C), a known inhibitor of DNA polymerases, inhibits BoDV replication (Bajramovic JJ *et al*., 2002). Ara-C treatment inhibited BoDV RNA and protein synthesis and prevented BoDV cell-to-cell spread in vitro (Bajramovic JJ *et al*., 2002). However, Ara-C's cytotoxic side effects are the principal obstacles for its therapeutic use (Bajramovic JJ *et al*., 2002). In 2004, the nucleoside analogue 2'-fluoro-2'-deoxycytidine (2'-FdC) was a candidate as antiviral therapy against Borna disease virus. The nucleoside analogue 2'-fluoro-2'-deoxycytidine exhibits potent antiviral activity, and its associated cytotoxicity is negligible (Bajramovic JJ *et al*., 2004).

In 2017, a study reported that the Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide), an antiviral drug that selectively inhibits the RNA-dependent RNA polymerase of the virus effectively suppressed the replication of BoDV-1 in persistently infected Vero cells (Tokunaga T *et al*., 2017).

In experimental studies, with rats as a model of Borna disease, the intracerebral ribavirin showed to reduce the morbidity (Solbrig MV *et al*., 2002). Also, neonatal infected gerbils showed that the intracranial inoculation of ribavirin resulted in protection from the occurrence of fatal neurological disorders, by reducing the viral propagation in the acutely infected brains (Lee BJ *et al*., 2008). However, the clinical improvement occurred without changes in virus titres (Solbrig MV *et al*., 2002).

During the second decade of the 21st century, the first published studies that tested the antiviral effect of ribavirin against avian bornaviruses arouse. The ribavirin inhibited parrot bornaviruses replication, decreasing viral mRNA and viral protein load, in infected avian cell lines (Musser JM *et al*., 2015; Reuter A *et al*., 2016). However, after the interruption of ribavirin administration, the virus recovered its replication process. Therefore, the ribavirin-based treatment does not eliminate the virus from persistently infected cells (Tokunaga T *et al*., 2017). Moreover, the ribavirin combined with type I interferon (IFN) strongly enhanced the antiviral efficiency, compared to either drug alone. Therefore, it resulted in a promising combination to treat the persistent infection (Reuter A *et al*., 2016). Additional studies provided an alternative to treat persistent bornavirus infection showing that favipiravir (T-705) effectively suppressed viral replication of avian bornavirus (Tokunaga T *et al*., 2017). Therefore, T-705 suppressed viral replication of mammalian and an avian bornavirus, suggesting that T-705 can have antiviral activity against a broad range of bornaviruses (Tokunaga T *et al*., 2017). However, until date, no published studies are reporting the antiviral effect of ribavirin/type I IFN combination, U0126, and favipiravir in naturally infected animals.

Naturally infected animals, with Borna disease virus, were treated with amantadine, at the end of the 20th century and during the first decade of the 21st century (Dietrich DE *et*

al., 2008; Ohlmeier MD *et al*., 2008; Bode L *et al*., 1997), despite its questioned antiviral effect on cell lines infected with Borna disease virus (Cubitt B *et al*., 1997; Hallensleben W *et al*., 1997). Another therapeutic approach was with corticosteroids, which reduced the clinical signs induced by the inflammatory response in feline Borna disease, (Wensman JJ, 2011). The treatment with corticosteroids was reported to be beneficial when used in the early stage of the disease (Wensman JJ, 2011), however, the immunosuppressive effects could lead to increased virus replication (Wensman JJ, 2011).

Previous studies evaluated the therapeutic effect of amantadine in neuropsychiatric and seropositive patients for bornavirus. The studies reported that amantadine reduced clinical symptoms, as the BoDV-infection in depressive patients, patients with affective disorders and bipolar patients (Dietrich DE *et al*., 2008). Another study, found similar findings, revealing that the use of amantadine reduced severe hypomania and the moderate mania in bipolar patients seropositive for bornavirus (Ohlmeier MD *et al*., 2008). However, in 2013, a study reported the ineffectiveness of amantadine in Borna disease (Herden C *et al*., 2013). More recently, in 2018, neuropsychiatric patients, who were seropositive for bornavirus, showed treatment-resistant symptoms improved after treated with ribavirin orally administered (Matsunaga H *et al*., 2018).

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Chapter 2 – Aims | Objectives

The present Thesis aims to contribute to the development of knowledge about the epidemiology of bornavirus infection, addressing the following specific objectives:

1) Develop a cost-effective real-time PCR assay, SYBR dye-based, to detect bornavirus reservoirs.

2) Develop a cost-effective real-time PCR assay, TaqMan probe-based to test animals suspected to be infected by bornavirus, to assist the clinical diagnosis.

3) Screen captive flocks of psittacines housed in breeding projects and zoos, to detect reservoirs.

4) Characterize the bornavirus detected in captive parrots in Portugal and Sweden.

5) Evaluate the relationship between bornavirus worldwide detected in captive psittacines and those detected in Portugal and Sweden.

6) Evaluate the relationship between bornavirus worldwide detected in captive psittacines and those detected in wild birds worldwide distributed.

Chapter 3 - Development of real-time PCR assays for bornavirus detection and quantification

3.1. The SYBR® Green-based assay & TaqMan®-based assay

3.1.1. Introduction

Bornaviruses are members of the order *Mononegavirales* belonging to the family *Bornaviridae* (Amarasinghe GK *et al*., 2019). So far, the genus *Carbovirus*, *Cultervirus* and *Orthobornavirus* have been identified, which comprise 11 species (Amarasinghe GK *et al*., 2019). Bornaviruses are enveloped entities with 80 to 100 nm in diameter, having a non-segmented genome of single-stranded negative-sense RNA of around 8900 nucleotides in length (Lipkin WI *et al*., 2011). Their genome has six open reading frames (ORFs) for the nucleoprotein (N), X protein (X), phosphoprotein (P), matrix protein (M), the glycoprotein (G) and the RNA-dependent RNA-polymerase (L) (Lipkin WI *et al*., 2011). Bornaviruses replicate in the nucleus of the nerve cells infected, of various organs, often establishing persistent non-cytolytic infections (Lipkin WI *et al*., 2011).

The infection by bornaviruses can cause the development of severe neurological and, or gastrointestinal disease and death of its hosts (Malkinson M *et al*., 1993; Berg A-L *et al*., 1998; Berg AL *et al*., 1999; Wensman JJ *et al*., 2012; Björnsdóttir S *et al*., 2013; Delnatte P *et al*., 2013; Guo J *et al*., 2014; Wensman JJ *et al*., 2014; Thomsen AF *et al*., 2015; Ando T *et al*., 2016; Tappe D *et al*., 2019). The disease occurs in humans, in several species of pets, in production animals and wild animals (Malkinson M *et al*., 1993; Berg A-L *et al*., 1998; Berg AL *et al*., 1999; Wensman JJ *et al*., 2012; Björnsdóttir S *et al*., 2013; Delnatte P *et al*., 2013; Guo J *et al*., 2014; Wensman JJ *et al*., 2014; Thomsen AF *et al*., 2015; Ando T *et al*., 2016; Tappe D *et al*., 2019). Two species caused disease in mammals, five in birds and three in reptiles (Amarasinghe GK *et al*., 2019). Among species that infects mammalians, there are viruses identified with zoonotic potential (Hoffmann B *et al*., 2015; Tappe D *et al*., 2018; Tappe D *et al*., 2019). Specifically, the Borna disease virus 1 (BoDV-1) and variegated squirrel bornavirus 1 (VSBV-1)) were the etiologic agents of lethal encephalitis in humans (Hoffmann B *et al*., 2015; Tappe D *et al*., 2018; Tappe D *et al*., 2019). However, some genotypes of Borna disease virus 1 (BoDV-1)) also shows the capacity to infect farmed ostriches and wild birds (mallards and jackdaws) (Malkinson M *et al*., 1993; Berg M *et al*., 2001), and besides, the wild birds can host a wide range of avian bornaviruses (Delnatte P *et al*., 2013; Guo J *et al*., 2014; Sassa Y *et al*., 2015; Thomsen AF *et al*., 2015).

Among captive birds, parrot bornavirus 1 to 8 (PaBV-1 to 8) caused severe effects on psittacines health (Tizard I, *et al*., 2016), having repercussions at economic and biodiversity

level; and therefore, becoming relevant in breeding projects of rare species, private collections and zoos. Asymptomatic infections with avian bornaviruses in captive and wild animals have been reported (Staeheli P *et al*., 2000; Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013; Zhang L *et al*., 2014). Also, the absence of seroconversion in some captive and naturally infected psittacines also occurred (Gancz AY *et al*., 2010). The virus is released through excretion in faeces, urine and saliva in an intermittent way (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013), as in captive flocks of psittacines, the avian bornaviruses infection have persisted for periods of years (Gancz AY *et al*., 2010).

The laboratory tools reported to be the gold-standard to detect bornaviruses infections were the histologic and immunohistochemistry examination of the tissues from the central nervous system of animals (Staeheli P *et al*., 2000; Richt JA *et al*., 2001; Gancz AY *et al*., 2010; Hoppes SM *et al*., 2020). However, the collection of samples in living animals, from wild and captive flocks, by very invasive methods to identify reservoirs is controversial from a feasibility and ethical issue. Even in clinical practice, from a practical point of view, the use of central nervous system tissues from living animals to confirm the suspected infection is challenging. Besides, the invasive procedures for the animal inherently have a high risk. There are diagnostic tools developed to evaluate samples collected by non-/less-invasive methods (e.g., faeces, urine, saliva and blood) (Staeheli P *et al*., 2000; Wensman JJ *et al*., 2014; Schlottau K *et al*., 2017; Hoppes SM *et al*., 2020). However, the available serological assays produced inconsistent results (Staeheli P *et al*., 2000; Dauphin G *et al*., 2002; Wensman JJ *et al*., 2014; Hoppes SM *et al*., 2020). The viral isolation methods are laborious, expensive and time-consuming (Guo J *et al*., 2014). The accurate quantification of the viral load in samples is difficult with conventional polymerase chain reaction (PCR) assays (Navarro E *et al*., 2015). Besides, they are laborious and time-consuming compared to real-time PCR-based diagnostic tools (Navarro E *et al*., 2015). However, the viral load quantitative systems used in the real-time PCR assays, previously reported in the literature, are expensive, require a laborious preparation and have short expiration dates (Schindler AR *et al*., 2007; Wensman JJ *et al*., 2007). A cost-effective diagnostic tool will be an asset in the surveillance of captive and wild animals, as in the context of clinical practice.

This study aimed to develop cost-effective real-time PCR assays, addressing the following specific objectives: 1) develop a real-time PCR assay, SYBR® Green-based, for screening large populations, with the purpose to identify reservoirs of bornavirus, 2) develop a real-time PCR assay, TaqMan®-based to test animals suspected to be infected by bornavirus, to assist the clinical diagnosis.

3.1.2. Materials and methods

3.1.2.1. Cell culture and Borna disease virus 1, strain He/80

A Vero cells line used, was infected with the Borna disease Virus 1, strain He/80 (BoDV-He/80), (the virus was made available by Section of Virology, Department of Biomedical Science and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden). The cells culture procedures were based in a previously published protocol (Ammerman NC *et al*.; 2008). Briefly, the propagation of Vero cell culture from frozen stocks were performed according to the following steps.

1) A cryovial of Vero cells (1 ml) was thawed by gently swirling in a 37ºC water bath.
2) In a laminar flow hood, the Vero cells suspension was transferred into a 15 ml conical tube containing the growth medium (10 ml of Dulbecco's modification of Eagle medium (DMEM) (Invitrogen™, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen™, Paisley, UK) and antibiotics (penicillin (100 U/ml) and streptomycin (100 µ/ml) (Sigma-Aldrich, Steinheim, Germany)).
3) Vero cells were pelleted by centrifugation at 200 X g for 5 minutes at room temperature, followed of cells resuspension in 5 ml of growth medium.
4) Vero cells suspension was transferred to a tissue culture flask of 25 cm^2 with vented cap and incubated at 37ºC with 5% CO_2.
5) Cells were monetarized daily, and media was changed every 3 days.

The maintenance of Vero cell cultures and the inoculation with BoDV-1, strain He/80 was performed according to the following procedures.

1) When cells reached more than 90% of confluence monolayer, the growth medium was removed, and the Vero cells were washed with 10 ml 1X PBS (phosphate buffered saline).
2) Cells were incubated with 5 ml of 1X trypsin-EDTA (Sigma-Aldrich, Steinheim, Germany) at 37ºC for 2 minutes.
3) Growth medium (5 ml) was added to inactivate the trypsin-EDTA and to wash the Vero cells.
4) The Vero cell suspension was transferred to a sterile 15 ml conical tube and centrifuged at 200 X g for 5 minutes at room-temperature.
5) The Vero cells pellet was resuspended in 10 ml growth medium.

6) From the resuspended Vero cells, dilutions (2:5) in a total of 5 ml of growth medium were performed and added to 25 cm^2 cell culture flasks with vented caps.
7) The cell culture flasks were incubated at 37ºC with 5% CO_2 and cells were monitored daily.
8) When cells reached about 90% of confluence monolayer, they were inoculated with BoDV-1, strain 80/He.
9) The cryovial containing the virus (1 ml) was thawed by gently swirling in a 37ºC water, filtered at 0.2 µm (Sarstedt, Nümbrecht, Germany), added to the cell culture flasks.
10) And the inoculated cell culture flasks were incubated at 37ºC with 5% CO_2. Uninoculated cell culture flasks were also maintained as negative controls during the virus propagation.

3.1.2.2. RNA extraction

The extraction of total RNA was with NYZol (NzyThec, Lisbon, Portugal) according to the manufacturer's instructions/protocol from Vero cells persistently infected with BoDV-He/80. Total RNA solutions were processed using the GeneJET RNA cleanup kit (Thermoscientific, Lithuania). The NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) was used to evaluate the purity and the concentration of total RNA according to the manufacturer's instructions. The RNA solutions with a ratio of absorbance at A260/280nm higher or equal to 2.0 (NanoDrop ND-1000) were used for cDNA synthesis. The total RNA solutions were maintained in ice if processed immediately or were stored at -20ºC until further processing.

3.1.2.3. Reverse transcription (RT)

The purified total RNA was reverse transcribed into cDNA following a protocol developed and optimized, using the RevertAid First Strand cDNA Synthesis Kit (Thermoscientific, Lithuania), based on the manufacturer's instructions. Briefly, one µg of total RNA was incubated with the Random Hexamer primer (50 µM) at 65ºC for 5 min. (Thermal Cycler PTC-100™, USA). Then, it was placed on ice for 5 min following the addition of: 1) Reaction Buffer (1X), 2) RiboLock RNase inhibitor (1U/µl), 3) dNTP Mix (100 Mm), 4) RevertAid M-MuLVRT (10 U/µl). The previous mix prepared was incubated for 5 min at 25ºC followed by 60 min at 45ºC, and the reaction finished with the enzyme

inactivation at 70 ºC for 5 min (using the Thermal Cycler PTC-100™, USA). The reverse transcription reaction products were maintained on ice if processed immediately or stored at -20ºC until further processing.

In the experiments, positive and negative control reactions were included and prepared according to manufactures instructions (RevertAid First Strand cDNA Synthesis Kit (Thermoscientific, Lithuania)). Briefly, for each experimental condition the following was considered: 1) reverse transcriptase minus (RT-) negative control, which contained every reagent for the reverse transcription reaction except for the reverse transcriptase enzyme; 2) no-template negative control (NTC), which included every reagent for the reverse transcription reaction except for the RNA template and 3) the human GAPDH (1.3 kb) as a positive control RNA template.

3.1.2.4. *In-house* prepared standard solutions

3.1.2.4.1. Standard solutions of RNA from Borna disease virus 1, strain He/80

The total RNA extracted (according to section 3.1.2.2.) from Vero cells persistently infected with BoDV-He/80 was used to prepare standard solutions. From 1 µg of total RNA extracted, ten standard-solutions of 10-fold serial dilutions were prepared. The concentration of standard-solutions ranged from 10^{3} ng/µl to 10^{-6} ng/µl of total RNA. Then, of each standard-solutions, five aliquots were prepared. Therefore, a total of fifty standard-solutions were prepared. All aliquots were stored at -80ºC and used only once.

3.1.2.4.2. Standard solutions of DNA from Borna disease virus 1, strain He/80

Stock solutions containing segments of the P gene, from BoDV-He/80 strain, were prepared. To produce segments of the P gene, with 456 base pairs, the conventional polymerase chain reaction (PCR) was used. Each PCR reaction contained a total volume of 25 µl (including 2 µl of template cDNA, 1.25 µl of primers (Berg M *et al*.; 2001) and Dream Taq Green Master Mix (Thermo Scientific)). The thermal cycle parameters used were the following: 95.0ºC for 10 min, followed by 40 cycles at 95ºC for 15 sec and 60.0ºC for 1 min.

The specific amplification was confirmed by electrophoresis, loading 2 µl of the PCR products per well on a 2.0% (W/V) agarose gel, stained with Midori Green™ (Nippon Genetics Europe) and observed under the UV light (Bio-Rad, California, USA). The products were purified using the Gene Jet Gel Extraction Kit (Thermo Scientific) and sequenced by

Sanger sequencing (Eurofins Europe). The Sanger sequencing results were used as a query sequence in a BLAST® search at GenBank® (www.ncbi.nlm.nih.gov) to ensure the amplification of a segment of the P gene. The solutions, containing segments of the P gene, with a ratio absorbance at A260/280nm higher or equal to 2.0 (estimated by NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) were used to produce standard solutions. Therefore, the prepared stock solutions of the P gene segment were 10 ng/µl. Then, stock solutions obtained were used to prepare ten standard-solutions of 10-fold serial dilutions (between 10 ng/µl and 1 $x10^{-8}$ ng/µl). Each standard-solutions of 10-fold serial dilutions, distributed into five aliquots, were stored at -80ºC. From the fifty standard solutions prepared each aliquot was used only one time.

3.1.2.5. Estimation of copy numbers of the P gene segment per standard solution

For each DNA standard-solution (prepared according to section 3.1.2.4.2) the number of the P gene copies was estimated. Briefly, following these procedures:

1) the mass estimation of the P gene segment using the formula: *(n) X (K)* (Applied Biosystems, 2003), where *n* was the segment size of 456 bp and *K* is the mass of each base pair (a constant value of $1.096X10^{-21}$g/bp) (Table 1 and Table 2) (Applied Biosystems, 2003);
2) the mass estimation of the P gene segment containing 1 copy of the respective gene (Table 1 and Table 2);
3) estimation of the mass of DNA to achieve copy numbers of P gene between $2x10^9$ and 2, using the formula: *(copies nº of P gene) X (m)* (Applied Biosystems, 2003), where *m* is the mass of the segment of the P gene containing 1 copy of the gene (Table 1 and Table 2);
4) estimation of the DNA concentration to achieve copy numbers of the P gene between $2x10^9$ and 2 (Table 1 and Table 2);
5) preparation of 10 standard solutions of 10-fold serial dilutions of DNA with a final number of P gene copies between $2X10^9$ and 2 (Table 1 and Table 2).

Table 1. Estimation of the DNA concentration needed to achieve a copy number between $2X10^9$ and 2 of the P gene

Segment of the P gene (456 bp) from Borna disease virus 1, strain He/80				
DNA mass with 1 copy of P gene (ng) [A]	**P gene copies of interest per standard dilutions [B]**	**DNA mass with P gene copies of interest [C]**	**Volume per PCR reaction [D]**	**[DNA] with P gene copies of interest [E]**
(456 bp) X (*1.096X10^{-21}g/bp*) = 5.00 X 10^{-9} ng of the segment of the P gene contains 1 copy of the gene.	$2X10^9$	10 ng	2 µl	5 ng/µl
	$2X10^8$	1 ng		5 X 10^{-1} ng/µl
	$2X10^7$	1 X 10^{-1} ng		5 X 10^{-2} ng/µl
	$2X10^6$	1 X 10^{-2} ng		5 X 10^{-3} ng/µl
	$2X10^5$	1 X 10^{-3} ng		5 X 10^{-4} ng/µl
	$2X10^4$	1 X 10^{-4} ng		5 X 10^{-5} ng/µl
	$2X10^3$	1 X 10^{-5} ng		5 X 10^{-6} ng/µl
	$2X10^2$	1 X 10^{-6} ng		5 X 10^{-7} ng/µl
	20	1 X 10^{-7} ng		5 X 10^{-8} ng/µl
	2	1 X 10^{-8} ng		5 X 10^{-9} ng/µl

A - The mass of the segment of the P gene was estimated using the formula: *(n) X (K)*, where *n* is the P gene segment size of 456 bp and *K* is the mass of each base pair (a constant value of *1.096X10^{-21}g/bp*) (Applied Biosystems, 2003). **B** - The number of copies of the P gene defined per standard dilutions. **C** - Estimation of the DNA mass needed per standard solution to have copy numbers of P gene between $2x10^9$ and 2. **D** - Volume of each standard-solution used per real-time PCR reaction. **E** - The final DNA concentration to achieve a copy number of P gene between $2X10^9$ and 2 per real-time PCR.

Table 2. DNA standard solutions preparation, of 10-fold serial dilutions, with the copy number between $2X10^9$ and 2 of the P gene

[DNA] with P gene copies of interest [A]	Final volume of each standard solutions	Source of [DNA] to prepare standard solutions	Volume of DNA to prepare standard solutions	[DNA] per standard solutions [C]
5 ng/µl		Stock solution [B]	50 µl	10ng/ µl
$5 X 10^{-1}$ ng/µl		Diluition 1	5 µl	1 ng/µl
$5 X 10^{-2}$ ng/µl		Diluition 2	5 µl	$1 X 10^{-1}$ ng/µl
$5 X 10^{-3}$ ng/µl		Diluition 3	5 µl	$1 X 10^{-2}$ ng/µl
$5 X 10^{-4}$ ng/µl	100 µl	Diluition 4	5 µl	$1 X 10^{-3}$ ng/µl
$5 X 10^{-5}$ ng/µl		Diluition 5	5 µl	$1 X 10^{-4}$ ng/µl
$5 X 10^{-6}$ ng/µl		Diluition 6	5 µl	$1 X 10^{-5}$ ng/µl
$5 X 10^{-7}$ ng/µl		Diluition 7	5 µl	$1 X 10^{-6}$ ng/µl
$5 X 10^{-8}$ ng/µl		Diluition 8	5 µl	$1 X 10^{-7}$ ng/µl
$5 X 10^{-9}$ ng/µl		Diluition 9	5 µl	$1 X 10^{-8}$ ng/µl

A - The final DNA concentration with a copy number of P gene between $2X10^9$ and 2, according to Table 1. **B** - A stock solution of DNA at 10 ng/µl prepared according to described in section 3.1.2.4.2 was used to prepare 10-fold serial dilutions containing segments of the P gene from the BoDV-He/80 strain. **C** - Preparation of 10 standard solutions of 10-fold serial dilutions of DNA with a final number of P gene copies between $2X10^9$ and 2, which were used to construct the standard curves.

3.1.2.6. Characteristics of primers and probe

The primers and probe were previously described (Wensman JJ *et al*., 2007). Briefly, the primers target a conserved region of the phosphoprotein gene (P gene), covering several strains of bornaviruses (Wensman JJ *et al*., 2007). The PCR product covers the nucleotides 1426 to 1515 of the BoDV-He/80 strain. The specificity of primers was previously established (Wensman JJ *et al*., 2007). The probe was labelled at the 5`-end with 6-carboxyfluorescein (FAM) and the 3´-end with the non-fluorescent Black Hole Quencher Dye (BHQ) (Eurogentec, Seraing, Belgium).

3.1.2.7. Optimization of primers concentration

The establishment of optimal concentration of primers was by determining the minimum primer concentration required to produce the lowest threshold cycle (Ct) and the maximum magnitude of the signal (∆Rn) for a set of real-time PCR conditions (Applied Biosystems, 2011). In the SYBR-based assay, the ∆Rn was the ratio of the fluorescence emission intensity of the SYBR® Green dye to fluorescence emission intensity of the ROXTM dye (passive reference), removing the baseline fluorescence emission (Applied Biosystems, 2011). And in the probe-based assay, the ∆Rn was the ratio of the fluorescence emission intensity of the TaqMan® dye to fluorescence emission intensity of the ROXTM dye (passive reference), removing the baseline fluorescence emission (Applied Biosystems, 2011).

3.1.2.7.1. SYBR® Green-based assay

Nine concentrations of primers were prepared (Table 3) and each concentration evaluated in triplicate. As templates, cDNA (produced according to section 3.1.2.3.) and DNA (prepared according to section 3.1.2.4.2.) were used. Each reaction contained a total volume of 25 µl with: 2 µl of template (cDNA or DNA) and 1X SYBR® Green PCR Master Mix (Applied Biosystems, 2011). Additionally, for each condition assessed a no-template negative control (NTC) was included in the experiment. The thermal cycle parameters used for primer optimization were the following: 95.0ºC for 10 min, followed by 40 cycles at 95ºC for 15 sec and 60.0ºC for 1 min.

The verification of nonspecific amplification absence in the reactions was by using the melt curves produced during real-time PCR (Applied Biosystems, 2016). The melt curve protocol included three stages: 1st) was at 95ºC for 15 sec; 2nd) was at 60ºC for 1 min, with a ramping time of 1.6ºC/sec and 3rd) was at 95ºC for 15 sec, with a ramping time of 0.15ºC/sec (according to the instrument user guide) (Applied Biosystems, 2011).

Table 3. The nine conditions evaluated during the optimization of primer concentration

	Templates from genomic material of BoDV-He/80					
	cDNA dilutions [A]		**[DNA] [B] ng/µl**			
[Set of primers]	**10^{-3}**	**10^{-2}**	**1.0**	**10^{-1}**	**10^{-2}**	**10^{-3}**
Concentrations evaluated (µM)	0.05	0.05	0.05	0.05	0.05	0.05
	0.10	0.10	0.10	0.10	0.10	0.10
	0.15	0.15	0.15	0.15	0.15	0.15
	0.20	0.20	0.20	0.20	0.20	0.20
	0.30	0.30	0.30	0.30	0.30	0.30
	0.35	0.35	0.35	0.35	0.35	0.35
	0.40	0.40	0.40	0.40	0.40	0.40
	0.45	0.45	0.45	0.45	0.45	0.45
	0.90	0.90	0.90	0.90	0.90	0.90

A - The RNA standard-solution of 10^3 ng/µl, reverse transcribed (prepared according to section 3.1.2.3 and 3.1.2.4.1, respectively) was used to prepare de cDNA dilutions evaluated. **B** - The preparation of the DNA solutions evaluated were according to section 3.1.2.4.2.

The production of the specific amplification was additionally confirmed by electrophoresis, loading 12.5 µl of the real-time PCR products per well, on a 2.0% (W/V) agarose gel, stained with Midori Green™ (Nippon Genetics Europe) and observed under the UV light (Bio-Rad, California, USA). The products were purified using the Gene Jet Gel Extraction Kit (Thermo Scientific) and sequenced by Sanger sequencing (Eurofins Europe).

The Sanger sequencing results were used as a query sequence in a BLAST® search at GenBank® (www.ncbi.nlm.nih.gov) to ensure the amplification of a segment of the P gene. The StepOne™ Systems (Applied Biosystems) was used to perform real-time PCR experiments, and the data produced were analysed using the StepOne software V 2.1. (Applied Biosystems).

All experimental conditions included a no-template negative control (NTC), which had all reaction components except the DNA or cDNA sample, and a no-reverse transcriptase control (no-RT) that included all reaction components except the nucleic acid samples. Namely, the nucleic samples were substituted by 2 µl of the product of the RT minus (RT^-), obtained according to section 3.1.2.3.

3.1.2.7.2. TaqMan®-based assay

Nine concentrations of primers (Table 4) were prepared and assessed in triplicate. The cDNA and DNA prepared according to section 3.1.2.3, and section 3.1.2.4.2 were used (Table 4). Each reaction contained a total volume of 30 µl containing: 2 µl of template (cDNA or DNA) and TaqMan® Environmental Master Mix 2.0 (Applied Biosystems). Additionally, for each condition evaluated, there was no-template negative control (NTC) inclusion in the experiment. The thermal cycling parameters used for primer optimization were the following: 95.0ºC for 10 min, followed by 40 cycles at 95ºC for 15 sec and 60.0ºC for 1 min. (Applied Biosystems, 2011).

