



Short communication

Newcastle disease virus in penguins from King George Island on the Antarctic region

Luciano M. Thomazelli^a, Jansen Araujo^a, Danielle B. Oliveira^a, Luiz Sanfilippo^a, Carolina S. Ferreira^a, Liana Brentano^b, Vivian H. Pelizari^a, Cristiane Nakayama^a, Rubens Duarte^a, Renata Hurtado^a, Joaquim O. Branco^c, David Walker^d, Edison L. Durigon^{a,*}

^a Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

^b Brazilian Agricultural Research Corporation (EMBRAPA), Concordia, Santa Catarina, Brazil

^c UNIVALI – University of Itajai, Santa Catarina, Brazil

^d Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA

ARTICLE INFO

Article history:

Received 22 September 2009

Received in revised form 27 April 2010

Accepted 3 May 2010

Keywords:

Newcastle disease virus

APMV-1

Antarctic

Serology

Real-time PCR

ABSTRACT

Here we report the isolation of Newcastle disease virus (NDV) from cloacal swabs obtained from penguins in the South Atlantic Antarctic region (62°08S, 58°25W). Samples of 100 penguins from King George Island were tested by real-time PCR, of which 2 (2%) were positive for NDV. The positive samples were isolated in embryonated chicken eggs and their matrix and fusion proteins genes were partially sequenced. This was complemented by the serological study performed on the blood of the same specimens, which resulted in a 33.3% rate of positivity.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Previous studies on subantarctic and antarctic penguins have shown that this region may become infected with a variety of avian viruses including paramixoviruses, influenza viruses and Newcastle disease virus (NDV) (Morgan et al., 1981; Wallensten et al., 2006; Morgan and Westbury, 1981, 1988). Although some studies demonstrated the presence of antibody to NDV, fewer detected the presence of the virus (Alexander, 1989). This paper reports the results of sampling from penguins around the Brazilian station (Comandante Ferraz Antarctic Base - EACF), Keller peninsula at King George Island. Keller peninsula (62°08S, 58°25W) is situated in the Admiralt Bay, near the tip of the Antarctic Peninsula (Fig. 1). The coastal areas of the island

are home to a comparatively diverse selection of vegetation and animal life, including elephant seals, weddell and leopard seals, chinstrap (*Pygoscelis antarcticus*), adelie (*Pygoscelis adeliae*) and gentoo penguins (*Pygoscelis papua*).

In order to better understand the epidemiology of the NDV and its possible reservoir, further knowledge is needed of the pool of NDV strains that exist in wild birds from which new strains may disperse. There is little sequence data available in the public domain regarding this virus in the southern hemisphere, especially in birds inhabiting such distant places as the Antarctic.

2. Materials and methods

2.1. Sample collection

The study was conducted in several penguins located at Keller peninsula. Penguins of three different species (gentoo, adelie and chinstrap) were caught on the beach using night nets. A tracheal/cloacal swabs sample and a

* Corresponding author. Tel.: +55 11 3091 7293; fax: +55 11 3091 7354.
E-mail address: eldurigo@usp.br (E.L. Durigon).

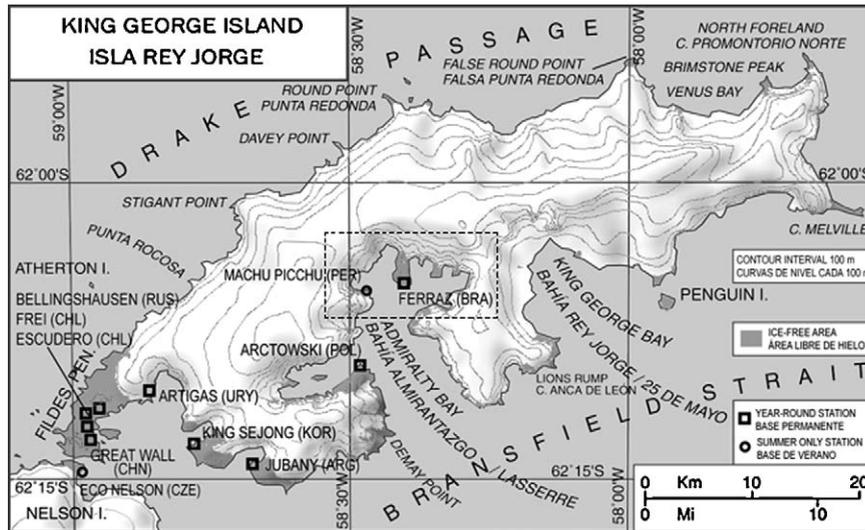


Fig. 1. Location of penguin colonies sampled near Brazilian station (FERRAZ). Withdraw Wikipedia.

