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Efficient isolation of avian bornaviruses (ABV) from naturally infected psittacine birds and identification of a new ABV genotype from a salmon-crested cockatoo (*Cacatua moluccensis*)

D. Rubbenstroth [a,*], M. Rinder [b], B. Kaspers [c], P. Staeheli [a]

[a] *Department of Virology, University of Freiburg, Hermann-Herder-Str. 11, D-79104 Freiburg, Germany*
[b] *Clinic for Birds, Reptiles, Amphibians and Ornamental Fish, Center for Clinical Veterinary Medicine, University of Munich, Sonnenstr. 18, D-85764 Oberschleissheim, Germany*
[c] *Department of Veterinary Sciences, University of Munich, Veterinärstr. 13, D-80539 München, Germany*

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ABSTRACT

Avian bornaviruses (ABV) have been discovered in 2008 as the causative agent of proventricular dilatation disease (PDD) in psittacine birds. To date, six ABV genotypes have been described in psittacines. Furthermore, two additional but genetically different ABV genotypes were recognized in non-psittacine birds such as canary birds and wild waterfowl. This remarkable genetic diversity poses a considerable challenge to ABV diagnosis, since polymerase chain reaction (PCR) assays may fail to detect distantly related or as yet unknown genotypes. In this study we investigated the use of virus isolation in cell culture as a strategy for improving ABV diagnosis. We found that the quail fibroblast cell line CEC-32 allows very efficient isolation of ABV from psittacine birds. Isolation of ABV was successful not only from organ samples but also from cloacal and pharyngeal swabs and blood samples collected *intra vitam* from naturally infected parrots. Importantly, using this experimental approach we managed to isolate a new ABV genotype, termed ABV-7, from a salmon-crested cockatoo (*Cacatua moluccensis*). Phylogenetic analysis showed that ABV-7 is most closely related to the psittacine genotypes ABV-1, -2, -3, and -4 and clearly distinct from genotypes ABV-5 and -6. Our successful identification of ABV-7 emphasizes the necessity to consider the high genetic diversity when trying to diagnose ABV infections with high reliability and further shows that classical virus isolation may represent a useful diagnostic option, particularly for the detection of new ABV genotypes.

1. Introduction

Avian bornaviruses (ABV) are enveloped negative-stranded RNA viruses of the family *Bornaviridae* within the order *Mononegavirales* (Honkavuori et al., 2008; Kistler et al., 2008). Epidemiological and experimental data demonstrated that ABV is the causative agent of proventricular dilatation disease (PDD) in psittacine birds (Gray et al., 2010; Honkavuori et al., 2008; Kistler et al., 2008; Mirhosseini et al., 2011; Ouyang et al., 2009; Payne et al., 2011b; Piepenbring et al., 2012). PDD has been known as a severe and often fatal neurodegenerative disease in captive psittacine populations for more than 30 years (Gancz et al., 2010; Staeheli et al., 2010). However, PDD-like diseases were also reported in several other avian species (Daoust et al., 1991; Gancz et al., 2010; Perpinan et al., 2007).

In contrast to classical mammalian Borna disease virus (BDV) strains, which can be assigned to only two genotypes (Nowotny et al., 2000), ABV strains appear to have a much higher genetic variability. Since the first discovery in 2008, six ABV genotypes were identified in psittacine species

* Corresponding author. Tel.: +49 761 203 6583; fax: +49 761 203 6562.
E-mail address: Dennis.Rubbenstroth@uniklinik-freiburg.de (D. Rubbenstroth).

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exhibiting about 70–80% nucleotide identity (Honkavuori et al., 2008; Kistler et al., 2008; Weissenbock et al., 2009a). Two additional ABV genotypes were identified in a captive canary bird (ABV-C) in Austria and in wild Canada geese in Northern America (ABV-CG), respectively (Delnatte et al., 2011; Payne et al., 2011a; Weissenbock et al., 2009b).