The purity of real-time PCR products was confirmed by electrophoresis, loading 15 µl of the products per well on a 2.0% (W/V) agarose gel, stained with Midori Green™ (Nippon Genetics Europe) and observed under UV light (Bio-Rad, California, USA). Additionally, the produced amplicons were sequenced (by Sanger sequencing (Eurofins Europe)). The Sanger sequencing results were used as a query sequence in a BLAST® search at GenBank® (www.ncbi.nlm.nih.gov) to ensure the amplification of a segment of the P gene. The StepOne™ Systems (Applied Biosystems) was used to perform real-time PCR experiments, and the data produced were analysed using the StepOne software V 2.1. (Applied Biosystems). All experimental conditions included a no-template negative control (NTC), which had all reaction components except the DNA or cDNA sample, and a no-reverse transcriptase control (no-RT) that included all reaction components except the nucleic acid samples. Namely, the nucleic acid samples were substituted by 2 µl of the product of the RT minus (RT^-), obtained according to section 3.1.2.3.

Table 4. The nine conditions evaluated during the optimization of primer/probe concentration

	Templates from genomic material of BoDV-He/80					
	cDNA dilutions [A]		[DNA] [B] ng/µl			
	10^{-3}	10^{-2}	1.0	10^{-1}	10^{-2}	10^{-3}
Set of primers/probe concentrations (µM)	0.30	0.30	0.30	0.30	0.30	0.30
	0.35	0.35	0.35	0.35	0.35	0.35
	0.40	0.40	0.40	0.40	0.40	0.40
	0.70	0.70	0.70	0.70	0.70	0.70
	0.75	0.75	0.75	0.75	0.75	0.75
	0.90	0.90	0.90	0.90	0.90	0.90

A - The RNA standard-solution of 10^3 ng/µl, reverse transcribed (prepared according to section 3.1.2.3 and 3.1.2.4.1, respectively) was used to prepare de cDNA dilutions evaluated. **B** - The preparation of the DNA solution evaluated were according to section 3.1.2.4.2.

3.1.2.8. The real-time PCR: the chemistry and thermal cycle parameters

The StepOne™ Systems (Applied Biosystems) was used to develop the real-time PCR, and data were analysed using the software (StepOne software V 2.1., Applied Biosystems). The thermal cycling parameters were: 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 sec and 60ºC for 1 min. The threshold was set in the exponential phase of the reactions, and the cut-off for the threshold cycle (Ct) values was 40. Consequently, we considered a positive result when at least one of the four replicates, for each sample assessed, had a Ct value less than or equal to 40.

3.1.2.8.1. SYBR® Green-based assay

The total volume of each reaction was 25 µl containing: 2 µl of cDNA/DNA template, 0.3 µM of each primer and 1X SYBR® Green PCR Master Mix (Applied Biosystems). Additionally, for each condition evaluated in an experiment, a no-template negative control

(NTC) was included. The NTC controls had all reaction components except the DNA or cDNA sample. Also, a no-reverse transcriptase control (no-RT) was included, which had all reagents and 2 µl of the product of the RT minus (RT^-), obtained according to section 3.1.2.3.

The absence verification of nonspecific amplification was by melt curve analysis (Applied Biosystems, 2016). The melt curve protocol was: 95ºC for 15 sec, 60ºC for 1 min, with a ramping time of 1.6ºC/sec and 95ºC for 15 sec, with a ramping time of 0.15ºC/sec (Applied Biosystems, 2011).

3.1.2.8.2. TaqMan®-based assay

The total volume of each reaction was 30 µl containing: 2 µl of cDNA/DNA template, 0.7 µM of each primer/probe and 1X TaqMan® Environmental Master Mix (Applied Biosystems, 2010). Additionally, for each condition evaluated in an experiment, a no-template negative control (NTC) was included. The NTC controls had all reaction components except the DNA or cDNA sample. Also, a no-reverse transcriptase control (no-RT) was included, which had all reagents and 2 µl of the product of the RT minus (RT^-), obtained according to section 3.1.2.3. The thermal cycling parameters were: 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 sec and 60ºC for 1 min. (Applied Biosystems, 2010).

3.1.2.9. Evaluation of the reverse transcription reaction

In the reverse transcription chemistry three parameters (the linear range, capacity and efficiency of the system) to evaluate its performance were considered. For that, a dynamic range test was performed using the relative standard curve method. For that, nine standard-solutions of total RNA (prepared according to section 3.1.2.4.1) were reverse transcribed (according to section 3.1.2.3.) and evaluated by real-time PCR. The evaluation of each of the nine standard-solutions was done four times, and a result was identified as an outlier if obtained a threshold cycle (Ct) standard deviation > 0.5. Standard-solutions that produced more than one outlier, at the evaluation procedure, were excluded from data analysis. Four runs of the relative standard curve were conducted, obtaining 16 threshold cycle (Ct) values per standard solution. The produced data (in the four runs) was used to construct the final relative standard curve as a semi-log regression line plot of mean threshold cycle (Ct) values versus log10 of RNA concentration. The efficiency (Ɛ) was estimated using the

formula: $\mathcal{E} = (10^{(-1/-\alpha)}-1) \times 100\%$, in which α is the slope of the final standard curve (Applied Biosystems, 2016).

When the efficiency (Ɛ) was constant within a range of RNA mass values added to the system the linearity range of the reverse transcription chemistry was defined (Applied Biosystems, 2016). The maximum mass of RNA added to the system without loss of efficiency established the system capacity (maximum value within the linear range) (Applied Biosystems, 2016). The correlation coefficient (R^2) value of the final standard curve >0.99 assessed the system precision (Applied Biosystems, 2016).

3.1.2.10. The performance of the quantitative real-time PCR

The real-time PCR assay performance evaluation was achieved through the standard curve method.

The BoDV-1 strain He/80 was used to construct a system for absolute quantitation in real-time PCR assays. For that, ten standard-solutions of DNA from the BoDV-He/80 (prepared according to section 3.1.2.4.2.) were used to construct a standard curve. The prepared standard-solutions ranged from $2X10^9$ to 2 copies of P gene/µl (Table 1 and Table 2). The ten standard 10-fold serial dilutions were divided into aliquots, stored at -80ºC and thawed only once before use. For the construction of the standard curve, four replicates were analysed for each of the ten DNA standard-solutions used (Table 1 and Table 2), and performed four runs of the standard curve, obtaining 16 results per DNA standard-dilution points. Therefore, the final standard curve consisted of a semi-log regression line plot of mean threshold cycle (Ct) values versus log10 of each DNA standard solution. When the threshold cycle (Ct) values showed a standard deviation value higher than 0.5, there was no inclusion in the standard curve.

The efficiency, lower detection limit (sensitivity), reproducibility and precision of the quantitative system developed were parameters used to evaluate its performance. Namely, the Ct values with standard deviation <0.5 assessed the system reproducibility (Applied Biosystems, 2016). The slope (α), of the standard curve, were used to estimate the efficiency (Ɛ) using the formula: $\mathcal{E} = (10^{(-1/-\alpha)} - 1) \times 100\%$ (Applied Biosystems, 2016). The correlation coefficient (R^2), of the standard curve, were used to estimate the precision (Applied Biosystems, 2016). The linearity range of the quantitative system was established as the range of DNA mass values in which the detection efficiency was constant (Ɛ), with increasing DNA mass added to the assay (Applied Biosystems, 2016). The maximum DNA mass detected within the linear range, of the standard curve, was the capacity of the system

found (Applied Biosystems, 2016). The lower detection limit (sensitivity) was the minimum mass of DNA detected within the linearity range (the last dilution that produced a specific amplification signal) (Applied Biosystems, 2016). The production confirmation of specific amplicons (segment of the P gene) during the experiment was according to the procedure described in 3.1.2.7.

3.1.2.10.1. SYBR® Green-based assay

To run the standard curves the StepOne™ Systems (Applied Biosystems) was used, and the data produced were analysed using the software StepOne software V 2.1., (Applied Biosystems). The experimental conditions of the real-time PCR were according to sections 3.1.2.8 and 3.1.2.8.1.

3.1.2.10.2. TaqMan®-based assay

To run the standard curves the StepOne™ Systems (Applied Biosystems) was used, and the data produced were analysed using the software StepOne software V 2.1., (Applied Biosystems). The experimental conditions of the real-time PCR were according to sections 3.1.2.8 and 3.1.2.8.2.

3.1.3. Results

3.1.3.1. Optimization of primer concentration

3.1.3.1.1. SYBR® Green-based assay

The primer concentration, giving the lowest threshold cycle (Ct) and the maximum magnitude of fluorescence signal (∆Rn), without nonspecific amplification, was established at 0.3 µM for both types of samples evaluated (cDNA and DNA). Electrophoretic analysis of the real-time PCR products showed the formation of an expected amplicon, per reaction, without diffuse bands below, confirming the absence of primer-dimers formation revealed by melting curves analyses.

3.1.3.1.2. TaqMan®-based assay

The primer and probe concentration, giving the lowest threshold cycle (Ct) and the maximum magnitude of the fluorescence signal (ΔRn), without nonspecific amplification, was 0.7 µM for both types of samples evaluated (cDNA and DNA). Electrophoretic analysis of the products from the real-time PCR showed the formation of an expected amplicon, per reaction.

3.1.3.2. Reverse transcription reaction performance

3.1.3.2.1. SYBR® Green-based assay

The reverse transcription system showed to convert a high percentage of RNA molecules into cDNA (Table 5) (Figure 1). Additionally, the reverse transcription system revealed an ample linear dynamic range and high capacity (Table 5) (Figure 1). Moreover, the system showed accuracy since a high correlation coefficient value of the relative standard curve was found (Table 5) (Figure 1). Therefore, the above-reported parameters show that the reverse transcription system established had a high performance (Table 5) (Figure 1). Therefore, the cDNA produced by the reverse transcription yields real-time PCR results within the linear dynamic range of the SYBR® Green-based assay (Table 5) (Figure 1).

Table 5. The performance parameters of the reverse transcription reaction within the linear dynamic range of the SYBR® Green-based assay

Dynamic linear range		Capacity [B] (ng/µl)	Precision [C] (R^2)	Efficiency [D] (%)
Ct Mean [A]	[RNA] ng/µl			
(12.12 – 32.25)	(10^2 – 10^{-4})	100	0.999	98.92

A - The mean threshold cycle (Ct) values range. **B** - The maximum concentration of RNA that was added to the system without losing the reverse transcription efficiency. **C** - Correlation coefficient. **D** - Efficiency was estimated using the formula: $\mathcal{E} = (10^{(-1/-\alpha)} - 1) \times 100\%$, in which α is the slope of the final standard curve (Applied Biosystems, 2016).

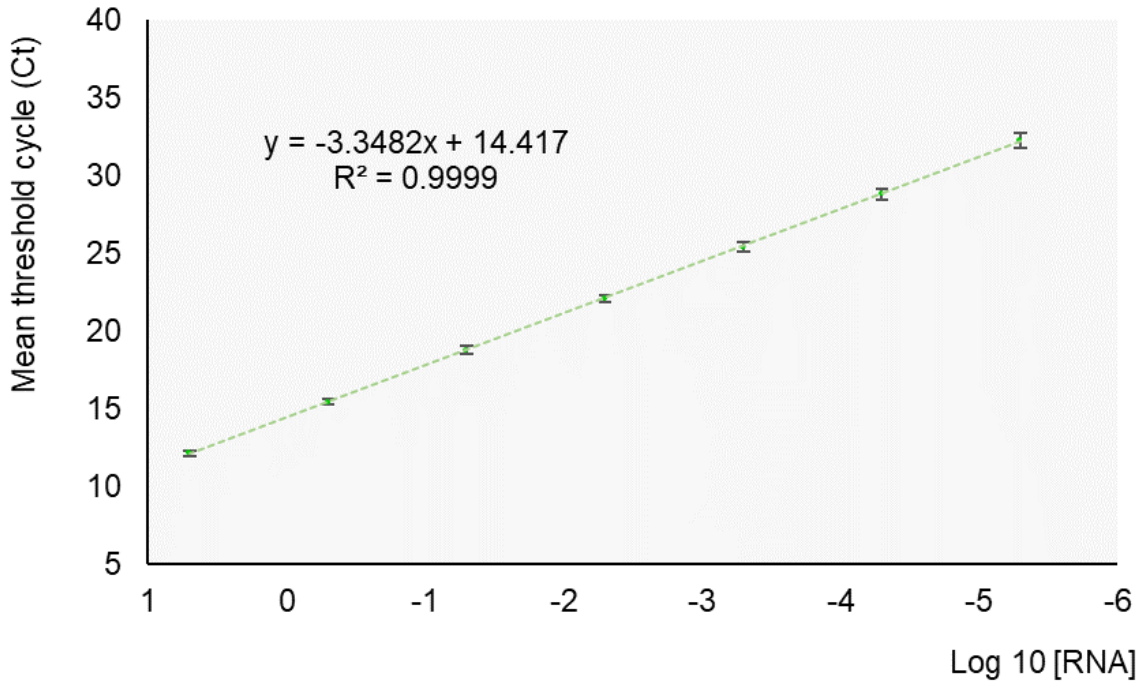

Figure 1. The relative standard curve, as a semi-log regression line plot of mean threshold cycle (Ct) values versus log10 of RNA concentration (SYBR® Green-based assay).

3.1.3.2.2. TaqMan®-based assay

The reverse transcription system showed high performance within the linear dynamic range of the TaqMan®-based assay (Table 6) (Figure 2). Namely, the reverse transcription system showed high capacity and efficiency, as well as accuracy (correlation coefficient (R^2) =0.999) (Table 6) (Figure 2). Therefore, the cDNA produced by the reverse transcription system yields real-time PCR results within the linear dynamic range of the TaqMan®-based assay (Figure 2).

Table 6. The performance parameters of the reverse transcription reaction within the linear dynamic range of the TaqMan®-based assay

Dynamic linear range		Capacity [B] (ng/µl)	Precision [C] (R^2)	Efficiency [D] (%)
Ct Mean [A]	[RNA] ng/µl			
(12.02 – 32.13)	(10^2 – 10^{-4})	100	0.999	99.10

A - The mean threshold cycle (Ct) values range. **B** - The maximum concentration of RNA that was added to the system without losing the reverse transcription efficiency. **C** - Correlation coefficient. **D** - Efficiency was estimated using the formula: $Ɛ = (10^{(-1/-α)} - 1) \times 100\%$, in which α is the slope of the final standard curve (Applied Biosystems, 2016).

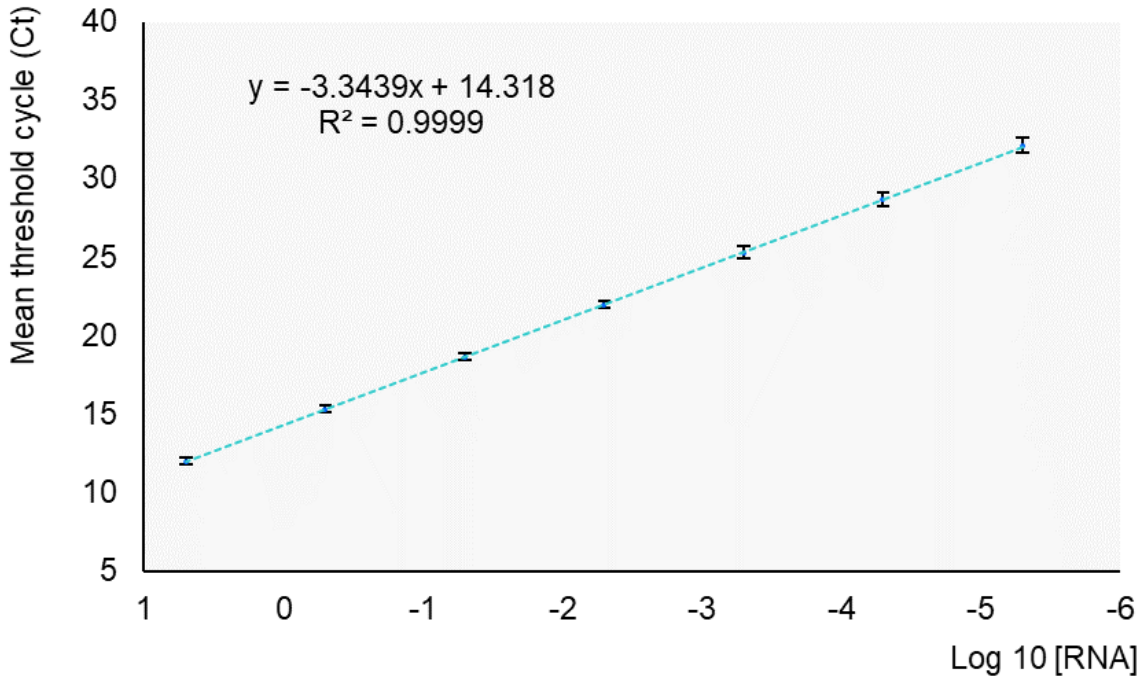

Figure 2. The relative standard curve, as a semi-log regression line plot of mean threshold cycle (Ct) values versus log10 of RNA concentration (TaqMan®-based assay).

3.1.3.3. The performance of the quantitative real-time PCR

3.1.3.3.1. SYBR® Green-based assay

The real-time PCR SYBR® Green-based assay developed showed high performance (by absolute standard curve method evaluation) (Table 7) (Table 8) (Figure 3). Namely, the assay showed an ample dynamic linear range of detection (Table 7) (Figure 3), as well as high capacity (upper detection limit), sensitivity (detected low nº target copies - lower detection limit), efficiency (detected a high percentage of target molecules) and showed accuracy (correlation coefficient (R^2) >0.99) (Table 7) (Table 8) (Figure 3).

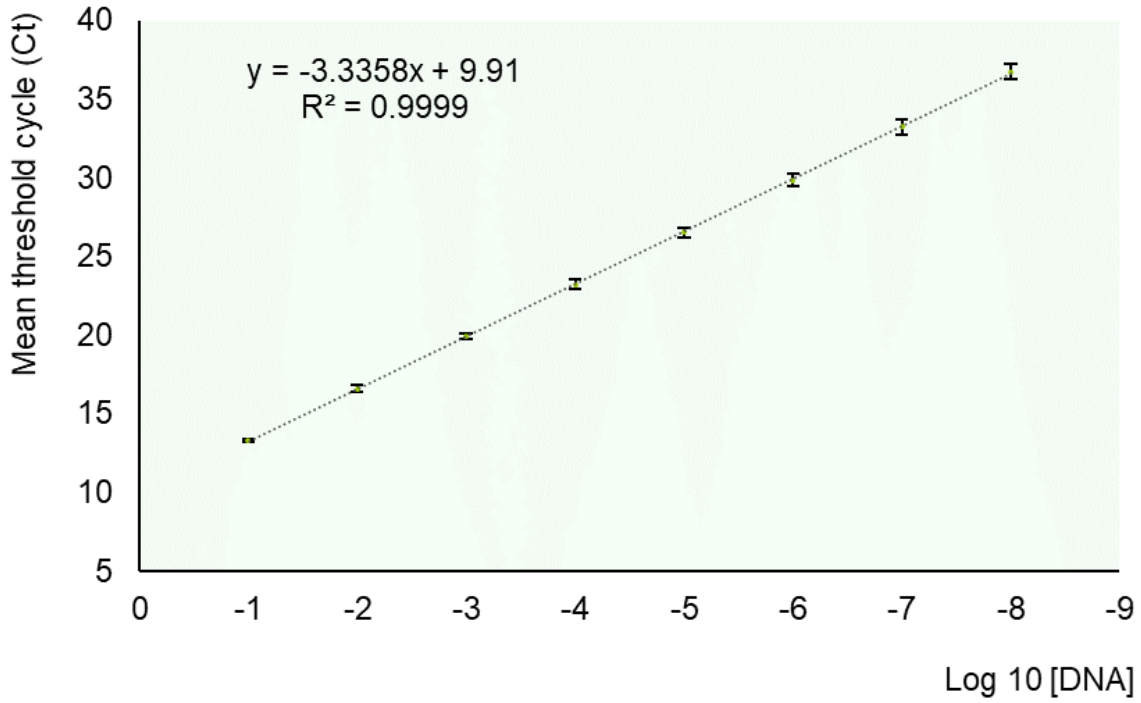

Figure 3. The standard curve used to assess the performance of the quantitative real-time PCR SYBR® Green-based assay.

Table 7. The performance parameters based on the dynamic range of the SYBR® Green-based assay developed

Dynamic linear range				
Ct [A] Mean	**[DNA] ng/µl**	**P gene**	**Capacity [B]**	**LDL [C]**
(13.29 – 36.73)	(10^{-1} – 10^{-8})	($2X10^{7}$ – 2)	$2X10^{7}$	2

A - The mean threshold cycle (Ct) values range. **B** - The maximum number of P gene copies detected by the assay without losing the efficiency (the upper limit of detection). **C** - Lower detection limit - the minimum number of P gene copies detected by the assay without losing the efficiency.

Table 8. The reproducibility, precision and efficiency of the real-time PCR SYBR® Green-based assay developed

	Threshold cycle range		Performance parameters	
	Mean [A]	**SD [B]**	**R^2 [C]**	**Ɛ % [D]**
Intra-assay				
Standard curve 1	(13.39 – 36.87)	0.10 ≤ SD ≤ 0.50	0.999	99.41
Standard curve 2	(13.38 – 36.82)	0.09 ≤ SD ≤ 0.45	0.999	99.41
Standard curve 3	(13.19 – 35.87)	0.10 ≤ SD ≤ 0.50	0.999	99.42
Standard curve 4	(13.20 – 35.92)	0.09 ≤ SD ≤ 0.45	0.999	99.42
Inter-assay				
Final standard curve	(13.29 – 36.37)	0.10 ≤ SD ≤ 0.50	0.999	99.42

A - The mean threshold cycle (Ct) values range. **B** - The range of standard deviation of the mean threshold cycle (Ct) per DNA standard dilution point. **C** - Correlation coefficient of the standard curve produced. **D** - Efficiency was estimated using the formula: $Ɛ = (10^{(-1/-\alpha)} - 1) \times 100\%$, in which α is the slope of the final standard curve (Applied Biosystems, 2016).

3.1.3.3.2. TaqMan®-based assay

The parameters evaluated by the absolute standard curve method showed that the real-time PCR TaqMan®-based assay developed had high performance (Table 9) (Table 10) (Figure 4). The assay showed an ample dynamic linear range of detection (Table 9) (Table 10) (Figure 4). It showed high capacity (upper detection limit), sensitivity (detected low nº target copies - lower detection limit) and efficiency (detection of a high percentage of target molecules) (Table 9) (Table 10), as well as the system developed showed accuracy (correlation coefficient (R^2) of the standard curve was >0.99) (Figure 4).

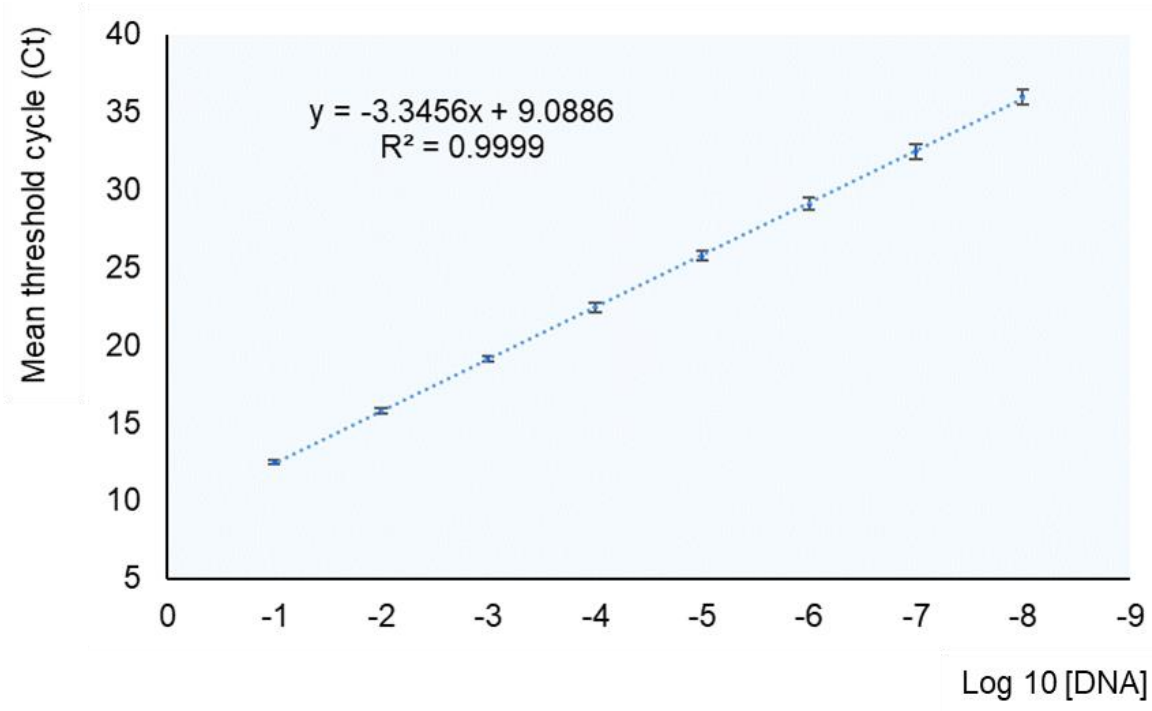

Figure 4. The standard curve used to assess the performance of the quantitative real-time TaqMan®-based assay.

Table 9. The performance parameters based on the dynamic range of the TaqMan®-based assay developed

Dynamic linear range				
Ct [A] Mean	**[DNA]ng/µl**	**P gene**	**Capacity [B]**	**LDL [C]**
(12.49 - 35.98)	(10^{-1} - 10^{-8})	($2X10^{7}$ – 2)	$2X10^{7}$	2

A - The mean threshold cycle (Ct) values range. **B** - The maximum number of P gene copies detected by the assay without losing the efficiency (the upper limit of detection). **C** - Lower detection limit - the minimum number of P gene copies detected by the assay without losing the efficiency.

Table 10. The reproducibility, precision and efficiency of the real-time TaqMan®-based assay developed

	Threshold cycle range		Performance parameters	
	Mean [A]	**SD [B]**	**R^2 [C]**	**Ɛ % [D]**
Intra-assay				
Standard curve 1	(12.59 - 36.47)	0.10 ≤ SD ≤ 0.49	0.999	99.02
Standard curve 2	(12.39 - 35.49)	0.10 ≤ SD ≤ 0.49	0.999	99.02
Standard curve 3	(12.60 - 36.48)	0.11 ≤ SD ≤ 0.50	0.999	99.01
Standard curve 4	(12.38 - 35.48)	0.11 ≤ SD ≤ 0.50	0.999	99.01
Inter-assay				
Final standard curve	(12.49 - 35.98)	0.11 ≤ SD ≤ 0.50	0.999	99.02

A - The mean threshold cycle (Ct) values range. **B** - The range of standard deviation of the mean threshold cycle (Ct) per DNA standard dilution point. **C** - Correlation coefficient of the standard curve produced. **D** - Efficiency was estimated using the formula: $Ɛ = (10^{(-1/-\alpha)} - 1) \times 100\%$, in which α is the slope of the final standard curve (Applied Biosystems, 2016).

3.1.4. Discussion and conclusions

3.1.4.1. SYBR® Green-based assay

The quantitative two-step real-time PCR SYBR® Green-based assay developed showed high performance. The reverse transcription system accurately reverse-transcribed an ample range of input RNA concentration into cDNA (having a dynamic range of 7 orders of magnitude) (Applied Biosystems, 2016). Namely, the dynamic range showed that the reverse transcription chemistry transcribes RNA concentrations between 10^2 ng/µl and 10^{-4} ng/µl. Therefore, the system revealed high capacity (10^2 ng/µl) and high sensitivity (the lower detection limit was 10^{-4} ng/µl) (Applied Biosystems, 2016). Moreover, the reverse transcription system converted 98.92% of target RNA molecules into cDNA along the entire transcript length, (an efficiency close to the theoretical value (100%)) (Applied Biosystems, 2016). Therefore, the evaluation conducted (assessing the parameters such as: orders of magnitude range, capacity, linearity range and efficiency) showed that the established reverse transcription system is not a limiting step in the estimation of the bornavirus RNA quantity, at the second step of the real-time PCR assay developed (Applied Biosystems, 2016).