blood sample from each 100 adults were collected during the summer period in November 2006. Each bird was sampled through the insertion of a sterile cotton wool swab into the cloacae and another into the trachea. The swabs were then combined in transport media (glycerol 5% in physiological saline with penicillin 2000 U/mL and fungizone 2000 U/mL) and processed just after the field-work. The blood was collected by medial metatarsal vein puncture with syringes and transferred to microtubes. At the station they were held for approximately 24 h at 20 °C before centrifugation. Serum was aspirated from the tubes and stored in individual sterile plastic vials at –20 °C at the station and during transport to Brazil.

2.2. RNA extraction, RT and real-time PCR

Extraction of viral RNA and primary viral detection was performed at the EACF. The tracheal/cloacal samples were divided into two aliquots, one for molecular analyses and other for isolation. RNA was extracted using NucliSens[®] Lysis' Buffer Reagent (Biomérieux) according to the manufacturers' guideline. The cDNA was obtained using random examers and the assay was processed with the High Capacity cDNA Archive kit (Applied Biosystems). Viral sequences were amplified in a real-time PCR (qPCR) using primers previously described by Wise et al. (2004) that targeting the matrix gene segment. The cycling was performed on a SmartCycler II, Cepheid (Portable thermocycle) with a program of 45 cycles of 94 °C/15 s, 60 °C/30 s and 72 °C/32 s.

The positive samples were isolated in embryonated chicken eggs and subjected to a new reaction of qPCR with the same conditions of reagents, but with primers and probe targeting the F gene of NDV (Seal et al., 1995) in order to molecularly check the pathogenicity of new isolates.

2.3. Virus isolation

The positive tracheal/cloacal samples were inoculated in embryonated chicken eggs for viral replication in the BSL

3 laboratory at University of Sao Paulo. The samples were centrifuged at 3000 × g for 30 min and the supernatant was collected and filtered with a filter membrane (0.22 μm). Then 0.2 mL was inoculated into the allantoic cavity of 10-day-old specific pathogen-free (SPF) embryonated chicken eggs. Each sample was inoculated into three eggs. After 72–96 h of incubation at 37 °C, the eggs were chilled and allantoic fluids were harvested and the undiluted allantoic fluids were tested for haemagglutination activity. Samples were re-passaged twice in embryonated eggs, and the allantoic fluids were tested for virus by (HA) and qPCR. Any fluids showing the presence of haemagglutinating activity were tested by the haemagglutination-inhibition (HI) test for the presence of Newcastle disease virus (NDV) using antiserum prepared against the B1 strain of NDV (New vac-B1[®] Fort Dodge).

2.4. Mean death time (MDT) in eggs

Fresh, sterile, infective allantoic fluids were diluted in sterile saline to give a tenfold dilution series between 10^{–6} and 10^{–9}. For each dilution, 0.1 mL was inoculated into the allantoic cavity of each of five 10-day-old embryonated SPF fowl eggs, which were then incubated at 37 °C. Each egg was examined twice daily for 7 days and the times of any embryo deaths were recorded. The MDT is the mean time in hours for the minimum lethal dose to kill all the inoculated embryos and has been used to classify NDV strains into the following groups: velogenic (taking under 60 h to kill); mesogenic (taking between 60 and 90 h to kill); and lentogenic (taking more than 90 h to kill) (OIE, 2004).

2.5. Serological analysis

The HI test was performed according to the technique described by OIE (2004) in U-shaped microtitre trays (Cooke Engineering Company, Alexandria, Virginia) using four haemagglutinating units of virus, twofold serial dilutions in 0.5% chicken erythrocyte suspension of serum

previously inactivated with RDE (1/3/6 of serum/Receptor-Destroyer Enzyme/Saline 0.85% NaCl) at 37 °C/over-night and 56 °C/30 min. Titers equal to or greater than $\log_2 2$ were considered positive results. The tests used the B1 strain of NDV (New vac-B1[®] Fort Dodge).