The considerable genetic heterogeneity as well as the supposed existence of as yet unidentified genotypes poses a major challenge to the diagnosis of ABV infections. ABV detection is mainly based on conventional or quantitative reverse transcriptase (RT) polymerase chain reaction (PCR) assays (Heffels-Redmann et al., 2011; Honkavuori et al., 2008; Kistler et al., 2008). However, with only about 70–80% sequence identity among the different ABV genotypes known to date, most assays may not be able to detect all ABV strains. Alternatively, ABV can be detected by virus isolation. In cell culture, bornaviruses establish persistent infections without causing a cytopathic effect. Thus, identification of the virus is performed by immunofluorescence staining for viral antigen. ABV isolation was reported in cell lines of quail, chicken or duck origin, whereas attempts to isolate the virus in mammalian cell lines were not successful. To date, the quail cell line CEC-32 and primary duck embryo fibroblasts (DEF) are most commonly used for ABV isolation (Gray et al., 2010; Herzog et al., 2010; Rinder et al., 2009). However, a thorough evaluation of ABV isolation using different cell lines has not yet been performed.

In this study we compare the efficacy of different cell lines for ABV isolation from organ samples, as well as from samples collected *intra vitam* from naturally ABV-infected birds. We further report the discovery and genetic characterization of a new ABV genotype, termed ABV-7, from a salmon-crested cockatoo (*Cacatua moluccensis*).

2. Material and methods

2.1. Cells

The stable cell lines CEC-32 (quail fibroblasts), QM7 (quail smooth muscle cells), DF-1 (chicken fibroblasts), LMH (chicken hepatocytes) and VERO (African green monkey kidney cells) were used in this study. In addition, primary DEF derived from Pekin ducks were included. All cells were propagated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). Following inoculation, cultures were kept in infection medium (DMEM containing 2% FCS).

2.2. Diagnostic samples

Organ samples were collected from ABV-positive psittacine birds euthanized in or submitted to our Clinic (Table 1). Organs were stored at −80 °C until prepared for virus isolation. Cloacal and pharyngeal swabs as well as blood samples were collected from six psittacines housed in the Clinic (Table 1). All six birds were known to shed ABV-4 ($n = 5$) or ABV-1 ($n = 1$) with their feces. All birds exhibited variable degrees of clinical signs, some of which were considered to be related to their ABV infection. Swabs and blood samples were stored on ice for up to 12 h before virus isolation was performed.

2.3. Isolation and titration of ABV

Organ samples were homogenized in 10-fold volume of infection medium. White blood cells (buffy coat) were harvested from heparinized blood samples and suspended in 1 ml infection medium. Swab samples were likewise suspended in 1 ml infection medium. All samples were ultrasonicated using a Branson sonifier 150 (Branson). Swab suspensions were subsequently passed through a 0.45 μm filter to remove bacterial contaminants. For virus isolation, nearly confluent cells were inoculated with 50–100 μl of sample preparations per well in 12-well plates. Inoculated cells were further propagated in 6-well plates by passaging them twice weekly for two weeks. Thereafter, ABV-positive cells were visualized by indirect immunofluorescence staining (iIFS) with a rabbit serum directed against the N protein of ABV (Reuter et al., 2010). ABV-positive cells were counted and the extent of infection was

Table 1
Origin of diagnostic samples.

Animal	Species	Sample type	ABV genotype
Organ samples			
#2082	Blue-fronted amazon (*Amazona aestiva*)	brain	4
#6609	Hispaniolan amazon (*Amazona ventralis*)	crop	2
#6758	Blue-and-yellow macaw (*Ara ararauna*)	brain	4
#11851	Rose-breasted cockatoo (*Eolophus roseicapillus*)	brain	4
#14400	African grey parrot (*Psittacus erithacus*)	brain	4
#15544	Eclectus parrot (*Eclectus roratus*)	brain	2
#16199	Kea (*Nestor notabilis*)	proventriculus	1
#16571	Blue-headed macaw (*Primolius couloni*)	organ mixture	4
#16667	Salmon-crested cockatoo (*Cacatua moluccensis*)	brain and proventriculus	4 + 7
#17684	Cockatiel (*Nymphicus hollandicus*)	liver	2
Intra vitam sampling			
#112	African grey parrot (*Psittacus erithacus*)	swabs and blood	4
#7041	Blue-fronted amazon (Amazona aestiva)	swabs and blood	4
#7044	African grey parrot (*Psittacus erithacus*)	swabs and blood	4
#16364	Kea (*Nestor notabilis*)	swabs and blood	1
#16489	Yellow-lored amazon (*Amazona xantholora*)	swabs and blood	4
#17234	Cuban amazon (*Amazona leucocephala*)	swabs and blood	4