Additionally, the second step-reaction showed high efficiency (99.42%) and operated at high efficiency between 10^{-1} and 10^{-8} ng/µl of DNA. Consequently, its dynamic range of detection was between $2X10^7$ and 2 copies of the phosphoprotein (P) gene. Also, the high correlation coefficient (R^2 >0.99) of the absolute standard curve showed that the assay accurately estimated the number of phosphoprotein gene copies in samples (Applied Biosystems, 2016). Therefore, the assay developed showed high capacity (upper detection limit) and sensitivity (lower detection limit), accurately estimating high and low amounts of the gene target, respectively (Applied Biosystems, 2016). Additionally, the developed system showed reproducibility; namely, the standard deviation of the cycle threshold means obtained for each of the 10-fold serial dilution points was less than 0.5 within and between experiments (Applied Biosystems, 2016). As a result, the real-time PCR SYBR® Green-based assay allows the achievement of reproducible results (Applied Biosystems, 2006; Applied Biosystems, 2016).

Therefore, the results obtained showed that the produced quantitative real-time PCR SYBR® Green-based assay is an advantageous alternative to both published real-time PCR TaqMan® probe-based assays, used to detect and quantify bornavirus (Schindler AR *et al*.,

2007; Wensman JJ *et al.,* 2007). Firstly, the real-time PCR assays for detection of nucleic acids using intercalary fluorescent dyes (as the SYBR® Green-based assays) reduce assay setup and running costs (once no probe is required) compared with probes-based assays (as the TaqMan®-based assays) (Applied Biosystems, 2016). Therefore, the SYBR® Green-based assay developed is suitable to conduct the screening of large populations. Secondly, the sensitivity of the SYBR® Green-based assay (2 copies of P gene per reaction) showed to be five times higher than the lower detection limit of the two reported TaqMan®-based assays (the lower limit of detection was 10 copies of the targeted gene) (Schindler AR *et al*., 2007; Wensman JJ *et al*., 2007). Thirdly, the commercial systems used to prepare the quantification processes of the real-time PCR assays (Schindler AR *et al*., 2007; Wensman JJ *et al*., 2007) are time-consuming (the preparation required one or more days) and have a short shelf life (because their instability over time) (Applied Biosystems, 2016). However, the *in-house* production of the quantification system used in the SYBR® Green-based assay required only two hours and was stable over time, making this approach less expensive (Applied Biosystems, 2003). Fourthly, a two-step real-time PCR approach is more suitable comparatively with a one-step reaction when specimens are rare (Applied Biosystems, 2016). For instances when samples only can be accessed once or are available in small quantities. Whereby, in clinical practice and academic research, usually, the maximization use of specimens is crucial. Therefore, the two-step real-time PCR assay is suitable for maximizing samples usage, allowing the production of high amounts of cDNA, to conduct multiple tests, and to make cDNA stock from a small amount of RNA (Applied Biosystems, 2016). Also, in the two-step assay, the reverse transcription and the amplification reactions occur in their individually optimized buffers, resulting in the lower detection limit improvement of the assay developed (Applied Biosystems, 2016). Fifthly, the developed diagnostics tool is an advantageous alternative to the histological and immunohistochemistry tests (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2020). Both tests imply collection of tissues by invasive methods and require tissue collection from the central nervous system (in mammalians) (Staeheli P *et al*., 2000; Richt JA *et al*., 2001) or the crop, proventriculus and or ventriculus (in birds) (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2020). Besides, in mammalians, the histological and immunohistochemistry tests are conducted at *post-mortem* (Staeheli P *et al*., 2000; Richt JA *et al*., 2001). In birds, it is possible to do histological tests at *ante-mortem* (Hoppes SM *et al*., 2020), but a challenging approach to perform on live animals. In the literature, there are descriptions of diagnostics tests that detect the infection using specimens (e.g., blood, faeces, urine and oral secretions) collected by non/less-invasive procedures from live animals (Staeheli P *et al*., 2000; Wensman JJ *et al*., 2014; Schlottau K *et al*., 2017; Hoppes SM *et al*., 2020).

There are serologic techniques (e.g., western blotting, enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA)) available, but they produced heterogeneous and unreliable results (Staeheli P *et al*., 2000; Dauphin G *et al*., 2002; Wensman JJ *et al*., 2014; Hoppes SM *et al*., 2020). The viral isolation methods, besides, to be expensive, are laborious and time-consuming (Guo J *et al*., 2014). An accurate quantification of the viral load in samples is challenging to perform with the conventional polymerase chain reaction (PCR) assays. Besides, they are laborious and time-consuming compared to real-time PCR SYBR® Green-based developed diagnostics tools. The expensiveness induce inequality in access to laboratory diagnostics. And the increased response-time by diagnostics tools reduce the chance of interventions in useful-time. Therefore, the assay developed is suitable for the surveillance of captive flocks, aiming to segregate the asymptomatic members, avoiding outbreaks. Thus, the segregation of infected animals allowed the improvement of the health and wellbeing of psittacines housed in breeding projects of rare species, private collections and zoos. The prevention of infection transmission by implementing prophylactic measures in captive flocks is currently the available approach since there are no effective antiviral drugs to treat the infected animals (Hoppes SM *et al*., 2020). For the reasons reported and discussed above, the SYBR® Green-based assay characteristics make it an added value diagnostic tool suitable for large populations' surveillance. In addition to the infection management in captive animals, the SYBR® Green-based assay will be a cost-effective approach in surveillance studies with wild birds' populations.

3.1.4.2. TaqMan®-based assay

The research conducted allowed the development of a cost-effective real-time PCR TaqMan®-based assay. The two-step real-time PCR assay developed to detect and quantify the RNA of bornaviruses in living animals with clinical signs compatible with infection showed high performance. The real-time PCR assay performance resulted from the high performance of the first-step (reverse transcription system) and second-step (detection and quantification system). That is, the established chemistry of the reverse transcription showed: 1) to operate in a wide dynamic range (7 orders of magnitude), 2) to have high capacity (10^2 ng/µl), 3) wide linearity range (RNA concentrations between 10^2 to 10^{-4} ng/µl of RNA were accurately reverse transcribed) and 4) a high efficiency (99.10% of target RNA molecules were reverse transcribed into cDNA along the entire transcript length). Therefore, the established system of the reverse transcription was not a limiting step in the estimation

of the bornavirus RNA quantity at the second step of the real-time PCR assay developed (Applied Biosystems, 2016).

Besides, the second step of the system showed a high performance, the efficiency of the developed TaqMan®-based assay found was 99.02% (Applied Biosystems, 2016). Moreover, the system showed a constant high efficiency throughout a wide dynamic range (within 8 orders of magnitude). Namely, a wide range of input cDNA concentration were detected (between 10^{-1} and 10^{-8} ng/µl). Therefore, the TaqMan®-based assay developed allows to detect and quantify between $2X10^7$ and 2 copies of the P gene, with high efficiency. Consequently, the developed assay allows to efficiently detect and quantify high numbers of the P gene in samples (the capacity was $2X10^7$ copies of the P gene). As well as TaqMan®-based assay showed high sensitivity (the lower detection limit), quantifying a low number of the target (2 copies of the P gene per reaction) in samples. Moreover, the standard deviation of cycle threshold (Ct) means values of each standard dilution showed reproducibility within and between assays (when evaluated 4 and 16 replicates, respectively). Also, the achieved high correlation coefficient (R^2 >0.99) indicated the development of an accurate real-time PCR TaqMan®-based assay (Applied Biosystems, 2016). The developed added value diagnostics tool TaqMan®-based showed to share advantages with the developed SYBR® Green-based assay. The developed added value diagnostics tool showed benefits comparatively with the TaqMan®-based assays, previously published (Schindler AR *et al*., 2007; Wensman JJ *et al*., 2007). In the following paragraphs, in brief, advantages are discussed. Firstly, the assay showed a lower detection limit improved (2 copies of the phosphoprotein gene per reaction). Secondly, it allows accurate quantification of the virus using an approach less expensive and less time-consuming. Once, the quantification system, in previously published assays (Schindler AR *et al*., 2007; Wensman JJ *et al*., 2007), was produced using plasmids or in vitro-transcribed RNA systems (which are more expensive, time-consuming to generate and difficult to maintain accuracy over time due to their instability) (Applied Biosystems, 2016). Thirdly, the developed assay allows for maximizing samples usage, comparatively with the one-step method (Applied Biosystems, 2016). Fourthly, it is more suitable to detect infection in living animals, which showed signs compatible with bornaviruses infection. Once the previously reported approaches in the literature (such as the histological, serological, immunohistochemistry, conventional polymerase chain reaction assays, as well as the viral isolation methods) required tissue samples collected using invasive procedures, or are time-consuming, expensive and produce heterogeneous results (Staeheli P *et al*., 2000; Richt JA *et al*., 2001; Dauphin G *et al*., 2002; Gancz AY *et al*., 2010; Guo J *et al*., 2014; Wensman JJ *et al*.,2014; Hoppes SM *et al*., 2020). Besides, the developed TaqMan®-based assay is

more suitable as a complement tool of clinical diagnosis than the developed SYBR® Green-based assay. Once SYBR® Green-based assays have more chances to detect eventual false-positives targets in the reaction; because the SYBR® dye binds to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA (Applied Biosystems, 2016). Consequently, genotyping the positive result obtained by real-time PCR SYBR® Green-based assays is crucial. Therefore, it implies additional time (which often takes more than a day) to confirm that the PCR products generated are from bornavirus genome and not an artefact. Consequently, the increased response time makes this approach disadvantageous within the scope of clinical diagnosis.

In conclusion, the TaqMan®-based assay developed is a suitable diagnostics tool to complement the clinical diagnosis. Once the neurological and gastrointestinal disorders caused by bornaviruses, in their hosts, are analogous to the clinical signs produced by other infectious agents (Richt JA *et al*., 2001; Dauphin G *et al*., 2002; Shivaprasad HL *et al*., 2012; Dhama K *et al*., 2013; Done LB *et al*., 2014; Guimarães MB *et al*., 2014; Dhama K *et al*., 2015; Mohammad Saleh MS *et al*., 2016). Therefore, the laboratory tests are indispensable to confirm the infection by bornaviruses, to choose the better clinical approach to follow.

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Chapter 4 - The screening of psittacines for bornavirus infection

4.1. The screening of psittacines from breeding projects and zoos

4.1.1. Introduction

The infection by PaBV-1 to 8 usually causes severe effects on psittacines health, mainly the illness known as Proventricular Dilatation Disease (PDD) (Honkavuori KS *et al*., 2008; Kistler AL *et al*., 2008; Weissenböck H *et al*., 2009; Heffels-Redmann U *et al*., 2011; Last RD *et al*., 2012; Philadelpho NA *et al*., 2014). The histopathologic lesions caused by the infection are usually described as: lymphoplasmacytic ganglioneuritis (in the vegetative nerve plexuses of the crop, proventriculus, gizzard, and duodenum), lymphoplasmacytic encephalomyelitis, and neuritis (in peripheral nerves of the myocardium and adrenal glands) (Tizard I *et al*., 2016). Therefore, the location of the tissue lesions define the clinical signs, which include impaired gastrointestinal function (a flaccid and dilated proventriculus unable to digest seeds, and variable distension of the remaining gastrointestinal tract) and disorders of the central nervous system (e.g. “uncoordinated movements, postural disorders, apathy, blindness, and behavioural disorders, such as loss of appetite and self-mutilation”) (Berhane Y *et al*., 2001, Weissenböck H *et al*., 2009; Hoppes SM *et al*., 2013; Tizard I *et al*., 2016). Therefore, the disease is characterized by a severe impairment of the neurologic and gastrointestinal system, leading to death (Tizard I *et al*., 2016).

Thus, the infection becomes relevant in breeding projects of rare species, private collections and zoos because of its repercussions at the economic and biodiversity levels, with consequences at a worldwide dimension. In several countries, the infection by members of the *Psittaciform 1 orthobornavirus* species (parrot bornavirus 1 to 8 (PaBV-1 to 8)) has been reported in captive psittacines (Honkavuori KS *et al*., 2008; Kistler AL *et al*., 2008; Weissenböck H *et al*., 2009; Heffels-Redmann U *et al*., 2011; Last RD *et al*., 2012; Philadelpho NA *et al*., 2014).

In captive flocks, the avian bornaviruses infection may persist for years. One of the proposed explanations for the persistence of the infection in captive contexts for long time was the presence of psittacines that do not develop clinical signs, despite being infected (Gancz AY *et al*., 2010). The occurrence of infection with the absence of clinical picture by psittacines is unusual, and the factors causing these events are still unknown. Consequently, the infected psittacines, apparently healthy, (Gancz AY *et al*., 2010) can be the foci of infection in captive flocks of psittacines, as asymptomatic psittacines can spread the virus releasing it through excretion in faeces, urine and saliva (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013). As the spread of bornaviruses through faeces, urine and saliva

occur intermittently (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013), the surveillance of bornaviruses in captive flocks should be considered to occur at all times (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013).

Effective antiviral drugs to treat infected psittacines are not available, and the vaccines for control are under study (Olbert M *et al*., 2016; Runge S *et al*., 2017; Hameed SS *et al*., 2018). Therefore, the surveillance of captive psittacines aiming to segregate asymptomatic members is essential to avoid the occurrence of outbreaks. Besides, the characterization of bornaviruses that infect psittacines, which do not develop clinical signs, is crucially to improve the knowledge about bornaviruses infection in companion animals.

This study intended to screen captive flocks of healthy psittacines aiming to detect reservoirs and characterize the genetic and proteins profiles of potential bornaviruses; to further evaluate any link between the profiles of the detected bornaviruses and the asymptomatic infections.

4.1.2. Material and methods

4.1.2.1. Samples and data collection

4.1.2.1.1. Animals included in the study

The captive psittacines included in the study were from private breeding projects and a Zoological collection in Portugal. The flocks were from the following Districts: Aveiro, Coimbra, Lisboa and Porto.

The inclusion criteria of psittacines in the study was to be identified as apparently healthy by the veterinarian. The owners gave their informed consent for the inclusion of birds in the study. The samples collected from the birds consisted of excretions (urine and faeces from cloacae), secretions (swabs from oropharynx) and blood (when possible). Additional information was collected by the Veterinarian using a questionnaire designed for the study (appendix, at the section “Epidemiologic questionnaires” of the present Thesis). Briefly, the collected information was on demographic variables, clinical history, clinical signs, the type and the number of the biologic samples.

The transport of biological samples to the laboratory was under refrigerated conditions and stored at -20ºC.

4.1.2.1.2. Positive controls: cell culture and Borna disease virus 1, strain He/80

The preparation of positive controls was according to the procedures established in "*Chapter 3 - Development of real-time PCR assays for bornavirus detection and quantification*" in the present Thesis. Briefly, an inoculum of Borna disease virus 1, strain He/80 (BoDV-He/80), was used to infect Vero cells line persistently. The preparation and expansion of inoculated Vero cells cultures were according to the section *3.1.2.1. Cell culture and Borna disease virus1, strain He/80*.

4.1.2.2. Bornavirus nucleic acid extraction

Blood (peripheral), secretions and excretions (oropharyngeal swabs and cloaca swabs) were evaluated for RNA bornaviruses detection. To perform the extraction of total RNA from samples the methods of organic lysis (phenol/chloroform method) with silica-binding were combined. The extraction of total RNA from samples was with NYZol (NzyThec, Lisbon, Portugal) according to the manufacturer's instructions. Then, RNA solutions extracted were processed using the GeneJET RNA cleanup kit (Thermo Scientific, Lithuania), according to the instructions recommended by the manufacturer. The NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to evaluate the purity and the concentration of total RNA, following the manufacturer's instructions. The RNA solutions with a ratio of absorbance at A260/280nm higher or equal to 2.0 were included in the experiments. The total RNA solutions were maintained in ice if processed immediately or were stored at -20ºC until further processing. Additionally, the production of positive controls was by extracting and preparing total RNA from Vero cells infected with BoDV-He/80 according to the procedures described above.

4.1.2.3. Bornavirus nucleic acid detection

The total RNA extracted from all collected samples was reverse transcribed according to the protocol developed and established in "*Chapter 3 - Development of real-time PCR assays for bornavirus detection and quantification*" in the present Thesis. Briefly, the reverse transcription of the total RNA was by using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Lithuania), according to the instructions of the manufacturer. The cDNA

produced was used to prepare pools containing cDNA synthesized from different specimens of the same psittacine.

Two microliters of the cDNA produced were evaluated by real-time polymerase chain reaction (real-time PCR), using the SYBR® Green-based assay developed (according to *Chapter 3 - Development of real-time PCR assays for bornavirus detection and quantification*). Briefly, each reaction was carried out in a total volume of 25 µl, containing 1X SYBR® Green PCR Master Mix (Applied Biosystems) and 0.3 µM of each primer. The thermal cycling parameters of real-time PCR were the first step at 95ºC for 10 min and 40 cycles during each round of the second step at 95°C for 15 s and the third step at 60°C for 1 min (data collected on hold). The thermal cycling parameters of the melt curve protocol was 95ºC for 15 s, 60ºC for 1 min (with a ramping time of 1.6ºC) and 95ºC for 15 s (with a ramping time of 0.15ºC/s). The performing analyses were in a One-Step Plus thermal cycler (Applied Biosystems). The establishment of the threshold was in the exponential phase of the reactions, and the cut-off for the cycle threshold (Ct) values was 40 cycles. Consequently, a result was defined as positive when, at least, one of the four tests replicates had a Ct value less than or equal to 40 cycles for each sample.

The reverse transcription and the real-time PCR protocols above described were also used to produce cDNA and conduct analysis of positive controls, respectively.

4.1.3. Results

4.1.3.1. Samples and data collected

The study included 300 psittacines, 33.33% (n=100) were from a zoological collection in the District of Porto, and 66.67% (n=200) of birds were from private breeding projects at the Districts of Aveiro, Coimbra and Lisboa. From the psittacines of the zoo, 250 hundred samples were collected and evaluated. Forty % of samples were cloaca swabs (n=100), 40% were oropharyngeal swabs (n=100), and 20% were blood (n=50)). From the psittacines of the breeding projects, 400 samples were collected and evaluated. Fifty % of samples were cloaca swabs (n=200) and 50% were oropharyngeal swabs (n=200).

4.1.3.2. Bornavirus nucleic acid detection

All tested samples (n=650) were shown to be negative as they did not produce nucleic acid amplification signals during the 40 cycles (the cut-off for the cycle threshold (Ct) values) of the real-time PCR SYBR® Green-based assay developed.

4.1.4. Discussion and conclusions

All samples evaluated by real-time PCR SYBR® Green-based assay were negative for the presence of bornaviruses RNA. However, this study may have had weaknesses that could have introduced a bias in the results obtained, namely that the samples collection from each bird was done once in time (blood, swab and oropharyngeal swabs). According to published studies, the virus release through secretions or excretions may be intermittent (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013), what may suggest that there were some infected birds, which at the time of sampling were not releasing viruses through secretions (saliva) or excretions (urine and faeces), in this study. Therefore, surveillance of captive flocks must involve a plan, for collecting several types of samples, from each bird over some time but, the ideal frequency and time range to perform the sampling was undefined in the literature (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013). However, based on the Veterinarians experience that contributed to the study, the sampling process is stressful for psittacines. For the reasons above described, it will be useful to conduct studies in future to produce evidence that allows the establishment of a standard protocol with the ideal frequency and time range of sampling. Therefore, the collecting process of specimens in the flocks included in the study is done once a year for monitoring the animal health status. However, due to the unavailability of financial resources the follow-up of the birds included in the study was not possible.

Usually, the infection by bornavirus do not induce viremia, and commonly the infected animals do not have the virus in their blood (Guo J *et al*., 2014). If the virus eventually infects the blood tissue, it is expected to be at low concentrations, making the detection of viral RNA difficult (Guo J *et al*., 2014). On the other hand, the eventual detection of high concentrations of the virus in the bloodstream is most likely to occur at the late stage of the disease, when almost certainly the psittacine will show clinical signs (Guo J *et al*., 2014). Consequently, blood is not a sample of choice for virus nucleic acid screening aiming to segregate asymptomatic psittacines in captive flocks to avoid the occurrence of outbreaks.

However, by the end of the studies within the present Thesis, we found that all the parrots evaluated remained healthy, without clinical signs compatible or suggestive of infection by bornaviruses. From the time they tested negative, psittacines remained without clinical signs for about 4 years (the end of the research studies within the present Thesis). Therefore, the most plausible explanation of the negative results was that the captive flocks of psittacines were free of parrot bornavirus (PaBV-1 to 8). Otherwise, some of the

psittacines would have shown clinical signs compatible with infection by bornaviruses within 1 to 4 years after the screening performed.

Due to the results obtained in the first objective of the study it was not possible to further proceed with the other objective namely, to study a possible a link between genetic and proteins profiles of detected bornaviruses and the occurrence of asymptomatic infections.

It is essential to conduct future cross-sectional studies to gather preliminary data in an inexpensive and fast way, which may allow the directing or redirecting of further research aiming to improve the knowledge of bornaviruses infection in companion animals.

4.1.5. References

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Chapter 5 - Studies on bornavirus infection in psittacines in Sweden

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RESEARCH ARTICLE

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Detection and phylogenetic analysis of parrot bornavirus 4 identified from a Swedish Blue-winged macaw (*Primolius maracana*) with unusual nonsuppurative myositis

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ABSTRACT

Background: The genus Orthobornavirus comprises RNA viruses infecting humans, mammals, birds and reptiles, where parrot bornavirus 1 to 8 causes fatal neurological and/or gastrointestinal syndromes in psittacines. There is, to the best of our knowledge, no publication describing avian bornaviruses in pet parrots in Sweden. We aimed to identify and to produce epidemiologic knowledge about the etiologic agent associated with a history of severe weight loss and death of a Primolius maracana.

Methods and results: The results of histopathology, immunohistochemistry and real-time RT-PCR were compatible with avian bornavirus infection. Sequencing indicated infection by parrot bornavirus 4 (PaBV-4). The genotype reported shared high identity with PaBV-4 identified from pet psittacines and from wild birds in several countries. The N gene and X protein showed genotype clusters formation. P protein revealed to be more conserved within and between species of bornaviruses. Findings suggest horizontal transmission within and between avian orders and species.

Conclusion: There seems to be a worldwide trading without biosafety measures, hence, further disease transmission could be avoided. For screening purposes, the P gene is a good candidate as a universal target in molecular diagnostics. Wild birds may be key pieces in the puzzle of bornavirus epidemiology.

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5.1. Detection and phylogenetic analysis of parrot bornavirus 4 identified from a Swedish Blue-winged macaw (*Primolius maracana*) (manuscript I)

5.1.1. Introduction

Bornaviruses are enveloped, 80 to 100 nm in diameter with a non-segmented genome of single-stranded negative sense RNA of around 8900 nucleotides in length (Tizard, I *et al*., 2016). In captive psittacines, parrot bornavirus 1 to 8 cause proventricular dilatation disease (PDD) (Tizard, I *et al*., 2016), a chronic and fatal disease characterized by a flaccid and dilated proventriculus impacted with feeds, and variable distension of the remaining gastrointestinal tract (Payne, S L *et al*., 2012). The infection has become relevant in the context of psittacines housed in reserves, in breeding projects of rare species, in private collections and zoos.

In Sweden, there is extensive research published regarding mammalian bornaviruses (Lundgren AL *et al*., 1992; Lundgren AL *et al*., 1995; Berg AL *et al*., 1998; Dörries R *et al*., 1999; Degiorgis MP *et al*., 2000; Berg M *et al*., 2001; Johansson M *et al*., 2002; Wensman JJ *et al*., 2007; Wensman JJ *et al*., 2011; Wensman JJ *et al*., 2012). However, to date, there is to the best of our knowledge, no publication that identified and characterized avian bornaviruses infecting pet parrots in Sweden.

We aimed to identify and to produce epidemiologic knowledge about the etiologic agent associated with a history of severe weight loss and death of a *Primolius maracana*.

5.1.2. Material and methods

5.1.2.1. Case history

A female *Primolius maracana* died after a history of severe weight loss and was submitted a complete standardised necropsy to the Department of Pathology and Wildlife Diseases, National Veterinary Institute, Uppsala, Sweden. The macroscopic findings reported, by an experienced pathologist, during the necropsy were strongly compatible with avian bornaviruses infection. Therefore, our approach was directed to search for the presence of avian bornaviruses. Tissue samples (adrenal gland, brain, cecum, colon, crop, heart, ileum, jejunum, kidneys, liver, lung, ovary, proventriculus, tongue and trachea) were

formalin-fixed in 10% buffered formaldehyde, and a selection of tissues (brain, cecum, colon, ileum, jejunum, kidney and lung) were stored at -80°C.

5.1.2.2. Histopathology and immunohistochemistry

The formalin-fixed samples were stained with haematoxylin and eosin (HE). Immunohistochemistry to identify T-cells (polyclonal rabbit Anti-human CD3, DAKO, A00452, dilution 1:20) and B-cells (monoclonal rabbit Anti-human CD79a, Thermo Scientific, RM-9118, dilution 1:20) was performed on paraffin-embedded sections, which were mounted on Vectabond (Vector Laboratories, Inc., Burlingame, CA) treated glass slides, deparaffinised in xylene and rehydrated. After inhibition of endogenous peroxidase activity with 3% hydrogen peroxide for 20 min, heat-induced epitope retrieval followed. Unspecific antigen staining was blocked with 2% bovine serum albumin for 20 min prior to incubation with the primary antibodies at room temperature for 45 min. The detection was conducted with the dextran polymer method (DAKO EnVisionTM+/HRP, DakoCytomation), the colour was developed with 3,3'-Diaminobenzidine (DAB) substrate and followed by counterstained with haematoxylin.

5.1.2.3. Real-time polymerase chain reaction (PCR) and conventional PCR

From stored samples at -80ºC, total RNA was extracted as previously described (Wensman JJ *et al*., 2012) and Qubit® RNA BR Assay kit was used to estimate concentration (ThermoFisher Scientific/Life Technologies), according to the manufacturer's instructions. Five-hundred micrograms were reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific), with random hexamer primers, according to the manufacturer's instructions. Real-time PCR was carried out using previously reported primers (Wensman JJ *et al*., 2007; Wensman JJ *et al*., 2012) and the Power SYBR® Green Master Mix (Applied Biosystems), according to the manufacturer's instructions. Conventional PCR was conducted using primers previously described (Weissenböck H *et al*., 2009), (targeting the N (forward sense) and M (reverse sense) genes) and the Dream Taq Green Master Mix (Thermo Scientific), according to the manufacturer's instructions. The products were visualized using a 2% agarose gel, purified with the Gene Jet Gel Extraction Kit (Thermo Scientific) and analysed by Sanger sequencing (Macrogen Europe). The sequences obtained were submitted to GenBank® database under accession number MK192982.1.

5.1.2.4. Identification and phylogenetic analysis

Phylogenetic analyses were conducted based on genes (N, X, P and M) and proteins (X and P) from bornaviruses reported in GenBank®. Strains with a nucleotide identity ≥ 98%, were included into the same cluster and the relationship between each cluster and the host species, the time of sampling and the country origin of cases was evaluated. We used BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find regions of similarity between sequences obtained and the GenBank®, as to estimate the percent identity (id%) and E-values. Analyses were conducted in MEGA7 software (Kumar S *et al*., 2016).

5.1.3. Results

5.1.3.1. Necropsy, histopathology, immunohistochemistry, real-time and conventional PCR

The results of necropsy showed a reduced muscle mass, a severely dilated intestine and the liver and spleen were atrophic. The histopathological examination of the crop, proventriculus, gizzard and small intestine showed multifocal, mild to moderate lymphoplasmacytic intra- and perineural infiltrates (Figure 1 (a), Figure 1 (b)). There were multifocal, moderate to severe lymphoplasmacytic infiltrates in the muscularis layers of these organs (Figure 1 (c), Figure 1 (d), Figure 1 (e) and Figure (F)) associated with mild to moderate degeneration of smooth muscle cells seen as fractured cells, cell debris, pyknosis and granulated cytoplasm. In the cerebrum and cerebellum there were multifocal, mild to moderate, perivascular lymphoplasmacytic infiltrates and large areas of mild, diffuse gliosis in the white mater (Figure 2). Leucocytic infiltrates were also seen in the adrenal glands (not shown). In the other organs examined, no significant lesions were seen. Immunohistochemical detection of T- and B-cells showed that most leukocytes in the cerebrum (Figure 3) and other organs (not shown) were CD3-positive, i.e., T-cells. The samples of brain and lungs were positive by real-time PCR, and fragments of 1600 bp were obtained by conventional PCR and sequencing revealed a parrot bornavirus 4 (PaBV-4).

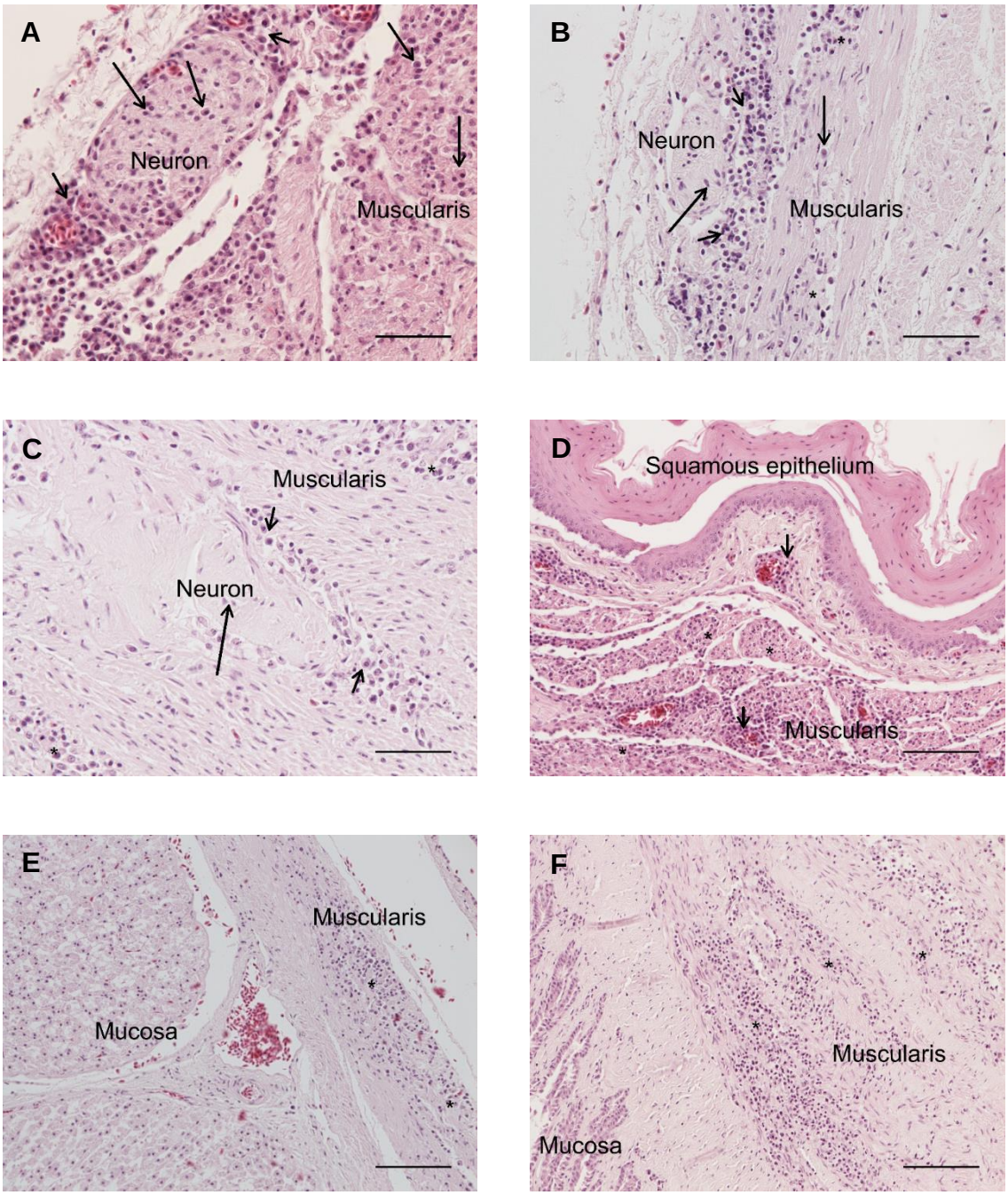

Figure 1. Photomicrographs of HE-stained sections. Bar = 50 µm (A, B, C) or 100 µm (D, E, F). Short arrows= perineural or perivascular lymphoplasmacytic infiltrates, long arrows = lymphoplasmacytic cells, * = diffuse intramuscular infiltrates. Lymphoplasmacytic neuritis, perineuritis and myositis in crop (A), proventriculus (B) and gizzard (C). Lymphoplasmacytic myositis in crop (D), proventriculus (E) and gizzard (F).

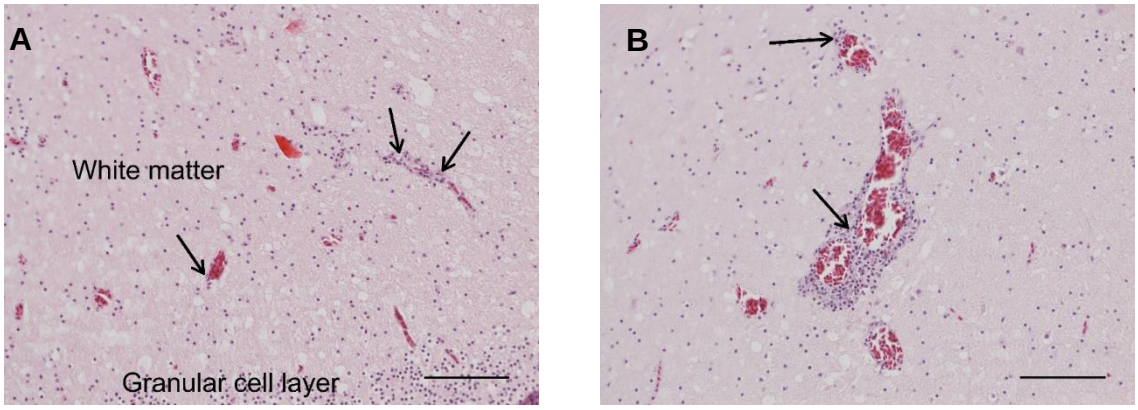

Figure 2. Photomicrographs of HE-stained sections, bar= 100 µm. Perivascular lymphoplasmacytic infiltrates and diffuse gliosis in the white matter of the cerebellum (A) and cerebrum (B).

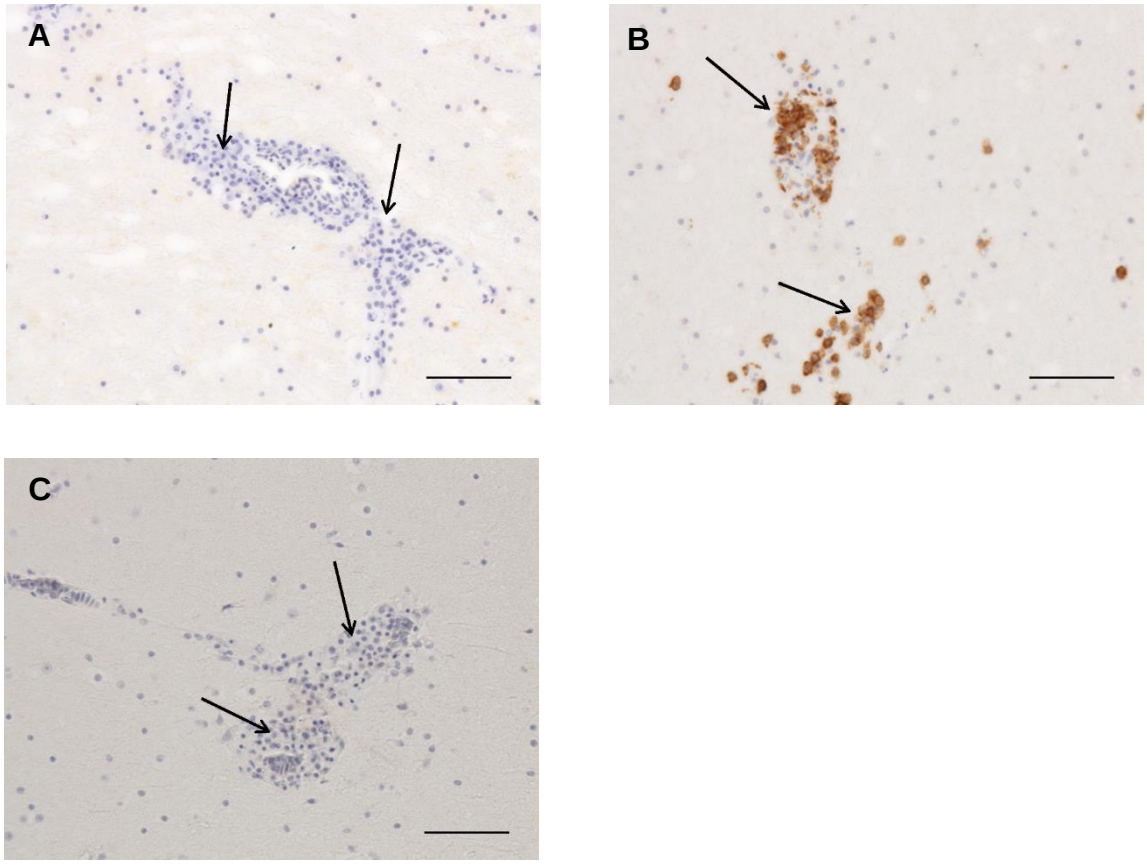

Figure 3. Photomicrographs of IHC to identify T and B cells. bar = 100 µm. Arrows = perivascular lymphoplasmacytic infiltrates. Cerebrum, negative control (A). Cerebrum, CD3-staining, numerous strongly positive (brown) cells (B). Cerebrum, CD79-staining, no positive cells (C).

5.1.3.2. Identification and phylogenetic analysis

Analysis based on N, X, P and M genes revealed that PaBV-4 identified in the present study shared a higher percent identity with *Mammalian 2 orthobornavirus* than with some *Waterbird 1 orthobornavirus* (Figure 4) (Table 1).

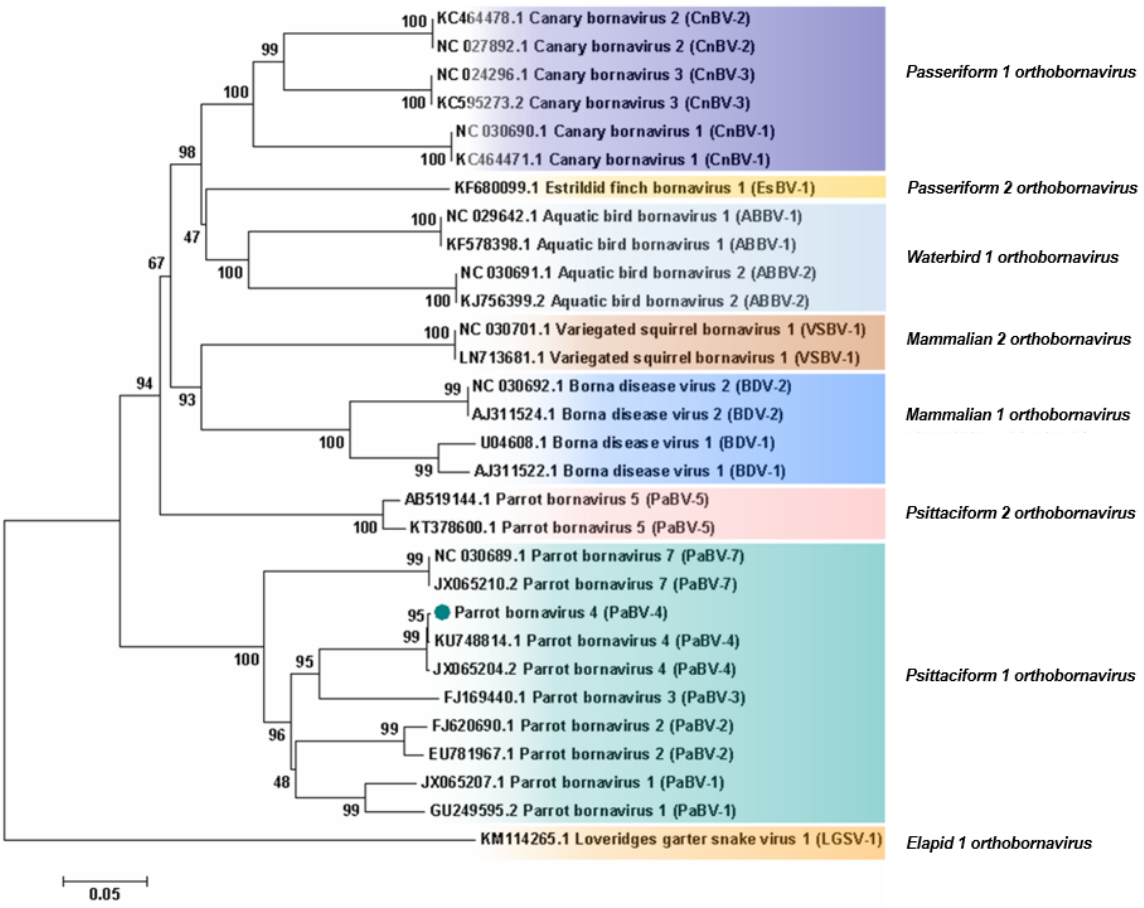

Figure 4. Phylogenetic relationships between the genotype identified in the present study and the selected genotypes from each bornavirus species, regarding the N, X, P and M genes sequences. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 30 nucleotide sequences. There were a total of 1527 bases in the final dataset. Evolutionary analyses were conducted in MEGA7. Sequences identified by GenBank® accession numbers, name of virus and its abbreviation name. The sequence marked with a circle was produced during this study.

Table 1. Identity between PaBV-4 reported and members of genus *Orthobornavirus*.

Bornaviruses	PaBV-4 identified in the present study					
	Genes		Proteins			
	"N", "X", "P", "M"		X protein		Phosphoprotein	
	Id. (%) [A]	E-value [B]	Id. (%) [A]	E-value [B]	Id. (%) [A]	E-value [B]
Avian [C]						
Psittaciform 1 orthobornavirus	82–99	0	75–100	$2e^{-63}$–$3e^{-37}$	94–100	$3e^{-148}$–$8e^{-82}$
Psittaciform 2 orthobornavirus	71–72	0	53	$1e^{-28}$–$1e^{-27}$	69	$6e^{-87}$
Passeriform 1 orthobornavirus	70–71	0	51–57	$4e^{-31}$–$3e^{-27}$	68–70	$5e^{-96}$–$8e^{-96}$
Passeriform 2 orthobornavirus	71	0	63	$3e^{-36}$	70	$8e^{-82}$
Waterbird 1 orthobornavirus	69–71	0–$6e^{-179}$	54–56	$9e^{-30}$–$4e^{-27}$	68–69	$4e^{-85}$–$3e^{-83}$
Mammalian [D]						
Mammalian 1 orthobornavirus	69	$2e^{-153}$–$9e^{-139}$	45–49	$5e^{-23}$–$3e^{-22}$	62–64	$4e^{-95}$–$6e^{-93}$
Mammalian 2 orthobornavirus	70	$5e^{-161}$	52	$1e^{-25}$	69	$1e^{-95}$
Reptilian [E]						
Elapid 1 orthobornavirus	68	$6e^{-31}$	37	$1e^{-8}$	43	$1e^{-50}$
Gaboon viper virus 1 [f]	---	---	---	---	43	$1e^{-45}$

A - The highest percent identity of all query-subject alignment given by Basic Local Alignment Search Tool (BLAST®). **B** - The best (lowest) Expect value (E-value) of all alignments from that database sequence given by BLAST®. **C** - Avian bornaviruses sequences considered in the analysis were the same used to construct the phylogenetic trees. **D** - Mammalian bornaviruses sequences considered in the analysis were the same used to construct the phylogenetic trees. **E** - Reptilian bornaviruses sequences considered in the analysis were the same used to construct the phylogenetic trees. **F** - The database of the GenBank® only reported information for the P gene/protein.

Analysis, based on P protein showed that PaBV-4 was more related with *Mammalian 2 orthobornavirus* than with some *Waterbird 1 orthobornavirus* and *Passeriformes 1 orthobornavirus* (Figure 5) (Table 1). The reptilian bornaviruses remain the most divergent (Figure 5) (Table 1).

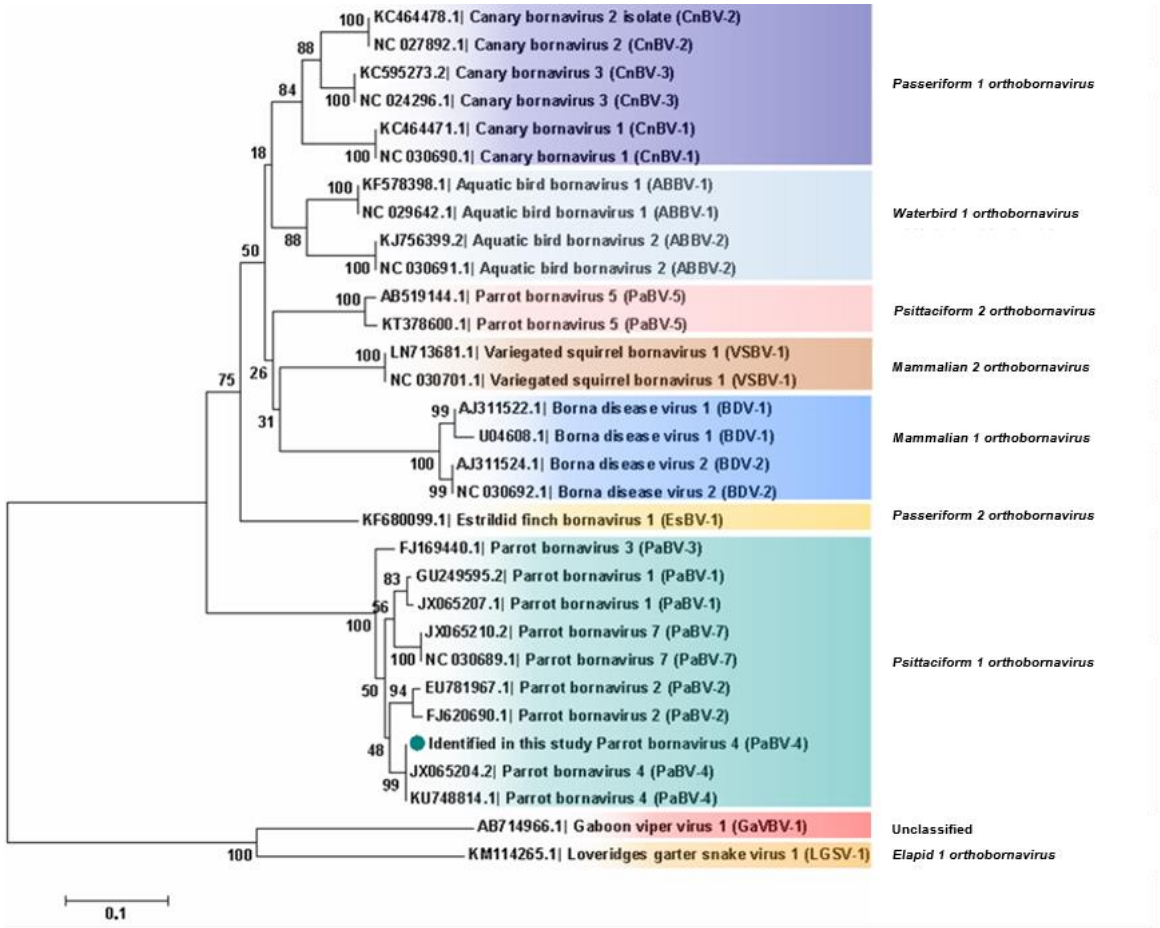

Figure 5. Phylogenetic relationships between the genotype identified in the present study and the selected genotypes from each bornavirus species, regarding the complete P protein sequences. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 31 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 192 amino acids in the final dataset. Evolutionary analyses were conducted in MEGA7. Sequences identified by GenBank® accession numbers, name of virus and its abbreviation name. The sequence marked with a circle was produced during this study.

When analysis was based on X protein, similar phylogenetic relationship was found, however, the X protein showed lower id% than the P protein (Table 1). Therefore, phylogenetic outcomes based on protein profiles indicate the possibility of cross-reactivity between antigens of avian bornaviruses and antibodies induced by mammalian bornaviruses. A published experimental study reported that polyclonal anti-BoDV-N and anti-BoDV-P antisera cross-react with ABV particles (Herzog S *et al*., 2010).

When analysis was restricted to PaBV-4 N gene, 16 sequences from Japan were excluded, avoiding bias introduction, due to the small size of the sequence in the final dataset. Therefore, 93 partial PaBV-4 N gene sequences analysed were divided in 5 genotype clusters (Table 2) (Figure 6). One sequence shared <97% of identity and was not included in the clusters (Figure 6) (Table 2). Analysis based on P protein showed absence of clusters, while based on X protein formed 4 clusters (Figure 7) (Table 2). One sequence was not included in any cluster because it shared <97% (Figure 6); however, according to the analysis based on the N gene, it was reported to belong to cluster 3 (Figure 7). In genetic and protein approach there was a homogeneous distribution of host species, sampling time and geographic origin of cases, by clusters (Figure 6 and 7).

Table 2. Identity between parrot bornavirus 4 (PaBV-4) worldwide distributed.

	Gene			Proteins					
	"N"			X protein			Phosphoprotein		
	Id. (%)[A]	E-value [B]	n [C]	Id. (%)[A]	E-value [B]	n [c]	Id. (%)[A]	E-value [B]	n [C]
PaBV-4 worldwide distributed [D]									
All sequences	95–100	$4e^{-51}$–$1e^{-48}$	93	93–100	$4e^{-51}$–$1e^{-48}$	23	99–100	$4e^{-51}$–$1e^{-48}$	23
Between clusters of PaBV-4									
Cluster 2 vs. 1	97	0–$1e^{-147}$	--	97–98	$1e^{-62}$–$1e^{-62}$	--	--	--	--
Cluster 3 vs. 1	96–97	0–$3e^{-149}$	--	95	$2e^{-52}$	--	--	--	--
Cluster 4 vs. 1	96	0	--	93–94	$4e^{-51}$–$1e^{-48}$	--	--	--	--
Cluster 5 vs. 1	95–97	0–$4e^{-152}$	--	--	--	--	--	--	--
Within PaBV-4 clusters									
Cluster 1	99–100	0–$1e^{-162}$	48	99–100	$2e^{-63}$–$3e^{-62}$	13	--	--	--
Cluster 2	99	0	4	100	$2e^{-63}$	2	--	--	--
Cluster 3	99–100	0–$7e^{-180}$	18	100	$2e^{-63}$	3	--	--	--
Cluster 4	99	0	4	100	$3e^{-63}$–$9e^{-61}$	4	--	--	--
Cluster 5 [E]	98–99	0–$8e^{-179}$	18	--	--	--	--	--	--

A - The highest percent identity of all query-subject alignment given by Basic Local Alignment Search Tool (BLAST®). **B** - The best (lowest) Expect value (E-value) of all alignments from that database sequence given by BLAST®. **C** - The sample size of each cluster. **D** - Several parrot bornavirus 4 (PaBV-4), available from GenBank®, with complete sequences on X and P proteins were the same used to construct the phylogenetic trees. **E** - The cluster 5 was formed only when the analysis was based on a segment of the N gene.

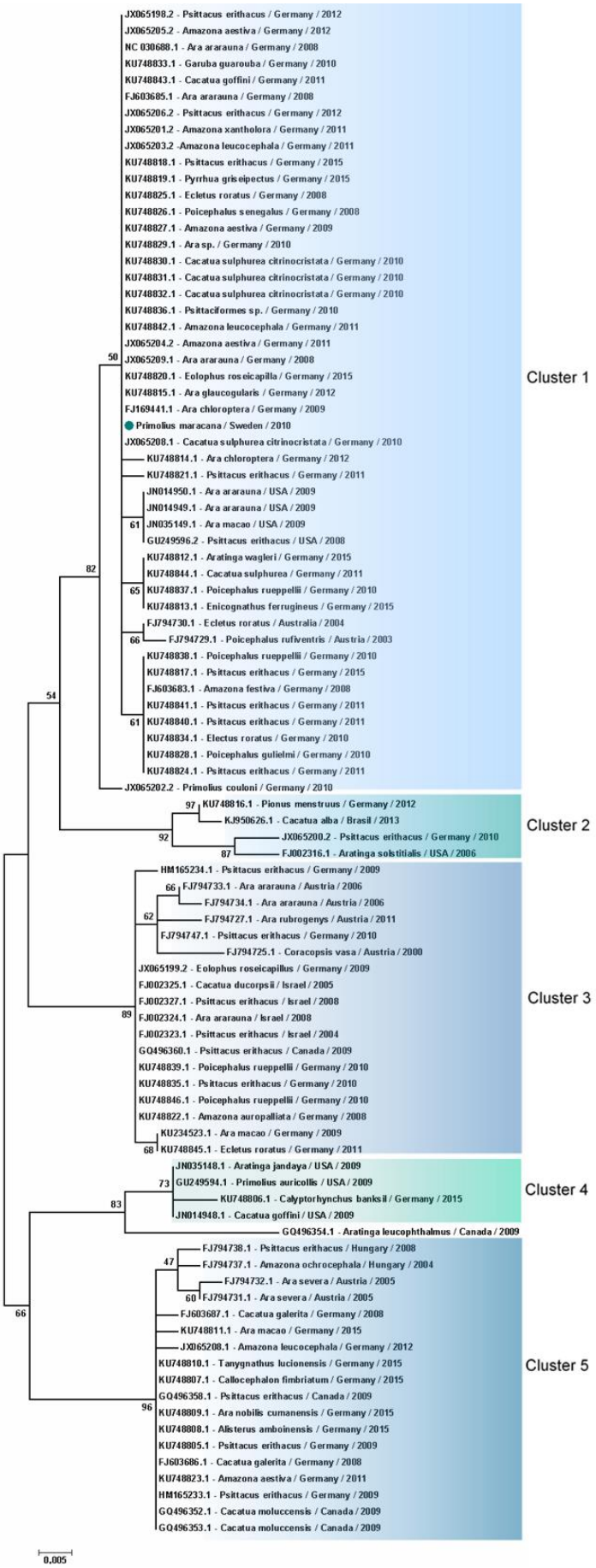

Figure 6. Clusters of PaBV-4 N nucleotide sequences identified from *Psittaciformes*.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There was a total of 305 bases in the final dataset. Evolutionary analyses were conducted in MEGA7. PaBV-4 sequences are identified with GenBank® accession numbers, with the name of the host in Latin, country origin and time of sampling. The sequence marked with a circle was produced during this study.

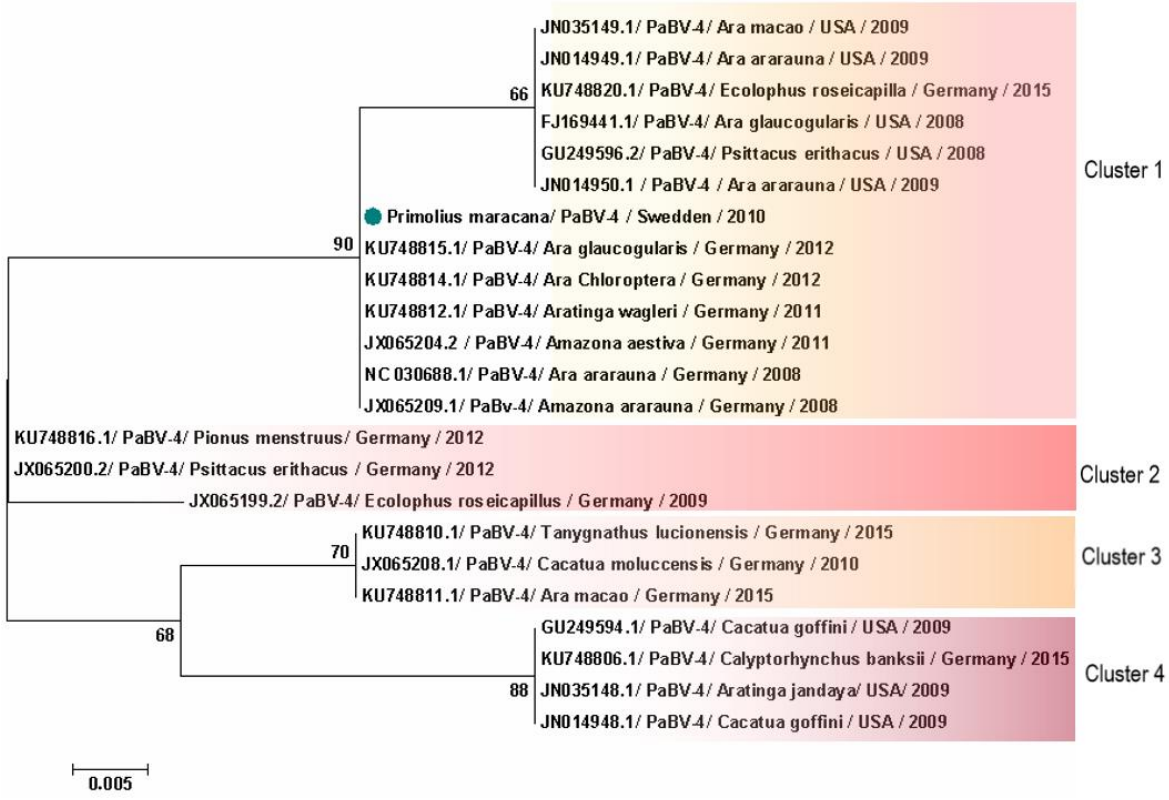

Figure 7. Clusters of PaBV-4 X protein sequences identified from *Psittaciformes*. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 23 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 85 amino acids in the final dataset. Evolutionary analyses were conducted in MEGA7. PaBV-4 sequences are identified with GenBank® accession numbers, with the name of the host in Latin, country origin and time of sampling. Sequences identified by GenBank® accession numbers, name of virus and its abbreviation name. The sequence marked with a circle was produced during this study.

5.1.4. Discussion and conclusions

This is the first published report that identifies and phylogenetically characterizes an avian bornavirus infecting a pet parrot in Sweden. The PaBV-4 identified in the present study is more related with the zoonotic *Mammalian 2 orthobornavirus* than with some avian bornaviruses.

We have not searched for other viruses, however, during the standardised necropsy the macroscopic findings observed were strongly compatible with infection by avian bornaviruses. Lymphoplasmacytic ganglioneuritis and mild encephalitis were observed during histopathological and immunohistochemistry examinations. This is like the gastrointestinal lesions seen in experimentally infected cockatiels, which had lymphoplasmacytic infiltrates in the tunica muscularis of the ventricles and mild encephalitis (Payne S *et al*., 2011). Moreover, in a published study, cockatiels were experimentally infected with a virulent PaBV-4 strain, and the authors propose that this may have contributed to the increased immune response and inflammation seen in these animals (Payne S *et al*., 2011). A similar pathogenesis is therefore possible in the presented case, since the Swedish bird, additionally, developed an unusual presentation characterized by a severe lymphoplasmacytic myositis in the gastrointestinal tract. However, the experimentally infected cockatiels did not have myocyte degeneration, and much less involvement of the remaining gastrointestinal tract, as compared to the current case. In mammals, both microglial activation and CD8+ T cells contribute to the development of brain lesions (Stitz L *et al*., 1992; Lundgren AL *et al*., 1995; Ovanesov MV *et al*., 2007; Ovanesov MV *et al*., 2008), which implies that a type IV hypersensitivity reaction is occurring, as previously suggested (Payne S *et al*., 2011). The dominant T cell inflammation seen in all lesions in the current case supports this suggestion.

Phylogenetic analyses based on N gene and X protein, showed that PaBV-4 clusters are not linked with geographic origin, host species and time of sampling of cases, since there is a homogeneous distribution of variables by cluster. Regarding P protein, no clusters were found, also it revealed to be more conserved within all *orthobornavirus* species, than X protein. Similar findings were reported for N gene by a previous study (Rubbenstroth D *et al*., 2016) however, our approach was more comprehensive. Additionally, phylogenetic analyses suggest horizontal transmission within and between avian orders and species (Figure 6 and Figure 7). The exact route of horizontal transmission and risk factors remain controversial (Rubbenstroth D *et al*., 2014), more studies are needed to fill this gap in knowledge, which will allow to establish preventive measures to manage the outbreaks.

Despite not meeting the criteria to be included in phylogenetic analysis, we evaluated PaBV-4 from captive psittacines of South Africa (accession no.: FJ002341, FJ002330 and GQ496351), Italy (accession no.: HM565487), Spain (accession no.: HQ728250 and HQ728251) and Japan (accession no.: AB744683 and AB744682), as well as from wild birds of Japan (accession no.: AB842432, AB842437, AB842439 to AB842441, AB842450 to AB842455, AB842464, AB842466, and AB842476 to AB842478). Therefore, the PaBV-4 identified in the present study shared a high genetic similarity with genotypes found in

captive psittacines (id%=93-100; E-value=0–$7e^{-82}$) and in wild birds (id%=80-98%; E-value= $9e^{-81}$–$5e^{-40}$). The findings suggest that wild birds could play a role in introduction of infection in captive flocks. A Swedish study suggested that wild birds are possible reservoirs of Borna disease virus 1 (Berg M *et al*., 2001), with recently recognised zoonotic potential (ECDC, 2018). Therefore, birds could favour the emergence of novel strains, since they are reservoirs of avian and mammalian bornaviruses.

In conclusion, this study describes the first PaBV-4 in Sweden, a virus with worldwide distribution in captive psittacines, because of extensive trading without biosafety measures. Preventive measures are required, to improve the management of outbreaks, which have severe impacts for zoos, breeding projects of rare species and psittacines trade. Wild birds can be key pieces in the puzzle of bornavirus epidemiology. For screening purposes, the P gene is a suitable candidate as universal target in molecular diagnostics.

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Chapter 6 - Studies on bornavirus infection in psittacines in Portugal

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Bornaviruses in naturally infected *Psittacus erithacus* in Portugal: insights of molecular epidemiology and ecology

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ABSTRACT

Background: The genus *Orthobornavirus* comprises non-segmented, negative-stranded RNA viruses able to infect humans, mammals, reptiles and various birds. Parrot bornavirus 1 to 8 (PaBV-1 to 8) causes neurological and/or gastrointestinal syndromes and death on psittacines. We aimed to identify and to produce epidemiologic knowledge about the etiologic agent associated with a death of two female *Psittacus erithacus* (grey parrot).

Methods and Results: Both parrots were submitted for a complete standardised necropsy. Tissue samples were analysed by PCR. The findings in necropsy were compatible with bornavirus infection. Analysis revealed PaBV-4 related with genotypes detected in captive and in wild birds. The N and X proteins of PaBV-4 were more related to avian bornaviruses, while phosphoprotein was more related to variegated squirrel bornavirus 1 (VSBV-1). Within the P gene/phosphoprotein a highly conserved region between and within bornavirus species was found.

Conclusions: Portugal is on the routes of the intensive world trade of psittacines. Broad screening studies are required to help understanding the role of wild birds in the emergence and spread of pathogenic bornaviruses. PaBV-4 phosphoprotein is closer to VSBV-1 associated with lethal encephalitis in humans than with some of the avian bornaviruses. The highly conserved P gene/phosphoprotein region is a good target for molecular diagnostics screenings.

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6.1. Bornaviruses in naturally infected *Psittacus erithacus* in Portugal: insights of molecular epidemiology and ecology (manuscript II)

6.1.1. Introduction

Bornaviruses are enveloped, 80 to 100 nm in diameter with a non-segmented genome of single-stranded negative sense RNA of around 8900 nucleotides in length, belonging to the order *Mononegavirales* (Lipkin WI *et al*., 2011).

Bornaviruses replicate in the nucleus of the nerve cells of various organs and establish persistent, non-cytolytic infections by exploiting the cellular splicing mechanisms to efficiently use its genome, organized into six open reading frames (ORFs) (Lipkin WI *et al*., 2011) (Figure 1). Alternative transcription *start* and *stop* sites and splicing produce mRNAs that are translated to produce the viral encoded proteins (Lipkin WI *et al*., 2011) (Figure 1). The first transcription unit contains an ORF for the nucleoprotein (N), the second transcription unit contains two overlapping ORFs for the phosphoprotein (P), and the X protein (X) (Lipkin WI *et al*., 2011) (Figure 1). The third transcription unit is spliced differently, and has different transcription initiation and termination signals, enabling polymerase read-through during transcription, which results in expression of the matrix protein (M), the glycoprotein (G), and the RNA-dependent RNA-polymerase (L) (Lipkin WI *et al*., 2011) (Figure 1). The P and X ORFs overlap; as well as the M and G ORFs (Lipkin WI *et al*., 2011) (Figure 1).

The immunogenicity of phosphoprotein (de Araujo JL *et al*., 2019), as well as the degree of conservation of the phosphoprotein and its gene, within and between bornavirus species, make them good candidates as universal targets in laboratory diagnosis (Pinto MC *et al*., 2018). The family *Bornaviridae* is expanding speedily (Amarasinghe G K *et al*., 2019), producing knowledge about highly conserved regions within phosphoprotein will be useful for the development of sensitivity laboratory diagnostic tools.

So far, genus *Carbovirus*, *Cultervirus* and *Orthobornavirus* have been identified, which comprise 11 species (Amarasinghe G K *et al*., 2019). From those, 10 species are associated with the development of severe neurological and/or gastrointestinal disease and death of its hosts (Malkinson M *et al.,* 1993; Berg A-L *et al.,* 1998; Berg AL *et al.,* 1999; Wensman JJ *et al.,* 2012; Björnsdóttir S *et al.,* 2013; Delnatte P *et al.,* 2013; Guo J *et al.,* 2014; Wensman JJ *et al.*, 2014; Ando T *et al.,* 2016; Thomsen AF *et al.,* 2015; Hyndman TH *et al.,* 2018; Tappe D *et al*., 2019) (Figure 2).

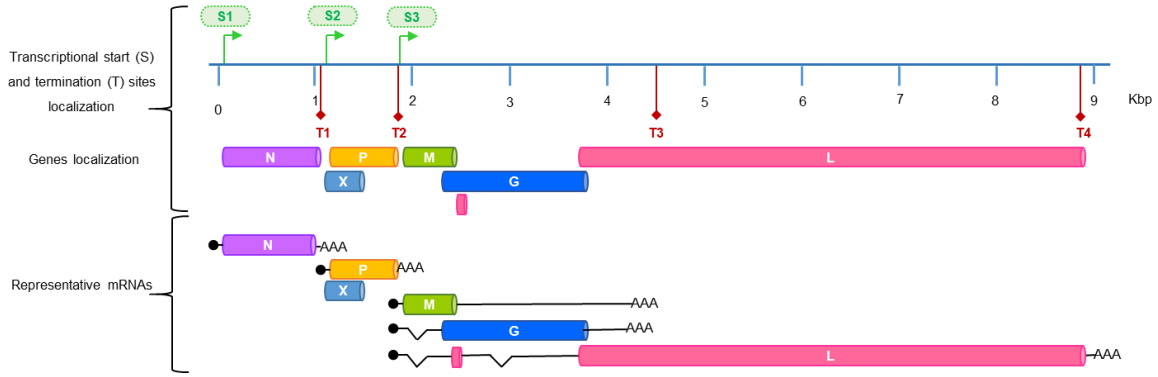

Figure 1. Borna disease virus 1 (BoDV-1) genomic map and protein-coding mRNA transcripts. BoDV uses alternative transcription strategies, like over-lapping open reading frames (ORFs) and the host cellular splicing mechanisms. Abbreviations: S1–S3 initiation sites of transcription; T1–T4 termination sites of transcription; (N) nucleoprotein gene; (X) X protein gene; (P) phosphoprotein gene; (M) matrix protein gene; (G) glycoprotein gene; (L) RNA-dependent RNA-polymerase gene (Lipkin WI *et al*., 2011). The genomic map is similar for all bornaviruses, as far as known, except for *Queensland carbovirus* and the *Southwest carbovirus*, which have the gene order 3'-N-X-P-G-M-L-5', representing a transposition of the G and M genes (Hyndman TH *et al.,* 2018).

The disease has been reported in humans, several species of pets, production and wild animals (Malkinson M *et al.,* 1993; Berg A-L *et al.,* 1998; Berg AL *et al.,* 1999; Wensman JJ *et al.,* 2012; Björnsdóttir S *et al.,* 2013; Delnatte P *et al.,* 2013; Guo J *et al.,* 2014; Wensman JJ *et al.*, 2014; Ando T *et al.,* 2016; Thomsen AF *et al.,* 2015; Hyndman TH *et al.,* 2018; Tappe D *et al*., 2019). Namely, two species infect mammals (*Mammalian 1 to 2 orthobornavirus*), five infect birds (*Passeriform 1 to 2 orthobornavirus*, *Psittaciform 1 to 2 orthobornavirus* and *Waterbird 1 orthobornavirus*) and three infect reptiles (*Queensland carbovirus*, *Southwest carbovirus* and *Elapid 1 orthobornavirus*) (Amarasinghe G K *et al*., 2019) (Figure 2). However, some *Mammalian 1 orthobornavirus* showed the ability to also infect farmed ostriches and wild birds (mallards and jackdaws) (Malkinson M *et al*., 1993; Berg M *et al*., 2001). Wild birds are hosts of avian bornaviruses (e.g., strains of *Waterbird 1 orthobornavirus* and *Psittaciform 1 orthobornavirus*) (Delnatte P *et al.,* 2013; Guo J *et al.,* 2014; Sassa Y *et al*., 2015; Thomsen AF *et al.,* 2015) and mammalian bornaviruses (e.g., genotypes of *Mammalian 1 orthobornavirus*) (Berg M *et al*., 2001). Therefore, co-infection may play a role in the emergence of new pathogenic and zoonotic bornavirus species; once X and P proteins of PaBV-4 look like to have different ancestors (Pinto MC *et al*., 2018).

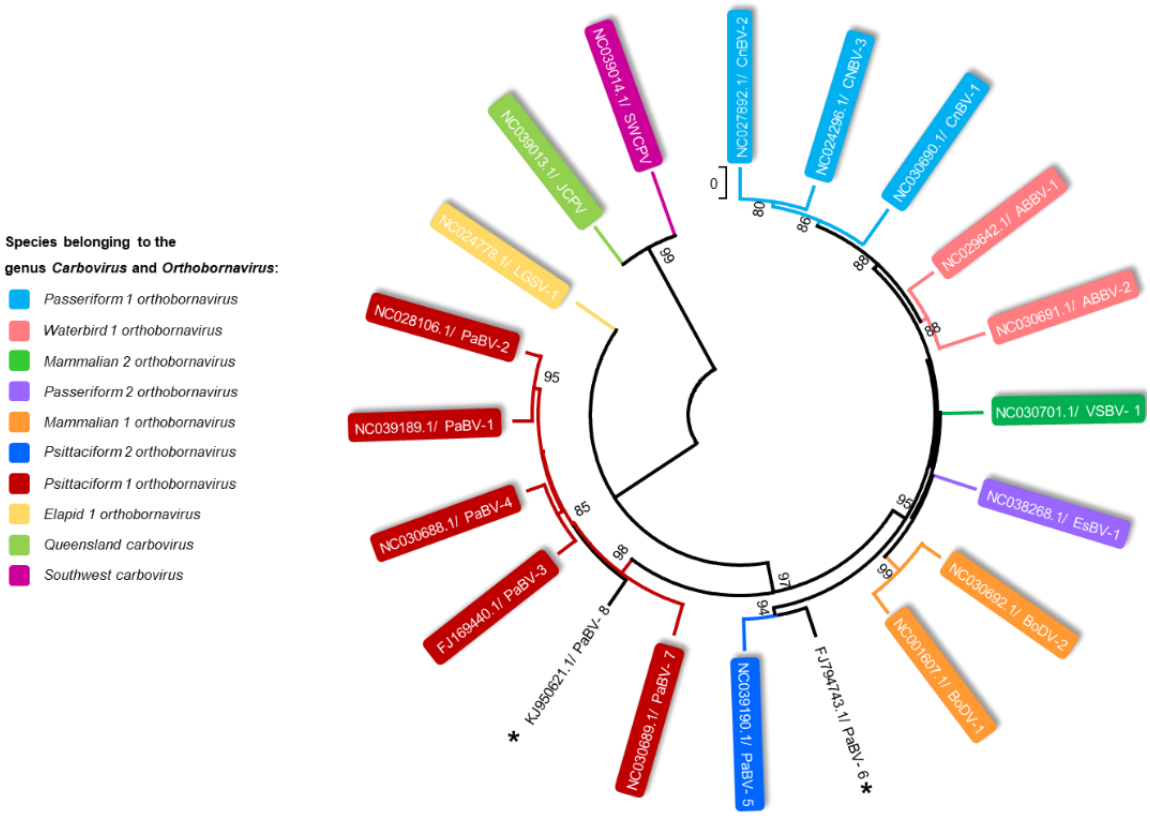

Figure 2. Phylogeny shows the species and strains belonging to the *Bornaviridae* family, which were associated with the development of neurological and/or gastrointestinal disease and/or death of its hosts. The phylogenetic relationship between species and strains was based on a segment of the M gene. Sequences identified by GenBank® accession numbers and abbreviated name of virus. Bornaviruses marked with asterisks are not classified following the currently accepted taxonomy (Amarasinghe G K *et al*., 2019). ABBV-1 to 2 = aquatic bird bornavirus 1 to 2, BoDV-1 to 2 = Borna disease virus 1 to 2, CnBV-1 to 3 = canary bornavirus 1 to 3, EsBV-1 = estrildid finch bornavirus 1, JCPV = jungle carpet python virus. LGSV-1 = Loveridge's garter snake virus 1, SWCPV = southwest carpet python virus, PaBV-1 to 8 = parrot bornavirus 1 to 8, VSBV-1 = variegated squirrel bornavirus 1.

In psittacines, parrot bornavirus 1 to 8 (PaBV-1 to 8) can cause proventricular dilatation disease (PDD), characterized by a flaccid and distended proventriculus impacted with feed, because of the inability of seeds' digestion (however throughout the gastrointestinal tract can occur variable distention) (Tizard I *et al*., 2016). Psittacines can also show uncoordinated movements, postural disorders, apathy, blindness, and behavioural disorders, such as loss of appetite and self-mutilation (resulting from lesions in the central nervous system) (Tizard I *et al*., 2016). Within captive birds, the virus has

become relevant for psittacines housed in reserves, in breeding projects of rare species, in private collections and zoos, because of the severe effects it may cause for bird welfare, economy, and biodiversity levels. Analyses of molecular epidemiology suggested that a world trade of psittacines without biosafety measures has been carried out (Pinto MC *et al*., 2018). There is, to the best of our knowledge, no publications identifying and characterizing the avian bornaviruses infecting pet parrots in Portugal. Moreover, there are still unresolved questions on the epidemiology of bornaviruses, such as the role of waterbirds in the emergence and dissemination of new pathogenic and zoonotic species, and the localization of highly conserved regions within P gene inter- and intra-species of bornaviruses.

The aim of this study was to identify and phylogenetically characterize the etiologic agent associated with clinical signs and necropsy findings consistent with avian bornavirus infection in two pet parrots in Portugal, as well as to produce molecular epidemiologic knowledge on bornaviruses.

6.1.2. Material and methods

6.1.2.1. Case histories and necropsy

Two females *Psittacus erithacus* (grey parrot) from different owners died with an interval of two months between them. One of the parrots (case number B24082018) did not show clinical signs prior to sudden death. The other parrot (case number B2610208) had a history of clinical signs compatible with bornavirus infection and had been examined (physical examination and X-ray) at the animal hospital prior to its death. The owners gave their informed consent for the necropsy, as well as for the collection of tissue samples and information on the clinical and life history of the parrots (by applying a structured questionnaire designed for the study). Both parrots were submitted for a complete standardised necropsy by a veterinarian specialized in exotic birds. Tissue samples from adrenal gland, brain, cecum, colon, crop, heart, ileum, jejunum, kidneys, liver, lung, ovary and proventriculus were collected and frozen at -80°C. The laboratory diagnosis was directed to search for avian bornavirus RNA based on: 1) the clinical history of the captive flock (weight loss, sudden death, undigested seeds in faeces, diarrhoea, poor condition of plumage) from which the parrot B24082018 originated; 2) the clinical signs, physical examination and X-ray of the parrot 26102018; and 3) the macroscopic findings reported during the necropsy, for both parrots.

6.1.2.2. Bornavirus nucleic acid detection by polymerase chain reaction (PCR)

We used tissue samples from brain, liver, kidney, spleen, ventricle, lung, heart, duodenum and pancreas, to search for avian bornavirus RNA. Extraction of total RNA from the tissue samples was done as previously described (Pinto MC *et al*., 2018). Briefly, the TRIzol/chloroform method (TRIzol reagent, Life Technologies) was used followed by purification with the RNeasy® Kit (Qiagen, Hilden, Germany), as recommended by the manufacturer's instructions. Estimation of the total RNA concentration was achieved using NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, USA), according to the manufacturer's instructions. Five hundred nanograms of total RNA were reverse transcribed into cDNA as previously described (Pinto MC *et al*., 2018). The cDNA was amplified by two conventional PCR protocols, one using a set of primers specific for a fragment of the N gene (the forward primer: 5'-CATGAGGCTATWGATTGGATTA-3' and the reverse primer (5'-TAGCCNGCCMKTGTWGGRTTYT-3') and the other was carried out with a set of primers specific for a fragment of the M gene (forward primer: 5'-CAAGGTAATYGTYCCTGGATGG-3' and the reverse primer: 5'-ACCAATGTTCCGAAGMCGAWAY-3'). In both protocols, the thermal cycling parameters and the reaction composition of PCR were carried out as previously described (Pinto MC *et al*., 2018). The expected length of the conventional products was 389 base pairs (bp) for the fragment of the N gene and 352 bp for the fragment of the M gene. From the positive samples, for both genes, selected tissues were used to conduct an additional PCR, combining the forward primer targeting the N gene and the reverse primer targeting the M gene, under previously reported experimental conditions (Pinto MC *et al*., 2018). The expected length of the PCR product was 1600 bp (covering a partial region of the N gene, a total region of the X and P genes and a partial region of the M gene). The visualization of the PCR products was in electrophoresis using a 2% agarose gel, staining with Gel Red™ (Biotium Glowing Products for Science™, Hayward, CA) at 0.002%. The PCR products from all tissue samples evaluated were purified using the GRS PCR & Gel Band Purification Kit (GRISP, Porto, Portugal) and both strands of DNA were analysed by Sanger sequencing. The nucleotide sequences obtained in this study were submitted to GenBank® database under accessions numbers MK192982 and MK192983.

6.1.2.3. Virus identification, phylogenetic analysis and protein similarity within bornaviruses

The identification of bornaviruses was conducted by finding regions of similarity between the nucleotide sequences obtained (from all tissues evaluated) and sequences from the GenBank® database. Throughout the X and P protein, conserved regions between bornavirus species were searched, comparing the amino acid sequences produced with the selected sequences (from GenBank® database we selected two amino acid sequences from each species of bornaviruses, when available). The identification of bornaviruses, as well as the search for conserved regions throughout the X and P proteins were conducted using the Basic Local Alignment Search Tool (BLAST®) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the percent identity (id) and its statistical significance (E-value) was collected.

The relationship between the genotypes detected in the present study and the parrot bornavirus 4 (PaBV-4) genotypes reported was evaluated by phylogenetic analysis, based on N gene segment. Therefore, some of the nucleotide sequences of PaBV-4 from each of the 5 previously identified genotype clusters (Pinto MC *et al*., 2018) were included, as well as the new sequences deposited in GenBank® database. The output of the analysis was divided in clusters, the strains were classified to belong to the same cluster when the percent identity between them were ≥ 98%. Since not all sequences identified by BLAST® search covered completely the PCR products produced, all sequences were trimmed to the length of the shortest sequence included in the respective phylogenetic analysis.

To evaluate the role of waterbirds in the emergence and dissemination of new pathogenic bornaviruses, the evolutionary relationship between the PaBV-4 and bornaviruses detected in wild birds (aquatic bird bornavirus 1 to 2 and Borna disease virus 1), as well as bornaviruses linked to infection in humans (Borna disease virus 1 and variegated squirrel bornavirus 1), was determined. Therefore, sequences representatives of virus genotypes were selected from GenBank® database and included in the analysis. Phylogenetic analyses were conducted in MEGA7 software (Kumar S *et al*., 2016) using nucleotide sequences deposited in GenBank®.

6.1.3. Results

6.1.3.1. Case histories and necropsy findings

One of the parrots (case number B24082018) had suddenly died without previous clinical history compatible with bornavirus infection. The parrot belonged to a private

collection in which infection by avian bornaviruses was the cause of death of several parrots three years ago. Since the outbreak, the parrot B24082018 was the only case of illness, reported by the owner. The parrot was born in captivity in Spain and lived in the northern region of Portugal until its death. The necropsy of the bird showed a severe cachexia, with splenomegaly and hepatomegaly. The other organs were grossly normal, including a normal proventriculus without dilatation.

The other parrot (case number B26102018) had a clinical history compatible with bornavirus infection, displaying regurgitation, seeds not digested in the faeces, cachexia and prostration. The X-ray revealed a severe dilatation of the proventriculus (Figure 3).

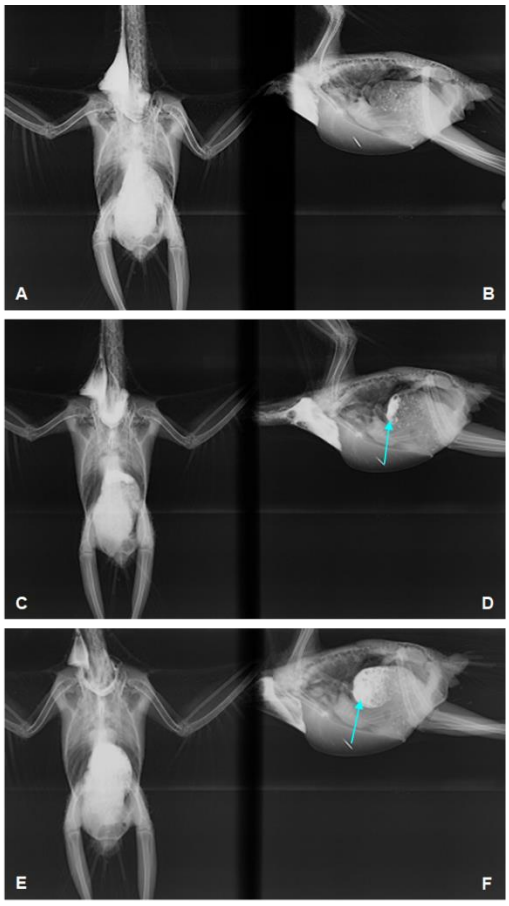

Figure 3. X-ray performed on the parrot case number B26102018, using barium contrast. (A) Dorsal-ventral view and **(B)** side-to-side view after the introduction of barium contrast in the crop (marked with the blue arrow). **(C)** Dorsal-ventral view and **(D)** side-to-side view of the diffusion start of barium contrast in the proventriculus. **(E)** Dorsal-ventral view and **(F)** lateral-lateral view allowing the visualization of the diffusion of barium contrast throughout the proventriculus, highlighting its delineation, which revealed a severe dilatation (marked with the blue arrow).

The bird, at necropsy, showed a reduced muscle mass and the proventriculus showed a severe dilatation with a ruptured wall (Figure 4). The other organs were grossly normal. The parrot had always lived apparently healthy, as a pet in the central region of Portugal for 20 years. The owner reported that the parrot had been donated to him and was unaware of its origin. The two parrots never had contact with each other during their lifetime.

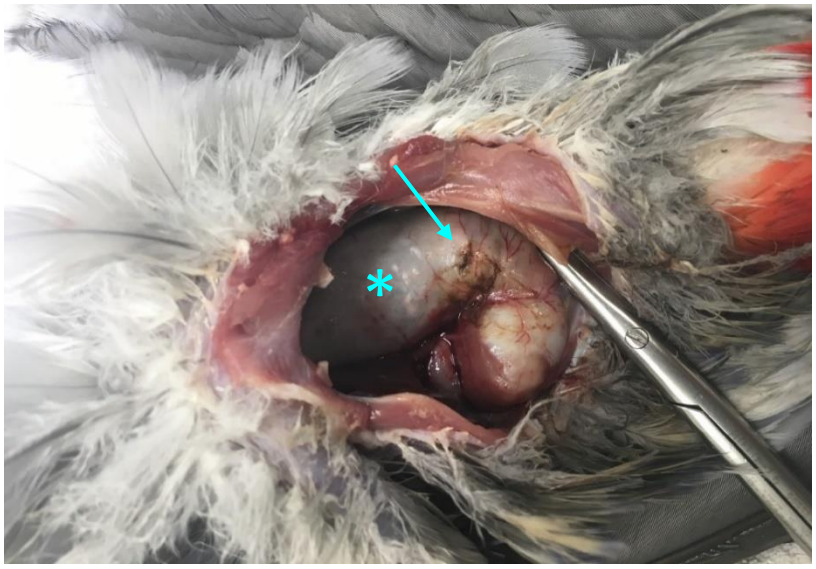

Figure 4. The proventriculus of parrot case number B26102018, at necropsy. The severe dilatation (asterisk) produced a small rupture in the proventriculus wall (arrow).

6.1.3.2. Bornavirus nucleic acid detection by polymerase chain reaction (PCR)

All tested tissue samples from the two *Psittacus erithacus* were positive for bornavirus RNA, with amplification of fragments of the N gene (with 389 bp) and of the M gene (with 352 bp). Additionally, from tissue samples of brain and lung of each *Psittacus erithacus*, nucleic acid fragments (with 1600 base pairs) comprising partially the N and M genes and totally the X and P genes were amplified.

6.1.3.3. Virus identification, phylogenetic analysis and protein similarity within bornaviruses

The bioinformatics analysis revealed that both parrots were infected with parrot bornavirus 4 (PaBV-4), based on the genetic segment that contained the partial N and M genes and the complete X and P genes (1543 base pairs for case number B24082018 and 1537 base pairs for case number B26102018). The highest percent identity (id) found

between PaBV-4 reported in the GenBank® database and the virus linked to B24082018 was 99% (E-value= 0.0) and 98% (E-value= 0.0) to B26102018. Additionally, the nucleotide composition of the two virus sequences in the present study, differed by 5% from each other (id= 95%, E-value=0.0). When the analysis was restricted to the X and P genes, they differed from each other by 2% (id= 98%, E-value= $1e^{-129}$) and 8% (id= 92%, E-value=0.0), respectively. The genetic differences found between the two PaBV-4 resulted into 5% and 2% of variation of amino acid composition of X protein (id= 95%, E-value= $1e^{-49}$) and P protein (id= 98%, E-value= $3e^{-42}$), respectively (Table 1).

Similar results were observed when comparing parrot bornavirus 4 (PaBV-4) genotypes detected in the present study with worldwide distributed bornaviruses (mammalian, reptile and avian bornaviruses) (Table 1). Specifically, the percent identity found for X protein was lower (id= (36 - 95) %, E-value= ($4e^{-48}$ - $3e^{-32}$)) than the identity found for P protein (id= (39 - 98) %; E-value=($7e^{-41}$-$1e^{-40}$)), within viruses of the same species (within *Psittaciform 1 orthobornavirus*) and between viruses of different species (*Psittaciform 2 orthobornavirus*, *Passeriform 1* and *2 orthobornavirus*, *Mammalian 1* and *2 orthobornavirus* and *Elapid 1 orthobornavirus*) (Table 1). Within X protein, the higher variation of identity occurred in the region shared with the P protein (id= (37 - 99) %, E-value= ($1e^{-40}$ - $9e^{-05}$)) (Table 1). Moreover, in the non-shared region of X protein the identity between the two PaBV-4 genotypes and mammalian bornaviruses (*Mammalian 1* and *2 orthobornavirus* (id= 75%, E-value= $2e^{-12}$)) was the same for some avian bornaviruses (*Passeriform 1 orthobornavirus* (canary bornavirus 1 and 2 (E-value= $2e^{-12}$)) and *Water 1 orthobornavirus* (aquatic bird bornavirus 1 (E-value = $2e^{-12}$)) (Table 1).

Concerning P protein, the amino acid sequence region non-shared with X protein revealed higher variation (id= (30 - 82) %, E-value= ($4e^{-53}$ - $1e^{-2.8}$)) than the shared region (id= (37 - 99) %, E-value = ($1e^{-40}$ - $9e^{-05}$)), within and between species of bornaviruses (Table 1). In the region shared between the two proteins, the identity found between the two PaBV-4 genotypes and members of *Mammalian 2 orthobornavirus* species was higher than the percent identity found with some members of avian bornaviruses (Table 1). Namely, the case number B24082018 showed a higher identity with *Mammalian 2 orthobornavirus* members (id= 74%, E-value= $5e^{-20}$) than with some members of *Waterbird 1 orthobornavirus* (Aquatic bird bornavirus 2, (id= 69%, E-value= $9e^{-18}$)) and *Passeriform 1 orthobornavirus* (Canary bornavirus 1, (id= 73%, E-value= $2e^{-20}$)) (Table 1). Also, the case number B26102018 showed a higher percent identity with *Mammalian 2 orthobornavirus* members (id= 69%, E-value= 2e-^{20}) than with some members of *Waterbird 1 orthobornavirus* (Aquatic bird bornavirus 2, (id= 64%, E-value= $4e^{-18}$)) (Table 1). Additionally, only in the region shared by both proteins, a statistically significant similarity

was found between the two PaBV-4 genotypes reported and members of reptilian bornaviruses (id= (37 - 45) %, E-value= ($5e^{-99}$ - $9e^{-05}$)), (Table 1).

The results obtained for X and P proteins, described above, were complemented by the phylogenetic analysis based on the segment of N gene, since the reported genotypes, in the present study, showed to be phylogenetically adjacent to PaBV-4 genotypes worldwide distributed (Figure 5). Namely, they were mostly related with PaBV-4 genotypes detected in several species of pet psittacines, in countries of Asia (Israel and Japan), Europe (Austria, Germany, Hungary, Sweden and Switzerland) and North America (Canada and USA) and South America (Brazil) (Figure 5). Moreover, the analysis of the N segment revealed that the detected PaBV-4 genotypes, in pet parrots sampled in Portugal, were also phylogenetically adjacent to those found in wild birds in Japan (such as *Anas* spp., *Emberiza* spp. and *Grus* spp.) (Figure 5). Besides, the analysis showed that PaBV-4 genotypes circulating in Portugal were closely related to genotypes which have been circulating in the last 19 years, all over the world (according to the earliest sampling date reported in GenBank® for PaBV-4) (Figure 5).

The second phylogenetic approach, based on the N gene segment, confirmed the results obtained for the relationship between the PaBV-4 genotypes and bornaviruses (of avian and mammalian) in bioinformatics analysis of X protein (Table 1); moreover, it added information about their evolutionary relationship (Figure 6 A). Namely, phylogenetic analysis revealed that PaBV-4 genotypes, detected in the present study, showed to be more closely related to avian bornaviruses than to mammalian bornaviruses (Figure 6). Within *Waterbird 1 orthobornavirus* species, the aquatic bird bornavirus 1 (ABBV-1) genotypes are phylogenetically closer to PaBV-4 than to aquatic bird bornavirus 2 (ABBV-2) genotypes (Figure 6 A). Regarding mammalian bornaviruses, the *Mammalian 2 orthobornavirus* members were more related to PaBV-4 than to *Mammalian 1 orthobornavirus* members (Figure 6 A). Specifically, the variegated squirrel bornavirus 1 (VSBV-1) genotypes detected in squirrels (from zoos and breeding collections) and in humans (with a fatal limbic encephalitis) are phylogenetically closer related to PaBV-4 than to Borna disease virus 1 (BoDV-1) detected in humans (associated with a lethal encephalitis) and in wild birds (Figure 6 A). When the phylogenetic analysis was conducted based on the amino acids encoded by the segment of N gene, the main evolutionary relationship persisted between parrot bornavirus (PaBV-4), aquatic bornavirus (ABBV-1, ABBV-2), variegated squirrel bornavirus (VSBV-1) and Borna disease virus (BoDV-1) (Figure 6 B).

The third phylogenetic approach, based on the P gene segment, revealed that members of *Mammalian 2 orthobornavirus* species are the most related to PaBV-4 genotypes than of all bornaviruses detected and reported in wild birds, other than parrots

(Figure 7 A). That is, the PaBV-4 genotypes are evolutionarily closer to the VSBV-1 genotypes, linked to the infection of squirrels (from zoos and breeding collections) and humans than members of *Waterbird 1 orthobornavirus* (ABBV-1 and ABBV-2) and of *Mammalian 1 orthobornavirus* (BoDV-1), detected in samples of wild birds (Figure 7). Namely, within genotypes detected in wild-bird samples, Borna disease virus (BoDV-1) is the most evolutionarily distant from parrot bornavirus (PaBV-4) (Figure 7 A). However, within Borna disease virus (BoDV-1) the genotype associated with lethal encephalitis in humans (accession nº MH190827.1) is evolutionarily more distant from PaBV-4 than the variants detected in samples of wild *Anas* spp. (Figure 7 A). When the phylogenetic analysis was conducted based on the amino acids encoded by the segment of the P gene, the main evolutionary relationship remained between bornaviruses (Figure 7 B).

The findings obtained through the several analyses conducted suggest that within parrot bornavirus (PaBV-4) genotypes the N and P gene had different ancestors. Since the N gene of PaBV-4 is evolutionarily closer to the N gene of avian bornaviruses (Figure 6 A and B), while P gene is more related to mammalian bornaviruses, specifically with the zoonotic variegated squirrel bornavirus (VSBV-1) genotypes (Figure 7 A and B). Additionally, the findings showed that the Borna disease virus (BoDV-1) detected in samples of wild birds (accession nº AF233071.1, AF232702.1 and AF232703.1) are intermediate variants between the two BoDV-1 genotypes (accession nº LT991983.1 and MH190827.1) linked with lethal encephalitis of humans, on the evolutionary line (Figure 6 B and Figure 7 A and B).

Table 1. The percent identity between the PaBV-4 genotypes detected in Portugal and bornaviruses worldwide distributed, regarding X and P protein

Bornaviruses A Worldwide distributed	Amino acid sequence of X to P protein (287 amino acid length) from bornaviruses worldwide distributed										Sequences compared		
	X protein (X)						Phosphoprotein (P)						
	Total		Non-shared region		Shared region		Non-shared region		Total				
	86 amino acids length		18 amino acids length		68 amino acids length		133 amino acids length		201 amino acids length		Present study *versus*		
	Id. (%)[B]	E-value [C]	Id. (%)[B]	E-value [C]	Id. (%)[B]	E-value [C]	Id. (%)[B]	E-value [C]	Id. (%)[B]	E-value [C]	Present study	GenBank® Accession nº	Hosts
Avian bornaviruses													
Psittaciform 1 orthobornavirus	74–95	$4e^{-48}$–$3e^{-32}$	81–100	$5e^{-17}$–$2e^{-12}$	91-99	$2e^{-43}$–$8e^{-21}$	50–82	$4e^{-53}$–$3e^{-08}$	92–98	$1e^{-40}$–$9e^{-20}$	B240818	KU748814.1; MK192982.1; JX065204.2; EU781967.1; FJ620690.1; JX065207.1; GU249595.2; JX065210.2; FJ169440.1; NC_030689.1	Parrot
	75–100	$6e^{-62}$–$1e^{-35}$	81–100	$5e^{-17}$–$2e^{-12}$	94-99	$1e^{-40}$–$1e^{-19}$	42–87	$2e^{-60}$–$1e^{-10}$	89–93	$6e^{-122}$–$2e^{-109}$	B260818		
Psittaciform 2 orthobornavirus	51–53	$1e^{-25}$–$1e^{-24}$	81	$2e^{-13}$	69–77	$5e^{-20}$–$2e^{-20}$	38–41	$2e^{-04}$	72	$4e^{-19}$–$2e^{-18}$	B240818	AB519144.1; KT378600.1	Parrot
	53	$1e^{-26}$–$1e^{-26}$	81	$2e^{-13}$	74	$1e^{-19}$–$2e^{-19}$	55–60	$1e^{-0.7}$–$1e^{0.3}$	64	$7e^{-74}$–$1e^{-72}$	B260818		
Passeriform 1 orthobornavirus	51–58	$1e^{-27}$–$7e^{-23}$	75	$2e^{-12}$	71–73	$3e^{-21}$–$1e^{-19}$	33–38	$5e^{-05}$–$1e^{-0.9}$	72–73	$5e^{-19}$–$3e^{-18}$	B240818	NC_030690.1; KC464471.1; NC_027892.1; KC464478.1; KC595273.2; NC_024296.1	Canary
	53–57	$1e^{-29}$–$2e^{-25}$	75–81	$2e^{-13}$–$2e^{-12}$	73–75	$2e^{-20}$–$1e^{-18}$	30–33	$5e^{-04}$–$1e^{-2.8}$	63	$1e^{-83}$–$7e^{-82}$	B260818		
Passeriform 2 orthobornavirus	64	$2e^{-23}$	81	$3e^{-12}$	73	$4e^{-20}$	33	$1e^{0.96}$	75	$2e^{-19}$	B240818	KF680099.1	Estrildid
	63	$1e^{-34}$	81	$3e^{-12}$	76	$2e^{-19}$	36	$1e^{-5.1}$	65	$2e^{-70}$	B260818		
Waterbird 1 orthobornavirus	56–57	$2e^{-27}$–$3e^{-26}$	75–81	$2e^{-13}$–$2e^{-12}$	64–69	$3e^{-19}$–$4e^{-18}$	33–37	$2e^{-04}$–$1e^{-1.1}$	67–72	$2e^{-16}$–$5e^{-16}$	B240818	KF578398.1; NC_029642.1; NC_030691.1; KJ756399.2	Duck Goose
	55–56	$7e^{-29}$–$3e^{-28}$	75–81	$2e^{-13}$–$2e^{-12}$	69–75	$9e^{-20}$–$9e^{-18}$	44–45	$1e^{-4.3}$–$1e^{0.8}$	63–65	$2e^{-73}$–$3e^{-72}$	B260818		

A - Bornaviruses identification as established by the International Committee on Taxonomy of Viruses (ICTV) *Bornaviridae* Study Group. **B** - The highest percent identity of all query-subject alignment given by Basic Local Alignment Search Tool (BLAST®). **C** - The best (lowest) Expect value (E-value) of all alignments from that database sequence given by BLAST®. ***** No significant similarity found.

Table 1. The percent identity between the PaBV-4 genotypes detected in Portugal and bornaviruses worldwide distributed, regarding X and P protein (continuation)

Bornaviruses [A] Worldwide distributed	Amino acid sequence of X to P protein (287 amino acid length) from bornaviruses worldwide distributed										Sequences compared		
	X protein (X)						Phosphoprotein (P)						
	Total		Non-shared region		Shared region		Non-shared region		Total				
	86 amino acids length		18 amino acids length		68 amino acids length		133 amino acids length		201 amino acids length		Present study *versus*		
	Id. (%)[B]	E-value [C]	Id. (%)[B]	E-value [C]	Id. (%)[B]	E-value [C]	Id. (%)[B]	E-value [C]	Id. (%)[B]	E-value [C]	Present study	GenBank® Accession nº	Hosts
Mammalian bornaviruses													
Mammalian 1 orthobornavirus	45–50	$6e^{-20}$–5.1	75	$2e^{-12}$–$7e^{-11}$	61–64	$9e^{-25}$–$2e^{-23}$	34–38	$1e^{-3.6}$–47	62–65	$5e^{-23}$–$5e^{-22}$	B240818	AJ311522.1; NC_030692.1; AJ311524.1; U04608.1	Horse
	46–49	$2e^{-21}$–$1e^{-20}$	75	$2e^{-12}$–$7e^{-11}$	65–67	$6e^{-24}$–$9e^{-23}$	37–44	$1e^{-4.6}$–$1e^{-0.4}$	56–58	$1e^{-81}$–$2e^{-80}$	B260818		
Mammalian 2 orthobornavirus	49	$9e^{-22}$	75	$2e^{-12}$–$1e^{-11}$	69	$2e^{-20}$	36	$2e^{-04}$	70	$4e^{-18}$	B240818	KY488729.1; MF597762.1; NC_030701.1; LN713681.1	Squirrel
	52	$5e^{-24}$	75	$2e^{-12}$–$1e^{-11}$	74	$5e^{-20}$	38	$1e^{-01}$	63	$1e^{-80}$	B260818		Human
Reptilian bornaviruses													
Elapid 1 orthobornavirus	37	$2e^{-05}$	53	$1e^{-6.2}$–$1e^{-5.8}$	44	$3e^{-11}$	41	$e^{4.29}$	46	$8e^{-09}$	B240818	KM114265.1; NC_024778.1	Snake
	37	$5e^{-07}$	53	$1e^{-6.2}$–$1e^{-5.8}$	45	$5e^{-09}$	41	$e^{4.29}$	39	$7e^{-41}$	B260818		
Queensland carbovirus	26	$1e^{0.74}$	*	---	34	$1e^{-1.5}$	30	$1e^{-6.9}$	30	$1e^{-6.2}$	B240818	NC_039013.1	Snake
	50	$1e^{1.2}$	*	---	34	$1e^{-1.6}$	*	---	26	$1e^{-1.1}$	B260818		
Southwest carbovirus	50	$1e^{1.5}$	*	---	50	$1e^{1.2}$	30	$1e^{-4.2}$	66	$1e^{-1.4}$	B240818	NC_039014.1	Snake
	50	$1e^{1.2}$	*	---	50	$1e^{0.9}$	*	---	*	---	B260818		

A - Bornaviruses identification as established by the International Committee on Taxonomy of Viruses (ICTV) *Bornaviridae* Study Group. **B** - The highest percent identity of all query-subject alignment given by Basic Local Alignment Search Tool (BLAST®). **C** - The best (lowest) Expect value (E-value) of all alignments from that database sequence given by BLAST®. * - No significant similarity found.

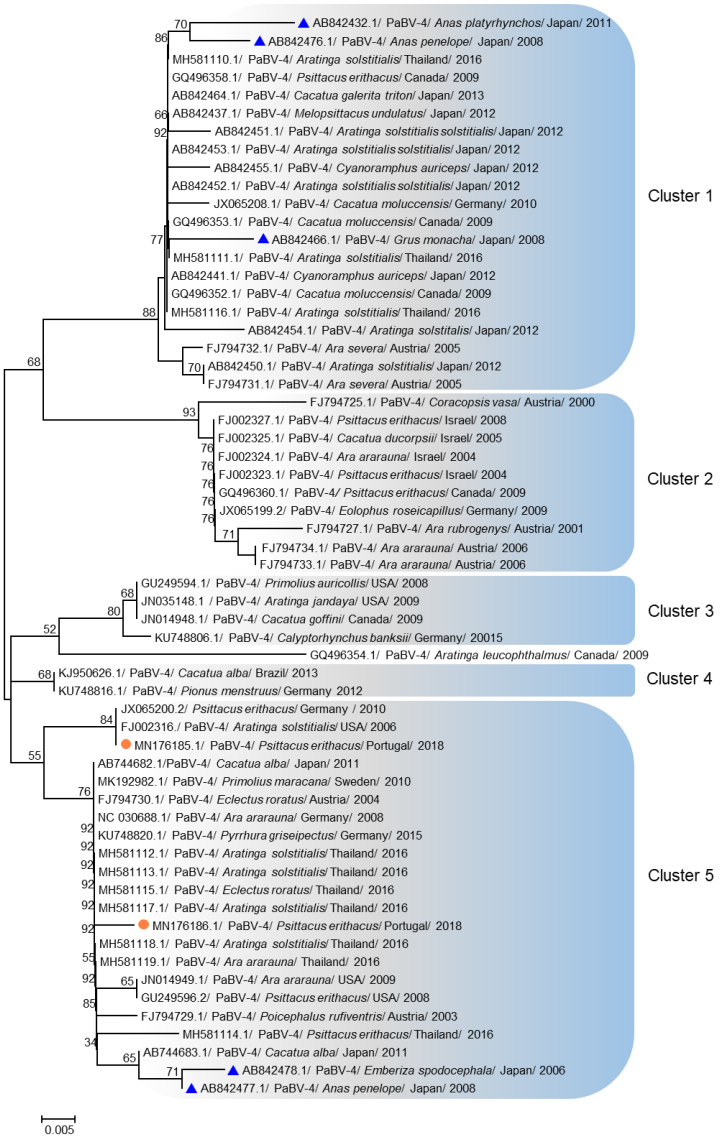

Figure 5. Phylogenetic relationships between PaBV-4 genotypes identified in the present study and PaBV-4 detected in wild birds as in pet parrots, regarding N gene. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N *et al*., 1987). The confidence probability was estimated using the bootstrap test (1000 replicates) and is shown above the branches (Dopazo J, 1994; Rzhetsky A *et al*., 1992). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura M, 1980) and are in the units of the number of base substitutions per site. The analysis involved 60 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 163 bases in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar S *et al*., 2016). Sequences identified by GenBank® accession numbers, abbreviation name of virus and its hosts, year and geographic origin of sampling. PaBV-4=parrot bornavirus 4. The sequences marked with a circle were produced during this study and triangles marked nucleotide sequences identified from wild birds.

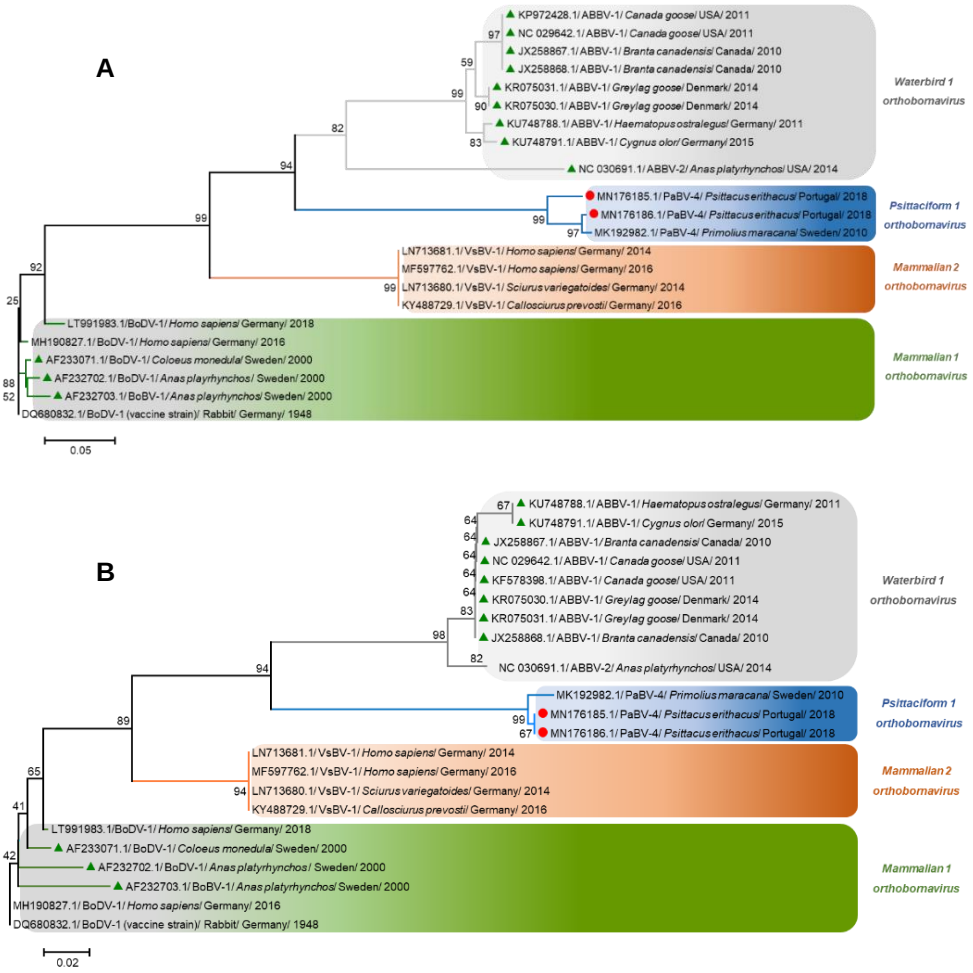

Figure 6. Phylogenetic relationships between PaBV-4 genotypes identified in the present study and bornaviruses detected in mammalian and in wild birds, based on N gene (A) and on N protein (B). The evolutionary history was inferred using the Neighbor-Joining method (Saitou N *et al*., 1987). The confidence probability (multiplied by 100) was estimated using the bootstrap test (1000 replicates) and is shown next to the branches (Dopazo J *et al*., 1994). Both trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Both trees were rooted with a vaccine strain (accession nº DQ680832.1). Evolutionary analyses were conducted in MEGA7 (Kumar S *et al*., 2016). **(A)** The evolutionary distances were computed using the Kimura 2-parameter method (Kimura M, 1980) and are in the units of the number of base substitutions per site. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 215 bases in the final dataset. **(B)** The evolutionary distances were computed using the Poisson correction method (Zuckerkandl E *et al*., 1995) and are in the units of the number of amino acid substitutions per site. The analysis involved 23 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 71 amino acids in the final dataset. Sequences identified by GenBank® accession numbers, abbreviation name of virus and its hosts, year and geographic origin of sampling. ABBV-1 = aquatic bird bornavirus 1, ABBV-2 = aquatic bird bornavirus 2, BoDV-1= Borna disease virus 1, PaBV-4 = parrot bornavirus 4, VSBV-1 = variegated squirrel bornavirus 1. The sequences marked with a circle were produced during this study and triangles marked nucleotide sequences identified from samples of wild birds.

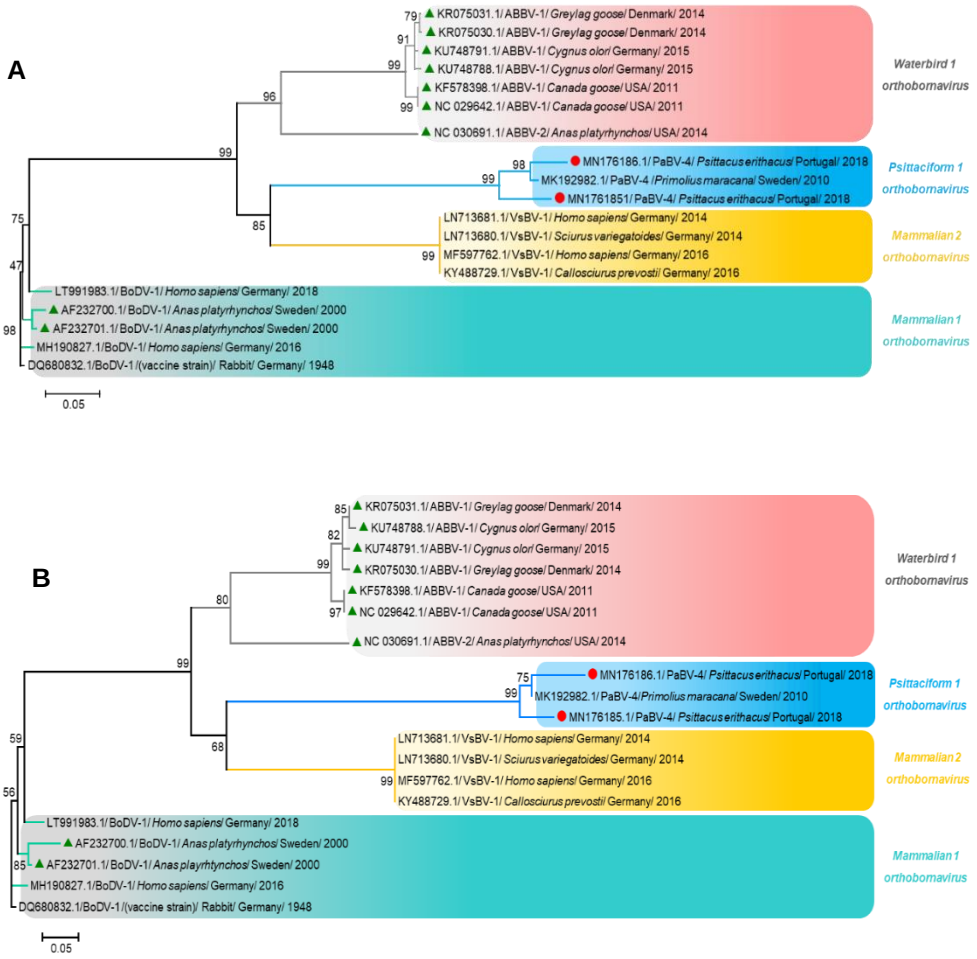

Figure 7. Phylogenetic relationships between PaBV-4 genotypes identified in the present study and bornaviruses detected in mammalian and wild birds, based on P gene (A) and on P protein (B). The evolutionary history was inferred using the Neighbor-Joining method (Saitou N *et al*., 1987). The confidence probability (multiplied by 100) was estimated using the bootstrap test (1000 replicates) and is shown next to the branches (Dopazo J *et al*., 1994). Both trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Both trees were rooted with a vaccine strain (accession nº DQ680832.1). Evolutionary analyses were conducted in MEGA7 (Kumar S *et al*., 2016). **(A)** The evolutionary distances were computed using the Kimura 2-parameter method (Kimura M, 1980) and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 386 bases in the final dataset **(B)**. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl E *et al*., 1995) and are in the units of the number of amino acid substitutions per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 110 amino acids in the final dataset. Sequences identified by GenBank® accession numbers, abbreviation name of virus and its hosts, year and geographic origin of sampling. ABBV-1 = aquatic bird bornavirus 1, ABBV-2 = aquatic bird bornavirus 2, BoDV-1= Borna disease virus 1, PaBV-4 = parrot bornavirus 4, VSBV-1 = variegated squirrel bornavirus 1. The sequences marked with a circle were produced during this study and triangles marked nucleotide sequences identified from samples of wild birds.

6.1.4. Discussion and conclusions

Although parrot bornavirus 1 to 8 (PaBV-1 to 8) are recognized as the etiologic agent of Proventricular Dilatation Disease (PDD) in psittacines, there are still unanswered questions regarding its epidemiology. This is the first study that characterizes, at genetic and protein level, genotypes of *Psittaciform 1 orthobornavirus*, found in Portugal. The sequencing of more than 1000 base pairs of the viral genome allowed the identification of parrot bornavirus 4 (PaBV-4) with statistically significant probability (percent identity (id) = (98.31–98.51) %, E-value=0.0), as the etiological agent associated with the death of two pet parrots (*Psittacus erithacus*). None of the PaBV-4 identified in Portugal share 100% identity with genotypes reported so far, when the recovered genome segment with more than 1500 nucleotides was analysed. The complete P protein of both PaBV-4 genotypes differs from proteins deposited in the GenBank® database (id= (73.13–92.54) %, E-value=$1e^{-91}$ - $2e^{-39}$). Regarding X protein, one of the identified PaBV-4 (accession nº B26102018) share 100% of identity (E-value=$2e^{-59}$) with genotypes detected in Germany and Sweden, according to GenBank® database. The X protein of the other PaBV-4 genotype differ from proteins deposited in GenBank® database (id= 97.56%, E-value=2 e^{-54}).

Regarding the parrot B24082018, the source of infection is unknown, however we suspect that parrot bornavirus 4 (PaBV-4) entered via the olfactory pathway and migrated to brain (Tizard I *et al*., 2016), causing a lethal encephalitis; without neurological signs perceptible by the owner. The absence of clinical signs in bornavirus-infected parrots with brain lesions has previously been reported (Tizard I *et al*., 2016). Therefore, in this case, probably the virus followed one of the postulated pathways, which consist of the centrifugal spread of virus from brain, passing down the spinal cord, affecting the parasympathetic and sympathetic nerves and the organs innervated by them (e.g., lung, heart, proventriculus and intestine) (Tizard I *et al*., 2016). Concerning the parrot B26102018, the source of infection is unknown, however, it is probable that virus entered through oral route and spread from proventriculus to the central nervous system, as postulated in the literature (Tizard I *et al*., 2016), based on the clinical picture characterized by the development of gastrointestinal signs followed by neurological signs at the final stage of disease.

In the present study we produced additional and detailed information to the previous reported insights about the degree of conservation of X and P protein within and between species of bornaviruses (Pinto MC *et al*., 2018). As previously reported, the P protein (phosphoprotein) showed to be more conserved between and within species of

bornaviruses than the X protein (Pinto MC *et al*., 2018). However, the present study showed that, within and between bornaviruses, the degree of conservation throughout the P protein was not constant (Table 1). The identity percent of the first 68 amino acid was higher than the following 133 amino acid of P protein (Table 1). The region showing a higher identity percentage is a shared amino acid sequence with X protein. In contrast, throughout the X protein the lower identity percent (intra- and inter-species of bornaviruses) comes from the region shared with P protein (Table 1). Therefore, the first 18 amino acid of the sequence revealed to be more conserved within X protein (Table 1). Nevertheless, when comparing both conserved regions of the two proteins, the X protein showed a smaller variability range of the amino acid sequence (Table 1). However, its most conserved region represents about 20.9% of the X protein, which corresponds to 54 nucleotides of the gene, making it less advantageous as a target region (in the design of tools) for molecular diagnostics, compared to the more conserved region of the P protein. In the case of P protein, the most conserved region corresponds approximately to 68 nucleotides, among species of family *Bornaviridae*, allowing more options in the design of molecular tools, aiming to decrease the occurrence of false negatives. In some cases, despite all findings of clinical diagnosis, the inability to amplify the viral nucleic acid might be a consequence of the presence of unknown species of bornaviruses in samples (Wensman JJ *et al*., 2012). Thus, a decrease in the number of false negatives is expected, since the highly conserved regions in evolutionarily more distant viruses will be more identical to the bornaviruses described so far. Therefore, the highly conserved region is a good candidate as a target in laboratory diagnostics for screening purposes of potential reservoirs or asymptomatic carriers, as well as to confirm the clinical diagnosis. The information produced about the highly conserved region is thus useful, since the diversity of members of the family *Bornaviridae* is expected to be higher than reported, considering the evolution of the scientific knowledge produced. In fact, between the end of 20th century and the beginning of 21st century, the family *Bornaviridae* comprised only the *Mammalian 1 orthobornavirus* species (Afonso CL *et al*., 2016). From then to the present, the family *Bornaviridae* includes 11 species and 19 strains (Amarasinghe G K *et al*., 2019). Moreover, within each of the 19 strains there are several genotypes described, as for example the taxonomic group PaBV-4 that integrates more than 30 genotypes (Pinto MC *et al*., 2018).

The evidence produced by the phylogenetic analysis restricted to the parrot bornavirus (PaBV-4) genotypes suggests that Portugal is on the trade route of psittacines; because both genotypes cluster together with genotypes worldwide distributed and detected from captive parrots (Pinto MC *et al*., 2018). Over the years, the international trade, without biosafety measures (Pinto MC *et al*., 2018), is expected to have had negative

implications on biodiversity. Whereas, in the last 19 years, (based on the oldest data collection (Figure 5)) bornavirus infection has contributed to the reduction of endangered species (Pinto MC *et al*., 2018; BirdLife International, 2018). The *Psittacus erithacus* is one of the endangered species (according to the International Union for Conservation of Nature (IUCN)) (BirdLife International, 2018) included in the list of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (The CITES species, 2019). In Portugal, the lawful trade of endangered species of parrots is supervised by several national entities working with CITES (National CITES Authorities, 2019).

The present study further considered parrot bornavirus 4 (PaBV-4) genotypes detected in wild birds (*Anas* spp., *Emberiza* spp. and *Grus* spp.) and in pet psittacines of Japan and Thailand (Figure 5), more inclusive and updated than in the previous study (Pinto MC *et al*., 2018), resulting in a broader view of the geographic dimension of the psittacines trade. Also, it suggests there are at least two factors (the wild birds and the world trade of psittacines) that, simultaneously or singularly, might be a factor for the dissemination of the PaBV-4 infection. Some of the PaBV-4 genotypes detected in pet parrots are phylogenetically adjacent to some genotypes detected in wild bird samples (Figure 5). In our analytical approach it was not possible to include the genotypes of PaBV-4 detected in wild parrots from Brazil (Encinas-Nagel N *et al*., 2014), because the nucleotide sequences were not available in the GenBank® database.

Over the last years, the evidence that wild birds could be reservoirs of bornaviruses has been accumulated (Berg M *et al*., 2001; Delnatte P *et al.*, 2013; Guo J *et al*., 2014; Sassa Y *et al*., 2015; Thomsen AF *et al*., 2015). In addition to the parrot bornavirus 4, cases of infection in wild birds with several genotypes of parrot bornavirus 2 (such as *Anas* spp., *Grus* spp., *Haliaeetus* spp., *Larus* spp., *Galidris* spp., *Calidris* spp. and *Emberiza* spp.), Borna disease virus 1 (such as *Anas* spp., and *Coloeus* spp.) and aquatic bird bornavirus 1 to 2 (such as *Cygnus* spp.*, Greylag* spp.*, Canda* spp. and *Anas* spp.) have been reported (Berg M *et al*., 2001; Delnatte P *et al.*, 2013; Guo J *et al*., 2014; Sassa Y *et al*., 2015; Thomsen AF *et al*., 2015). The co-infection was reported to occur in wild birds by different strains and genotypes belonging to *Psittaciform 1 orthobornavirus* (Sassa Y *et al*., 2015). It could then be expected that all members of the family *Bornaviridae* could be involved in co-infection events, since there are species of wild birds that may be hosting a wide range of genotypes from several species of bornaviruses (such as *Waterbird 1 orthobornavirus* (Delnatte P *et al.*, 2013; Guo J *et al*., 2014; Thomsen AF *et al*., 2015), *Psittaciform 1 orthobornavirus* (Sassa Y *et al*., 2015) and *Mammalian 1 orthobornavirus* (Berg M *et al*., 2001). Therefore, co-infected reservoirs can play a role in the emergence of new virus variants, providing conditions for the occurrence of changes in virulence and in host range

by two major ways: through recombination exchange between virus strains and through accumulation of amino acid changes that are under pressure by the immune system (antigenic drift event). Both events have been reported to occur within viruses belonging to the order *Mononegavirales* (Cane PA *et al*., 1995; Li J *et al*., 2012; Kondo H *et al*., 2017; Muñoz-Alía MÁ *et al*., 2017; Yuan C *et al*., 2017). A previous study reported findings of Borna disease virus 1 (BoDV-1) genome segments in samples from wild birds (such as *Anas* spp. and *Coloeus* spp.) in Uppsala, Sweden, which showed an intra-species divergence (Berg M *et al*., 2001). Namely, they revealed to be divergent from each other, as they were divergent from the Borna disease virus (BoDV-1) genotypes detected from naturally infected cat, lynx and horse in the same area (Berg A-L *et al*., 1998). In the present study, the bioinformatics approach suggests that the N and P genes of parrot bornavirus 4 (PaBV-4) have had different ancestors (Figure 6 A and B; and Figure 7 A and B). Namely, within PaBV-4 genotypes, the N gene showed to be evolutionarily close to avian bornaviruses (members of *Waterbird 1 orthobornavirus*) and P gene revealed to be closer to mammalian bornaviruses (members of *Mammalian 2 orthobornavirus*) (Figure 6 and 7). Moreover, the phylogeny conducted based on the P gene segment, revealed that the *Mammalian 2 orthobornavirus* members (VSBV-1 (accession nº LN713681.1 and MF597762.1)) associated with lethal encephalitis of humans in Germany are evolutionarily closest related to PaBV-4 (Figure 7 A). On the other hand, the *Mammalian 1 orthobornavirus* genotypes associated with death of humans in Germany, (accession nº MH190827.1 and LT991983.1), are evolutionarily closer to bornaviruses detected in wild birds (such as ABBV-1, ABBV-2 and BoDV-1 in Denmark, Germany, Sweden and USA) (Figure 7 A). Furthermore, the Borna disease virus 1 (BoDV-1) variants detected in samples of wild *Anas* spp. (accession nº AF232700.1 and AF232701.1) are phylogenetically adjacent to BoDV-1 genotypes associated with lethal outcomes in humans (accession nº LT991983.1 and MH190827.1), as well as to the vaccine strain (accession nº DQ680832.1/rabbit, Germany, 1948) (Figure 7 A). In fact, the Borna disease virus 1 (BoDV-1) detected in samples of wild *Anas* spp. are intermediate genotypes, positioned in the evolutionary line, between the two genotypes that were the cause of death of humans (accession nº MH190827.1 and LT991983.1) (Figure 7 A). When the analysis was conducted based on the amino acid sequence encoded by the P gene segment, the main phylogenetic relationship remained between aquatic bird bornavirus (ABBV-1 to 2), parrot bornavirus (PaBV-4), variegated squirrel bornavirus (VSBV-1) and Borna disease virus (BoDV-1) genotypes (Figure 7 B). However, their evolutionary relationship changed when the phylogenetic analysis was based on the segment of the N gene (Figure 6 A). The findings revealed that the N gene of PaBV-4 genotypes shared their ancestor with avian bornavirus (ABBV-1 and ABBV-2)

detected in wild birds (Figure 6 A). Furthermore, the parrot bornavirus (PaBV-4) and variegated squirrel bornavirus (VSBV-1) genotypes diverged earlier in the evolutionary line, regarding N gene (Figure 6 A). However, parrot bornavirus (PaBV-4) remains evolutionarily closer to variegated squirrel bornavirus (VSBV-1) than to the Borna disease virus (BoDV-1) detected in samples of wild birds (accession nº AF233071.1, AF232702.2 and AF232703.1) and in human tissues (accession nº MH190827.1 and LT991983.1) (Figure 6 A). Interestingly, from an evolutionary point of view, the Borna disease virus (BoDV-1) detected in wild birds remain as intermediate variants between the two genotypes linked to the death of humans, based on their nucleoprotein profile (Figure 6 B).

In addition, wild birds can also disseminate viruses along their migration routes (Ma Y *et al*., 2009**;** Kraus, RHS, 2011). According to published studies, the flow of migratory birds occurs within and between the four main migratory routes (Pacific North of America/Atlantic North of America, East Asian/Australian, Central Asia and Black Sea/Mediterranean) (Ma Y *et al*., 2009**;** Kraus, RHS, 2011), covering the geographical regions where the species *Waterbird 1 orthobornavirus* (e.g. ABBV-2), *Mammalian 1 orthobornavirus* (e.g. BoDV-1 strains) and *Psittaciform 1 orthobornavirus* (e.g. PaBV-2 and PaBV-4 strains) were detected in samples of *Anas* spp. (Berg M *et al*., 2001; Guo J *et al*., 2014; Sassa Y *et al*., 2015). Particularly the *Anas* spp. are considered long-distance transporters of virus, within and between continents (Ma Y *et al*., 2009**;** Kraus RHS, 2011). Therefore, more screening studies of some species of wild birds may provide better understanding of the contribution of these species in the epidemiology of bornaviruses.

In conclusion, this is the first study characterizing at genetic and amino acid level genotypes of parrot bornavirus 4 (PaBV-4) in Portugal. The characterized viruses were phylogenetically related with PaBV-4 worldwide distributed in captive psittacines, suggesting that Portugal is on the route of the international trading, which occurs without biosafety measures. Moreover, the PaBV-4 genotypes detected in Portugal are evolutionarily closer to some genotypes found in wild birds in Japan, than with some of those found in pet psittacines in Europe. More studies are needed to clarify the role of wild birds in the introduction of bornaviruses to captive parrots, if any. The N and X proteins of PaBV-4 are more related with avian bornaviruses detected in samples of wild migratory waterbirds, while phosphoprotein is phylogenetically closer to mammalian bornaviruses, as the variegated squirrel bornavirus (VSBV-1) genotypes linked to lethal encephalitis of humans. Wild migratory birds may be a key element in understanding the intra- and inter-continental emergence and dissemination of pathogenic variants of mammalian and bird bornaviruses.

The role of co-infected wild reservoirs in the emergence of new virulent variants and in the host range modification, should be addressed in future studies. For screening purposes, the highly conserved P gene/protein region is a good candidate as universal target in laboratory diagnostics of bornaviruses.

6.1.5. References

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Chapter 7 - General discussion and conclusions

7.1. Discussion

The infection by bornaviruses cause severe neurological or/and gastrointestinal disease, and the death of humans and animals including mammals, avian, reptiles and fish (pets, farmed and wild animals) (Berg AL *et al*., 1999; Berg AL *et al*., 1998; Berg M *et al*., 2001; Wensman JJ *et al*., 2012; Delnatte P *et al*., 2013; Guo J *et al*., 2014; Wensman JJ *et al*., 2014; Sassa Y *et al*., 2015; Thomsen AF *et al*., 2015; Korn K *et al*., 2018). Among mammalian bornaviruses, genotypes BoDV-1 and VSBV-1 have zoonotic potential (Tappe D *et al*., 2018; Niller HH *et al*., 2020). Besides, some BoDV-1 genotypes also infected ostriches (living in captivity) (Malkinson M *et al*., 1993) and wild birds (such as mallards and jackdaws) (Berg M *et al*., 2001). Several species of Wild birds were infected by a wide range of avian bornaviruses (several genotypes of ABBV-1 to 2 and PaBV-2 to 4) (Berg M *et al*., 2001; Delnatte P *et al.*, 2013; Guo J *et al*., 2014; Sassa Y *et al*., 2015; Thomsen AF *et al*., 2015). Also genotypes PaBV-2 and PaBV-4 coinfected some species of wild birds (Sassa Y *et al*., 2015). Besides, several PaBV-2 and PaV-4 genotypes were detected in captive psittacines (Sa-Ardta P *et al*., 2019). Despite the produced knowledge on bornaviruses there are remaining gaps of knowledge regarding its epidemiology. It is, for example, unknown if wild birds have a role in favouring the emergence and spread of novel pathogenic strains of bornaviruses, and in introducing the virus to captive flocks.

In addition, infection by PaBV-1 to 8 becomes a concern in the context of psittacine flocks from zoos, reserves, breeding projects for rare species and private collections (Kistler AL *et al*., 2010; Lierz M *et al*., 2011; Monaco E *et al*., 2012; Rubbenstroth D *et al*., 2016; Nielsen AMW *et al*., 2018; Sa-Ardta P *et al*., 2019). However, the reported geographic spectrum of the infection may have effects on the welfare, health and biodiversity of psittacines, and is most likely only the top of the iceberg; for instance, studies from Europe were conducted predominantly on the central region. Furthermore, the bornavirus can be a long-term problem because it can persist for several years in captive flocks, being that asymptomatic infected psittacines have been suggested as infectious focus (Gancz AY *et al*., 2010; Hoppes SM *et al*., 2013). However, there is no consensus regarding the route(s) of bornavirus transmission in captive flocks (Kerski *et al*., 2012; Rubbenstroth D *et al*., 2013; Rubbenstroth D *et al*., 2014; Heckmann J *et al*., 2017), as well as the risk factors associated with the occurrence of outbreaks (Kistler AL *et al*., 2010; Lierz M *et al*., 2011; Monaco E *et al*., 2012; Rubbenstroth D *et al*., 2016; Nielsen AMW *et al*., 2018; Sa-Ardta P *et al*., 2019). Therefore, the surveillance of captive psittacines aiming to segregate asymptomatic members is an essential preventive measure. Equally crucial is the laboratory confirmation

of the suspected clinical cases to choose the better clinical approach to follow; once other infectious agents produced the same outcomes (Richt JA *et al*., 2001; Dauphin G *et al*., 2002; Shivaprasad HL *et al*., 2012; Dhama K *et al*., 2013; Done LB *et al*., 2014; Guimarães MB *et al*., 2014; Dhama K *et al*., 2015; Mohammad Saleh MS *et al*., 2016). However, there is a lack of cost-effective diagnostic tools reported in the literature to detect bornavirus infection in live animals.

In the following paragraphs are discussed the produced knowledge on bornavirus epidemiology and their detection in living animals.

The quantitative real-time PCR SYBR® Green-based assay established showed high performance. The overall performance of the assay resulted from the combined performance of the first and the second reaction. Namely, the real-time PCR SYBR® Green-based assay showed a wide dynamic range (of 8 orders of magnitude), a high capacity (the upper detection limit was $2X10^7$ copies of the phosphoprotein (P) gene), a high sensitivity (the lower detection limit was 2 copies of the phosphoprotein (P) gene) and high efficiency (98.92%). It also showed accuracy (the absolute standard curve showed a correlation coefficient of $R^2>0.99$) and reproducibility (the standard deviation of the cycle threshold means was less than or equal to 0.5 within and between experiments).

The facts discussed above show that the produced quantitative real-time PCR SYBR® Green-based assay is an advantageous alternative to both described and published real-time PCR TaqMan® probe-based assays (Schindler AR *et al*., 2007; Wensman JJ *et al*., 2007). Firstly, the use of fluorescent dyes (as the SYBR® Green) instead of probes (as the TaqMan®) reduce assay setup and running costs (Applied Biosystems, 2016). Therefore, the SYBR® Green-based assay developed is suitable to screen large populations of animals. Secondly, the assay sensitivity established in this study was five times higher than the two published TaqMan®-based assays (Schindler AR *et al*., 2007; Wensman JJ *et al*., 2007). Thirdly, the quantification system developed has an increased lifespan, and its preparation is less time-consuming (Applied Biosystems, 2016). Fourthly, a two-step real-time PCR approach allows the maximization of samples usage, production of high amounts of cDNA, conducting multiple tests, and making cDNA stock from a small amount of RNA (Applied Biosystems, 2016). Fifthly, for bornaviruses detection in living animals, the assay developed is an advantageous alternative to the previously reported tests in the literature, such as the immunohistochemistry, histological, serological, conventional PCR assays and viral isolation methods. They required tissue samples (biopsies and blood) collected by invasive procedures (e.g. immunohistochemistry, histology and serology), produce inconsistent results (e.g. serologic techniques), are time-consuming (e.g. conventional PCR and viral isolation methods) and expensive (e.g. viral isolation methods,

immunohistochemistry, histology and serology) (Staeheli P *et al*., 2000; Richt JA *et al*., 2001; Dauphin G *et al*., 2002; Gancz AY *et al*., 2010; Wensman JJ *et al*., 2014; Hoppes SM *et al*., 2020).

Therefore, for the reasons reported and discussed above, the SYBR® Green-based assay characteristics make it an ideal diagnostics tool in the surveillance of psittacines housed in reserves, breeding projects of rare species, private collections and zoos. The SYBR® Green-based assay also will be a cost-effective approach for the surveillance studies of wild populations of birds.

Regarding the two-step real-time PCR TaqMan®-based assay developed, it also showed high overall performance. Namely, the assay showed a wide dynamic range (8 orders of magnitude), as high capacity ($2X10^7$ copies of the P gene), sensitivity (2 copies of the P gene) and efficiency (99.02%). As well as it showed accuracy (R^2>0.99) and reproducibility (Ct>= 0.5 within and between experiments). Therefore, the TaqMan®-based assay established showed to share advantages with the SYBR® Green-based assay developed in this study. Namely, the assay showed added-value comparatively with the published real-time PCR TaqMan®-based assays, regarding the reasons expressed in the previous paragraph (Schindler AR *et al*., 2007; Wensman JJ *et al*., 2007). However, compared to the SYBR® Green-based assay developed in this study, it is more suitable as a complementary tool for clinical diagnosis. SYBR® Green-based assays have more risk to detect eventual false-positives targets in the reaction; because the SYBR® dye binds to any double-stranded DNA, binding to nonspecific double-stranded DNA (Applied Biosystems, 2016). Consequently, it is critical to determine the genotype and melting curves of bornaviruses in samples testing positive by real-time PCR SYBR® Green-based assay. Furthermore, it implies additional time (which often takes more than a day) to confirm that the PCR products generated are from bornavirus and not an artefact. Consequently, the increased response time makes this approach disadvantageous within the scope of clinical diagnosis.

Therefore, the TaqMan®-based assay developed is a cost-effective tool to detect and quantify bornavirus-RNA in living animals with clinical signs compatible with infection.

Additionally, the research conducted allowed the identification and characterization of avian bornaviruses, circulating in Portugal and Sweden, at genetic and protein levels (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). The research also contributed to the molecular epidemiologic knowledge improvement about bornaviruses (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). Before the present research work (Pinto MC *et al*., 2018), no published studies of avian bornaviruses infecting pet parrots in Portugal and Sweden have been done.

The research allowed the identification of parrot bornavirus 4 (PaBV-4) as the cause of death of pet psittacines in Portugal and Sweden (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). The PaBV-4 genotypes identified in Portugal and Sweden belong to one of the five genetic clusters established, considering the N gene (nucleoprotein) of 100 PaBV-4 genotypes (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). Moreover, the PaBV-4 genotypes identified in both countries belong to the same genetic cluster (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). On the other hand, among PaBV-4, was observed four clusters for X protein and were not found clusters considering P protein (Pinto MC *et al*., 2018). Besides, the P protein (phosphoprotein) shown to be more conserved within the family *Bornaviridae* than the X protein (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). The degree of conservation was not constant throughout the P protein (Pinto MC *et al*., 2019). Namely, the region showing a higher degree of conservation intra-species and inter-species of bornaviruses is the shared amino acid sequence with X protein (Pinto MC *et al*., 2019). Thus, the highly conserved regions found in the virus genome became a suitable candidate as a universal target in laboratory diagnostics for screening purposes of potential reservoirs or asymptomatic carriers and to confirm the clinical diagnosis. In molecular diagnostic assays, a decrease in the number of false-negative results is most likely to occur when highly conserved genomic regions of the virus are used as targets, because they likely improve the detection odds of unknown bornaviruses that eventually are circulating. The eventual unidentified bornaviruses circulating most likely share with the identified species highly conserved genomic regions. Therefore, information produced about the highly conserved region is relevant since an expected diversity of members of the family *Bornaviridae* is likely to be higher than reported, given the advances achieved in the last two years (Amarasinghe GK *et al*., 2019). Furthermore, using highly conserved genomic regions as targets in molecular diagnostics tools allows searching simultaneously for several bornaviruses species described so far. Consequently, the time-consuming and costs linked with laboratory diagnostics become reduced by decreasing the number of setups and running assays required.

Bioinformatics analysis (considering PaBV-4 genotypes (n=100)) suggested that an intensive international trade of psittacines is going on (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019); as well as Portugal and Sweden are on the world trade route of psittacines (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). It also suggests that the horizontal transmission in the captivity context likely occurred (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). Therefore, the overall bioinformatics findings showed a worldwide distribution of PaBV-4 infection in captive psittacines flocks, suggesting that infection of bornavirus is a consequence of the extensive trading without biosafety measures (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019).

Over the years, international trading without biosafety measures likely had implications on psittacine biodiversity by spreading the infection leading to the death of captive psittacines and consequently reducing the genetic background diversity of populations (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). Whereas, at least, in the last 19 years, the trade of psittacines contributed to the death of members from endangered species by PaBV-4 infection (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019).

The bioinformatics results also showed that wild birds (*Anas* spp., *Emberiza* spp., and *Grus* spp.) and pet psittacines shared PaBV-4 genotypes (Pinto MC *et al*., 2018; Pinto MC *et al*., 2019). Therefore, findings suggested that the wild birds and the world trade of psittacines, simultaneously or singularly, contributed to the dissemination of PaBV-4 infection. Besides, results suggest that wild birds could play a role in the infection introduction in captive flocks.

The research conducted also showed that the N and the P gene of PaBV-4 had different ancestors. Specifically, the N gene of PaBV-4 likely shared an ancestral with members of *Waterbird 1 orthobornavirus* species (ABBV-1 and ABBV-2, detected in wild birds) (Pinto MC *et al*., 2019). On the other hand, the P gene more possibly shared an ancestor with members of *Mammalian 2 orthobornavirus* species (with the zoonotic VSBV-1, which was associated with lethal encephalitis of humans) (Pinto MC *et al*., 2019). Additionally, the X protein showed a greater degree of identity with the zoonotic VSBV-1 than with some members of avian bornaviruses (Pinto MC *et al*., 2019). Therefore, the findings suggest that events of gene recombination between ancestral entities of bornaviruses had occurred. Moreover, the present research showed that the BoDV-1 genotypes detected in wild birds (Berg M *et al*., 2001) were evolutionarily intermediate versions of BoDV-1 genotypes associated with lethal encephalitis of humans (Pinto MC *et al*., 2019). The gene recombination events between virus strains and the accumulation of amino acid changes due to the host pressure (antigenic drift event) induced changes in virulence and host range of viruses (Cane PA *et al*., 1995; Li J *et al*., 2012; Kondo H *et al*., 2017; Muñoz-Alía MÁ *et al*., 2017; Yuan C *et al*., 2017). And the gene recombination events as the antigenic drift events were described in members of the order *Mononegavirales* (Cane PA *et al*., 1995; Li J *et al*., 2012; Kondo H *et al*., 2017; Muñoz-Alía MÁ *et al*., 2017; Yuan C *et al*., 2017). Additionally, co-infection events by different genotypes of PaBV-2 and PaBV-4 had been documented to occur in wild birds (*Anas* spp.) (Sassa Y *et al*., 2015). Nevertheless, there were species of wild birds (*Anas* spp.) that hosted a wide range of genotypes from several species of bornaviruses (such as parrot bornavirus 2, Borna disease virus 1, aquatic bird bornavirus 1 to 2) (Berg M *et al*., 2001; Delnatte P *et al*., 2013;

Guo J *et al*., 2015; Sassa Y *et al*., 2015; Thomsen AF et al., 2015). Then, it is likely that all members of the family *Bornaviridae* can participate in co-infection.

Therefore, the findings suggest that wild birds can play a role in the emergence of novel virus variants of the family *Bornaviridae*. Besides, they can also play a role in spreading novel bornaviruses along their migration routes, within and between countries. Particularly the *Anas* spp. are considered long-distance transporters of virus, within and between continents (Ma Y *et al*., 2009; Kraus RHS *et al*., 2011). The four principal migratory routes (Ma Y *et al*., 2009; Kraus RHS et al., 2011) cover the geographical regions where wild *Anas* spp. infected with several strains of bornaviruses were found (Berg M *et al*., 2001; Delnatte P *et al*., 2013; Guo J *et al*., 2015; Sassa Y *et al*., 2015; Thomsen AF *et al*., 2015).

In summary, this Thesis provides new data on cost-effective real-time PCR assays to detect reservoirs of bornavirus and assist clinical diagnosis, as knowledge on bornavirus epidemiology. For instances, a highly conserved genome region was identified within the family *Bornaviridae*, becoming an ideal universal target for molecular diagnostics tools. The molecular epidemiology also positioned Portugal and Sweden on the world trade route of psittacines. And it suggested that world trade occurs without biosafety measures, leading to the death of endangered species, at least in the last 19 years. The horizontal transmission of bornavirus, within the order of *Psittaciformes* and between species of psittacines in captive flocks, was also supported by molecular epidemiology. The findings also suggested that wild *Anas* spp. most likely play several roles in the epidemiology of bornaviruses. Moreover, the research conducted allowed to hypothesize that genetic recombination events occur within and between different members belonging to the family *Bornaviridae*.

7.2. Conclusions

In the present Thesis, studies performed on bornavirus infection in companion animals are discussed in the light of the current knowledge, allowing the following conclusions:

- The two-step quantitative real-time PCR SYBR® Green-based assay developed showed high performance (revealing a wide dynamic linear range of detection, accuracy, reproducibility, high capacity, high sensitivity and high efficiency) and would be ideal as a cost-effective tool for screening large populations to detect bornavirus reservoirs.

- A cost-effective real-time PCR assay, TaqMan®-based with high range of detection, was developed, ideal to evaluate animals suspected to be infected by bornavirus to assist clinical diagnosis.

- The research allowed for the first time the characterization of avian bornaviruses found in Sweden and Portugal, regarding their genetic and proteins profiles. The research allowed to identify and characterize genotypes of parrot bornavirus 4 (PaBV-4) linked with the death of pet psittacines in Portugal and Sweden. The identified avian bornaviruses showed to be phylogenetically adjacent to the worldwide reported PaBV-4 that infected pet psittacines. Besides, the identified PaBV-4 genotypes revealed to be more related to some genotypes detected in wild birds than in pet psittacines. Moreover, the phosphoprotein of PaBV-4 showed a higher degree of similarity with mammalian bornaviruses (genotypes of the zoonotic VSBV-1) than with some avian bornaviruses.

- The genetic epidemiology suggests that horizontal transmission of bornavirus within and between different taxonomic groups of psittacines (orders and species) occurred in captive flocks. As well, it proposes that there is worldwide trading of psittacines without biosafety measures. Besides, it suggests that the world trade had consequences on biodiversity, reducing the number of psittacines of endangered species due to bornavirus infection.

- The molecular epidemiology showed that phosphoprotein is more conserved than X protein between and within species of bornavirus. Moreover, the degree of conservation was higher in the region shared with the X protein. Therefore, the high conserved regions of amino acid are a suitable as universal target for laboratory diagnosis. Also, findings showed that the X protein and nucleoprotein of PaBV-4 genotypes shared an ancestral with avian bornaviruses, while phosphoprotein shared an ancestor with *Mammalian 2 orthobornavirus*. Therefore, findings suggest that events of gene reassortment happened between members of the genus *Orthobornavirus*. The genetic and molecular epidemiology of bornavirus infection in wild birds suggest that wild birds may have a role in bornaviruses introduction at flocks of captive psittacines. Research findings also allowed the formulation of the hypotheses that wild birds can be involved in the emergence of novel pathogenic bornaviruses by favouring gene rearrangement events as co-infected reservoirs.

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Chapter 8 - Future perspectives

8. Future perspectives

The produced and reported results, in the present Thesis, contribute to the improvement of knowledge on bornavirus infection. The achieved findings allowed the identification of new research opportunities. In the following paragraphs the studies on bornaviruses that future research can address are identified.

- The knowledge about the effects and dimension of bornaviruses infections in health and wellbeing of animals, as well as, in Public Health is most likely only the top of the iceberg. Therefore, the search of infection and its outcomes in several species of companion and production animals should be continuously addressed.

- The achieved findings suggest that reassortment events occurred naturally between members of the *Bornaviridae* family. They suggest that recombination of genetic material between bornaviruses of mammalians and avian can occur. Namely, the findings suggest that the zoonotic VSBV-1 (variegated squirrel bornavirus 1) has a mosaic-like genome, containing regions from bornaviruses of mammalian and avian. Therefore, in the future, additional studies should be conducted to evaluate the occurrence of recombination events among members of the *Bornaviridae* family, as hypothesised in the present Thesis.

- The bioinformatics analyses, produced on the scope of the present Thesis, showed that wild birds (*Anas* spp.) hosted bornaviruses that, from an evolutionary point of view, turned out to be intermediate entities of the zoonotic BoDV-1 (Borna disease virus 1) genotypes. Also, *Anas* spp., in literature, were identified as hosts of several genotypes belonging to the species *Psittaciform 1 bornavirus* and *Waterbird 1 bornavirus*, virus that revealed to be pathogenic for birds hosted in zoos and pets. Moreover, in the literature, coinfection events by several members of the *Psittaciform 1 bornavirus* species have also been reported in wild *Anas* spp.. For the reasons stated above, the addressing of the role of migratory wild birds in the emergence and dissemination of novel bornaviruses, of public health and veterinary concern is to be considered in the future.

- The molecular diagnostics tools, routinely implemented in laboratories, should be continuously upgraded to decrease the occurrence of false-negative results. Thus, the search of universal targets in genomes/proteins of bornaviruses will be an added-value approach for the continuous improvement of molecular diagnostics tools in non-academic laboratories. For that reason, the future studies should address, the search of novel highly conserved regions, throughout genomes/proteins of bornaviruses.

Appendices

1) Epidemiologic questionnaire

Laboratório de Microbiologia e Doenças Infecciosas

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Aves

Questionário clínico

MÉDICO VETERINÁRIO QUE REALIZOU A CONSULTA E DATA		
Dia/mês/ano: __/__/____	Médico Veterinário: _____	Código (laboratório): _____

INFORMAÇÃO SOBRE A AVE E HISTÓRICO CLINICO			
Dados demográficos	Nome do animal: _____		N.º atribuído _____
	Espécie (nome comum): _____		Sexo: ♀ ❑ ♂ ❑
	Espécie (nome cientifico): _____		Idade (meses): _____
	Concelho de residência: _____		Peso (g): _____
	Ave importada? Não ❑ Sim ❑ Qual o pais de origem? _____		
	Proprietário	Columbófilo ❑ Coleccionador ❑ Criador/vendedor de aves ❑ Zoológico ❑ Outro/Qual? _____	
Histórico clínico	Patologias	Doenças infecciosas ❑ Microrganismo: _____	
		Parasitárias ❑ Agente: _____	
		Outras/Quais? _____	
		Observações: _____	

TIPO DE CONSULTA E DESFECHO	
Tipo consulta	1ª Consulta ❑ Rotina ❑ Vacinação ❑ Seguimento ❑ Internamento ❑ Emergência ❑ Outro/Qual? _____
Desfecho da consulta	Indicação para seguimento ❑ Data da próxima consulta (dia/mês/ano): __/__/____
	Indicação para internamento ❑ Morte ❑ Outro /Qual? _____
Observações: _____	

SINAIS NERVOSOS / ALTERAÇÕES COMPORTAMENTAIS OBSERVADAS			
Observado pelo médico veterinário ou reportado pelo proprietário / acompanhante da ave	Arranque de penas ❑	Agressividade ❑	Ataxia ❑
	Alterações da postura ❑	Perda de peso ❑	Dispneia ❑
	Défices proprioceptivos ❑	Défices motores ❑	Tremores ❑
	Sementes não digeridas fezes ❑	Perda de apetite ❑	Convulsões ❑
	Movimentos anormais da cabeça ❑	Depressão/apatia ❑	Automutilação ❑
	Outros /Quais: _____		

INFORMAÇÃO SOBRE AMOSTRAS RECOLHIDAS				
Tipo de amostra	Número/quantidade		Temp. armazenamento	Data de recolha
Fezes ❑	Quantidade: ≈ _____ g		[2-8] ºC ❑	(Dia/mês/ano): __/__/____
Sangue ❑	Volume: _____ml		[2-8] ºC ❑	
Zaragatoa da cloaca ❑	1 zaragatoa ❑	2 zaragatoas ❑	[2-8] ºC ❑ ou [-20] ºC ❑	
Zaragatoa orofaringe ❑	1 zaragatoa ❑	2 zaragatoas ❑	[2-8] ºC ❑ ou [-20] ºC ❑	

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2) Research work disseminated in the format of communications at scientific meetings

Congresso Hospital Veterinário Montenegro 2018 | 24 – 25 February | Europarque | Santa Maria da Feira | Portugal

EPIDEMIOLOGY OF PARROT BORNAVIRUS 4: GENOTYPE CLUSTERS, GEOGRAPHIC DISTRIBUTION, HOST SPECIES AND THE RELATIONSHIP WITH MAMMALIAN BORNAVIRUSES

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INTRODUCTION

The genus *Bornavirus* comprises non-segmented, negative-stranded RNA viruses able to infect humans (Hoffmann *et al.*, 2015), mammals (Tizard *et al.* 2016), birds and reptiles (Horie *et al.*, 2010). Within avian bornaviruses (ABV), parrot bornavirus 1 to 8 cause infection with clinical relevance in psittacines (Tizard *et al.* 2016). Until now, it has been unknown which ABV species/genotypes are circulating in Sweden and how they are related to reported strains from other countries.

The aim was to characterize, for the first time, the parrot bornavirus 4 (PaBV-4) from Swedish Blue-winged macaw (*Primolius maracana*), studying its relationship with isolates reported worldwide, based on genetic and protein profiles. In addition, we intended to evaluate the relationships between several ABV genotypes already reported and their geographic distribution, host species, time of sampling and mammalian bornaviruses.

METHODS

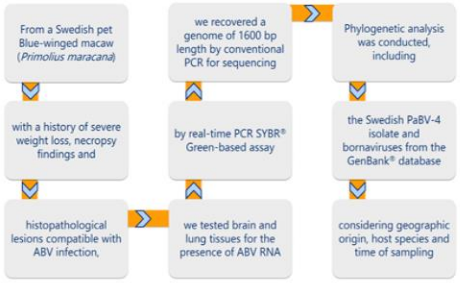

Figure 1. Flowchart of the material and methods used to conduct the study.

RESULTS

The nucleotide sequences obtained in this study were identified as parrot bornavirus 4 (PaBV-4), belonging to the *Psittaciform 1 bornavirus*. The X and P proteins of the Swedish isolate shared a higher homology with *Mammalian bornavirus 2* (VSBV-1) than with aquatic bird bornavirus 2 (*Waterbird 1 bornavirus*) and canary bornavirus 1 (*Passeriform 1 bornavirus*).

Table 1. Identity between the Swedish PaBV-4 and members of Genus *Bornavirus*.

Swedish PaBV-4	Genes "N", "X", "P", "M"		X protein		Phosphoprotein	
Bornaviruses	Id. (%) [a]	E value [b]	Id. (%) [a]	E value [b]	Id. (%) [a]	E value [b]
Avian [c]						
Psittaciform 1 bornavirus	82 – 99	0	75 –100	$2e^{-63}$ – $3e^{-57}$	94 – 100	$3e^{-148}$ – $8e^{-82}$
Psittaciform 2 bornavirus	71 – 72	0	53	$1e^{-28}$ – $1e^{-07}$	69	$6e^{-87}$
Passeriform 1 bornavirus	70 – 71	0	51 – 57	$4e^{-31}$ – $3e^{-27}$	68 – 70	$5e^{-96}$ – $8e^{-96}$
Passeriform 2 bornavirus	71	0	63	$3e^{-36}$	70	$8e^{-82}$
Waterbird 1 bornavirus	69 – 71	0 – $6e^{-179}$	54 – 56	$9e^{-30}$ – $4e^{-27}$	68 – 69	$4e^{-85}$ – $3e^{-83}$
Mammalian [d]						
Mammalian 1 bornavirus	69	$2e^{-153}$ – $9e^{-139}$	45 – 49	$5e^{-23}$ – $3e^{-22}$	62 – 64	$4e^{-95}$ – $6e^{-93}$
Mammalian 2 bornavirus	70	$5e^{-161}$	52	$1e^{-25}$	69	$1e^{-95}$
Reptilian [e]						
Elapid 1 bornavirus	68	$6e^{-91}$	37	$1e^{-6}$	43	$1e^{-50}$
Gabbon viper virus 1 [f]	---	---	---	---	43	$1e^{-45}$

[a] The highest percent identity of all query-subject alignment given by Basic Local Alignment Search Tool (BLAST®).
[b] the best (lowest) Expect value (E value) of all alignments from that database sequence given by BLAST®.
[c] Avian bornaviruses considered in the analysis were the same used to construct the phylogenetic trees.
[d] Mammalian bornaviruses considered in the analysis were the same used to construct the phylogenetic trees.
[e] Reptilian bornaviruses considered in the analysis were the same used to construct the phylogenetic trees.
[f] The database of the GenBank® only reported information for the P gene/protein.

Figure 2. Worldwide distribution of 93 parrot bornavirus 4 (PaBV-4) genotypes sampled between 2000 and 2015, reported in GenBank® database.

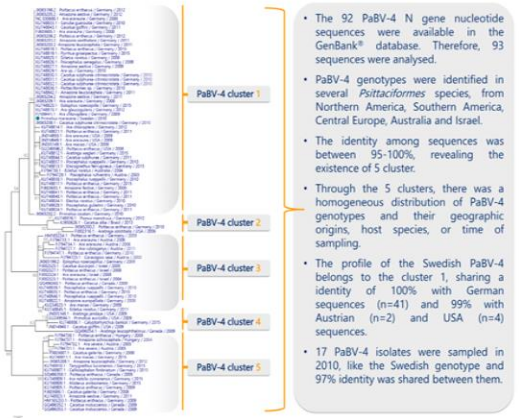

Figure 3. Phylogenetic analysis of partial PaBV-4 N sequences. Isolates identified with Genbank® accession no., name of the host, country origin and time of sampling. The sequence marked with a circle was produced during this study. Neighbor-Joining method, Maximum Composite Likelihood method and 1,000 bootstrap replicates were computed.

CONCLUSION

- **In Europe, PaBV-4 has been circulating**, at least, since 2000, eight years before the first published description of avian bornaviruses.
- **There was no relationship between PaBV-4 genotypes** and their geographic origins, host species, or time of sampling. Additionally, the high nucleotide identity (100%) between isolates of PaBV-4 suggests a spread within and between avian orders and species. These findings suggest horizontal and vertical transmission in captive flocks, as there is extensive trading without biosafety measures, which could avoid spreading.
- **Concerning X and P protein profiles**, the Swedish PaBV-4 isolate is more evolutionarily distant from some avian bornaviruses than from the German VSBV-1 isolate, reported in 2015, detected in the brain of squirrel breeders who died from neurological disorders.

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Real-time PCR TaqMan®-based detection assay of Bornavirus for challenging specimens

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Background

Bornavirus is a non-segmented, negative-stranded RNA virus, which can cause a severe neurological and/or gastrointestinal disease and death (Kinnunen et al., 2013). In 2015, the Center for Disease and Control recognized the zoonotic potential of some bornaviruses (Hoffmam et al.; 2015). The infection has been reported, worldwide, in humans, several species of pets, production and wild animals (Kinnunen et al., 2013). The facts point to the importance of its surveillance in public health issues as well as in the health and wellbeing of animals.

The diagnosis of Bornavirus of live animals is challenging, as the gold-standard diagnosis has been the histological examination and immunohistochemistry (Gancz et al., 2010); with the need of collect tissues from central nervous systems. Whereby, it is useful to develop specific, accurate, sensitive and specific tool for viral RNA detection from biological samples obtained by non-invasive methods.

We aimed to develop a two-step real-time PCR TaqMan®-based assay for the detection of bornavirus-RNA from cloacal/anal swabs or faeces.

Material & methods

- **Vero Cells infected with BDV–He/80 strain**
  - Isopropanol/chloroform method for RNA extraction.
  - RNA purified using a spin column technology.
  - Concentration and purity tested by spectrophotometry.
- **First strand cDNA synthesis**
  - Based on manufacturer's instructions.
  - With 1 ul of total RNA using random primers.
- **Optimal concentration probe/primer**
  - Probe/primers used to target the P gene of several Bornavirus strains. ‡
  - Several concentrations of the target and the probe/primers were tested.
  - The lowest C_T and maximum ΔRn used to define the optimal concentration.
- **The linearity & efficiency of the assay**
  - The capacity, linearity and efficiency of the TaqMan®-based assay were established by constructing a standard curve. ‡‡
- **Evaluation of animal samples**
  - Cloacal swabs and faeces of companion animals showing symptoms compatible with Bornavirus infection were evaluated.

Figure 1. The methodology flowchart: ‡ The specificity of the primers and probe was reported previously (Wensman et al., 2012). ‡‡ The standard curve constructed was a semi-log regression line plot of cycle threshold (C_T) values vs. $\log_{10}$ of 10-fold serial dilutions of the BDV-He/80 P gene copies. Sixteen results per dilution point obtained by performing four replicas of each 10-fold serial dilution and four runs of the standard curve were used. The last dilution in which all four replicas gave a positive and specific amplification, was considered the lower limit of detection (LOD4). The efficiency of the assay was calculated using the formula: $(10^{(-1/a)} - 1) \times 100\%$, where a is the slope of the standard curve (Applied Biosystems, 2014).

Results

- Optimal concentration of primers/probe

The optimal concentration of primer/probe was 0.7 µM, giving the lowest threshold cycle (C_T) and the maximum magnitude of fluorescence signal (ΔRn).

- The performance of the assay

The performance parameters of the TaqMan®-based assay were reported in table 1. The dynamic range, regression coefficient, inter assay standard variance of the C_T values (range from 0.21 to 0.99) and slope used to calculate the efficiency were reported in the standard curve (figure 2).

Table 1. The performance of the real-time PCR

Efficiency (Ɛ)	99.5%
Capacity	5 X 10^{10} copies §
LOD_4	5 copies §
Linearity	(5 X 10^{10} - 5) copies §

Legend: § - The copies number of P gene per reaction (in 30 µl). LOD_4 – The lower limit of detection.

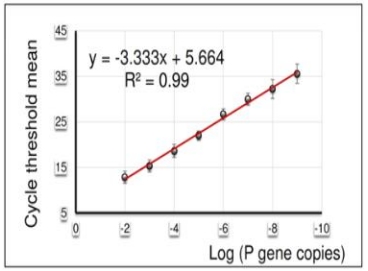

Figure 2. The standard curve (a semi-log regression line plot of cycle threshold (C_T) values Vs. $\log_{10}$ of 10-fold serial dilutions of BDV-He/80 P gene copies.

- Evaluation of the companion animal samples

The bornavirus-RNA were detected in faeces of a psittacine (C_T=37.13), (figure 3).

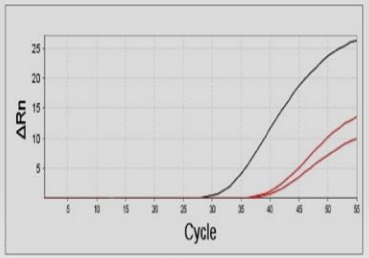

Figure 3. The magnitude of fluorescence signal Vs. C_T values. – BDV-He/80 strain. – Psittacine.

Conclusions

- This assay is highly specific, sensitive and accurate to detect RNA from cloacal/anal swabs or faeces; which are challenging samples, with real-time PCR inhibitors and limited genetic targets.
- Consequently, it is a tool of choice for clinical diagnosis and for the Bornavirus detection related to Public Health issues.

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European Congress of Virology 2016 | 19 – 22 October | Hamburg

Real-time PCR SYBR® Green-based detection assay for rapid screening and surveillance of *Bornavirus*

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INTRODUCTION

Bornavirus is a non-segmented, negative-stranded RNA virus, which can cause a neurological syndrome and/or gastrointestinal disease and death. The disease occurrence has been reported in humans, pets, production and wild animals, and the infection has also been described in apparently healthy pets and wild animals. In 2015, the European Center for Disease and Control recognized the zoonotic potential of *Bornavirus*. The facts point to the importance of its surveillance in public health issues as well as in the screening of animals housed in reserves, breeding projects of rare species, private collections of birds and zoos as a way to prevent severe effects at emotional, financial and biodiversity levels. Whereby, for surveillance and screening purposes it is useful to develop rapid, accurate, specific and cost-effective tools for viral RNA detection from biological samples preferably obtained by non-invasive methods.

In this study, we aimed to develop a real-time polymerase chain reaction (RT-PCR) assay for the detection of bornavirus-RNA from cloacal/anal swabs or faeces.

MATERIAL & METHODS

BDV-He/80 strain and specimens from animals

The Borna Disease Virus, strain He/80 was used in optimization and validation of the real-time PCR assay. The specimens tested were cloacal swabs collected from parrots in consultation in a veterinary clinic. The methodology used in optimization and validation of the assay, and to test specimens is described in the following flowchart:

Figure 1. Flowchart of the methodology: ● **RNA preparation:** RNA was extracted with NYZol (NzyThec, Lisbon, Portugal) according to the manufacturer's protocol, the concentration and the purity were estimated using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). ● **Reverse transcription (RT):** One µg of total RNA extracted was used for cDNA synthesis, in Thermal Cycler PTC-100TM, with the RevertAid RT Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA USA), according manufacture instructions. ● **Real-time polymerase chain reaction (PCR) design:** 1 µl of cDNA, a set of primers (Wensman *et al*, 2012) targeting a conserved region of the phosphoprotein (P) gene, covering several strains of *Bornavirus*, were used with the SyBr® Green Master Mix (Applied Biosystems, USA), under the concentrations and thermal cycling conditions established in manufacturer's protocol. ● **Optimisation of the primers concentration:** The cDNA, synthetized from total RNA extracted from Vero cells persistently infected with BDV-He/80, was used in a range of 10^{-1} to 10^{-4} dilutions. The primers were tested at 0.05 µM, 0.15 µM, 0.25 µM, 0.3 µM, 0.45 µM, 0.55 µM and 0.9 µM. All titration series were done in quadruplicate. ● **Performance of the reverse transcription:** The capacity, linearity and efficiency were established by constructing a standard curve; (a semi-log regression line plot of cycle threshold values *vs.* $\log_{10}$ of cDNA at dilutions 10^{-4} to 10^{-11}). It was performed 4 replicas of each cDNA dilution and 4 runs of the standard curve, obtaining 16 results per dilution point. The last dilution, in which all 4 replicates gave a positive and specific amplification, was considered the limit of detection (LOD_R) (Broeders *et al*; 2014). The efficiency (ε) was calculated using the formula: $(10^{(-1/a)} - 1) \times 100\%$, where a is the slope of the standard curve. ● **Absolute quantification of the phosphoprotein (P) gene:** Serial 10-fold dilutions of the segment of the P gene ((0.1–10^{-9}) ng/µl) were used to construct the standard curve (a semi-log regression line plot of cycle threshold values *vs.* $\log_{10}$ of [DNA]).

The capacity, linearity and efficiency were established as described in the item 5.

RESULTS

Optimization of primers concentration

The optimal primer concentration was 0.3 µM, giving the lowest threshold cycle (C_T) and the maximum magnitude of fluorescence signal (ΔRn), without nonspecific amplification.

Performance of the reverse transcription (RT)

The results of the linear dynamic range of the assay and its efficiency are reported in figure 2 and table 1.

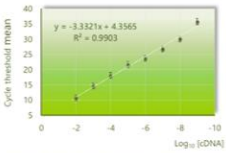

Table 1. Performance of the RT assay.

Efficiency (E)	99.58%
Capacity	39.8 pg/µl
Linearity	(39.8 – 39.9 X 10^{-5}) pg/µl

Figure 2. The Reverse Transcriptase linear range.

Absolute quantification of the phosphoprotein (P) gene

The real-time PCR SYBR Green-based detection assay had a linear dynamic range between $5x10^6$ and 1 copies of the P gene per reaction (figure 3). The lower limit of detection was 1 copy of the P gene per reaction (figure 3). These results were consistent in all 4 replicates of the lowest dilution (intra-assay) and in all 4 experiments conducted (inter-assay) (figure 3). The efficiency of the assay was estimated in 99,66% (table 2).

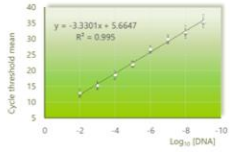

Table 2. Real-time PCR assay performance.

Efficiency (E)	99.66%
Capacity	5 X 10^6 copies *
Linearity	(5 X 10^6 - 1) copies *

Legend: * The copies number of P gene, in 25 µl.

Figure 3. The linear range of the real-time PCR assay.

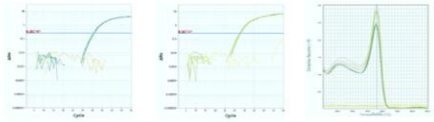

Figure 4. The amplification plot relative to psitaccid A (left) and psitaccid B (midle). The respective melting curves. (right).

CONCLUIONS

- ✓ This two-step RT-PCR assay is rapid, sensitive and accurate to detect RNA from cloacal/anal swabs or faeces. These are challenging specimens, with RT-PCR inhibitors and limited genetic targets.
- ✓ Consequently, this assay is a tool of choice for screening when compared with the laborious conventional PCR.
- ✓ In addition, this method can be used for the surveillance of *Bornavirus* infections in related Public Health issues, as a cost-effective tool, compared with TaqMan® based assays.

1st MEETING OF THE VETERINARY SCIENCES DOCTORAL PROGRAMME

15 de Abril 2016

3.2. *Bornavirus* infection in a psittacine with neurological and gastrointestinal disorders: a case report from Portugal

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Background: Proventricular dilatation disease (PDD) is a form of the progressive lethal neurologic disease of birds worldwide. PDD has been reported to occur in species of psittacines, toucans, honeycreepers, weaver finches, waterfowl, raptors, and passerines [1]. PDD was first recognized in the 1970's and twelve years later *Avian bornavirus* (ABV) was identified as its etiologic agent [2]. ABV belongs to the genus *Bornavirus* and shares with its relative, the zoonotic *Borna disease virus*, about 65% of nucleotide sequence identity [2,3]. PDD is considered an infectious disease based on its spread through aviaries and a threat to aviculture and private collections of endangered psittacines species [1]. The clinical presentation of PDD can be neurologic, gastrointestinal, or a combination of both [1]. The effects of *Bornavirus* infection on the health and wellbeing of animals and humans is still unknown in Portugal.

Aim: We report a case of *Bornavirus* infection in a psittacine with neurologic and gastrointestinal disorders.

Methods: The psittacine pet fulfilled the inclusion criteria based on clinical signs (neurologic and gastrointestinal). We developed two real-time polymerase chain reaction (PCR) assays to detect ABV in swabs from oropharynx and cloaca. A screening was performed using a real-time PCR SyBr® Green-based detection assay [4]. Confirmation was obtained using the high specificity real-time PCR TaqMan®-based detection assay [5] and by the sequencing of gene fragments, which were amplified by conventional PCR.

Results: We were able to detect the amplification of the *Bornavirus* p24 and p40 genes by real-time PCR from swabs of the cloaca. Additionally, the p24 gene fragment was amplified by conventional PCR and its sequencing revealed a 100% identity with genus *Bornavirus,* in agreement with our preliminary findings.