2.6. Sequencing of qPCR products and phylogenetic analysis

Resultant PCR products of isolates for both F and M genes were purified with Exosap. Purified DNA was then sequenced using the Big Dye terminator sequencing system (ABI) and sequencing reactions run and analyzed on an ABI 3100 Automated DNA Sequencer (ABI). Overlapping DNA fragments were sequenced in both directions from two independent RT-PCR reactions to generate a consensus sequence for each isolate. The sequences were aligned with others from GenBank using Blast in the NCBI Home page (<http://www.ncbi.nlm.nih.gov>). Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments were conducted by using Bioedit Sequence Alignment Editor software version 6.0.7. Additionally, phylogenetic trees were constructed by use of PAUP* software (Distance method, version 4.0) with a heuristic search and 1000 bootstrap replicates. The figure was obtained with the Tree Figure Drawing Tool FigTree version 1.2.1.

Nucleotide sequence accession numbers. The nucleotide sequences of the fusion protein and matrix protein genes from the RT-PCRs were submitted to GenBank as a single sense-strand contiguous sequence for each NDV isolate.

The accession numbers assigned and used are: AY845400, EU930424, EU930423, EU930425, AF309418, EU546165, AY444497, DQ097394, EU293914, AF431744, EF201805, DQ839397, DQ485248, DQ403244, EU167540, DQ485251, DQ485253, DQ485231, AY562989, AY562988, AY131275, AF473851, U25829, DQ486859, DQ485273, AY562990, EU346661, AF124442, AY444496, AY246047, AY444500, AY562986, AF124453, AF124451, AY438667, AY246048, U25836, AY865652, AY131279, AF089819, AY935493, AY935494, AY562991, AY935499, AY935498, AY935490, AY935489, HM143848, HM143849 and HM143850.

3. Results

3.1. Sample collection

One hundred adult penguins were sampled by swabs, it was difficult to collect blood samples from some of them (31%). This difficult was the same observed by Morgan and Westbury (1988) who described unsuccessful cases in which venepuncture produced a minimal blood flow because penguins may have had a reduced peripheral blood flow due to the cold conditions. No unusual mortality of birds was observed in any of the colonies sampled and no penguin exhibited clinical signals.

3.2. Detection of NDV

All cloacal/tracheal samples (100) were tested by qPCR targeting the matrix gene segment of APMV-1, of that 2

(2%) were positive. The positive samples were also sequenced and the phylogenetic analysis showed that these two strains are closer to the Ulster strain. Both sequences showed 100% homology and came from of the same species of penguin (*P. adeliae*) and the same penguin colony (EACF station).

3.3. Virus isolation and phylogenetic

Viral replication was performed through the inoculation into embryonated eggs of the two qPCR positive samples. In both cases HA of allantoic fluids and amplification of NDV matrix gene by qPCR gave negative results for the first passage. NDV was successfully isolated in the second passage, in which the virus was detected by both methods.

The isolates were then amplified using the same primers as the qPCR but without the probe (gene M and F). In both trees, phylogenetic analysis based on M gene fragment of 121 bp and F gene fragment of 172 bp, produced the same basic topology of an expected tree using full sequences of the main genes studied (Fig. 2).

3.4. MDT and pathogenicity

The Mean death time of both isolates, like the positive control strain, were more than 90 h (156 h for the positive samples and 96 h for the vaccine standard strain), showing that these isolates presumably pertained to the lentogenic strains and in accordance with the molecular results (data not shown), in which both isolates (02AntarBR and 39AntarBR) had the amino acid sequence “GKQGRL” at the F cleavage site, the same sequence of the avirulent strain Ulster67. The positive control (vaccine strain B1) had the amino acid sequence “GRQGRL” with an R for K substitution at position 113 as seen in the avirulent strain La Sota.

3.5. Serology

The detection limit for antibodies to NDV was a titer of 40 in the penguin serum. Titer values were expressed as the reciprocal of the highest dilution of serum in which antibody activity was observed. Twenty-three (33.3%) of the 69 sera collected from penguins in the King George Island were positive for antibodies to NDV. The range of titers was from 40 to 640 (Table 1).

4. Discussion

Even though there were no clinical signs of Newcastle disease in any of the penguins from which samples were collected, NDV was isolated from two cloacal/tracheal swab samples from penguins in the King George Island. The pathogenicity of these viral isolates was low by biological (MDT \geq 156) and molecular methods (sequence of cleavage site of F gene showing a amino acid sequence of avirulent strains) and, although the effects on penguins are unknown, they are likely to be asymptomatic.

Although the isolating of positive samples in embryonated chicken eggs, confirmation was achieved by qPCR in

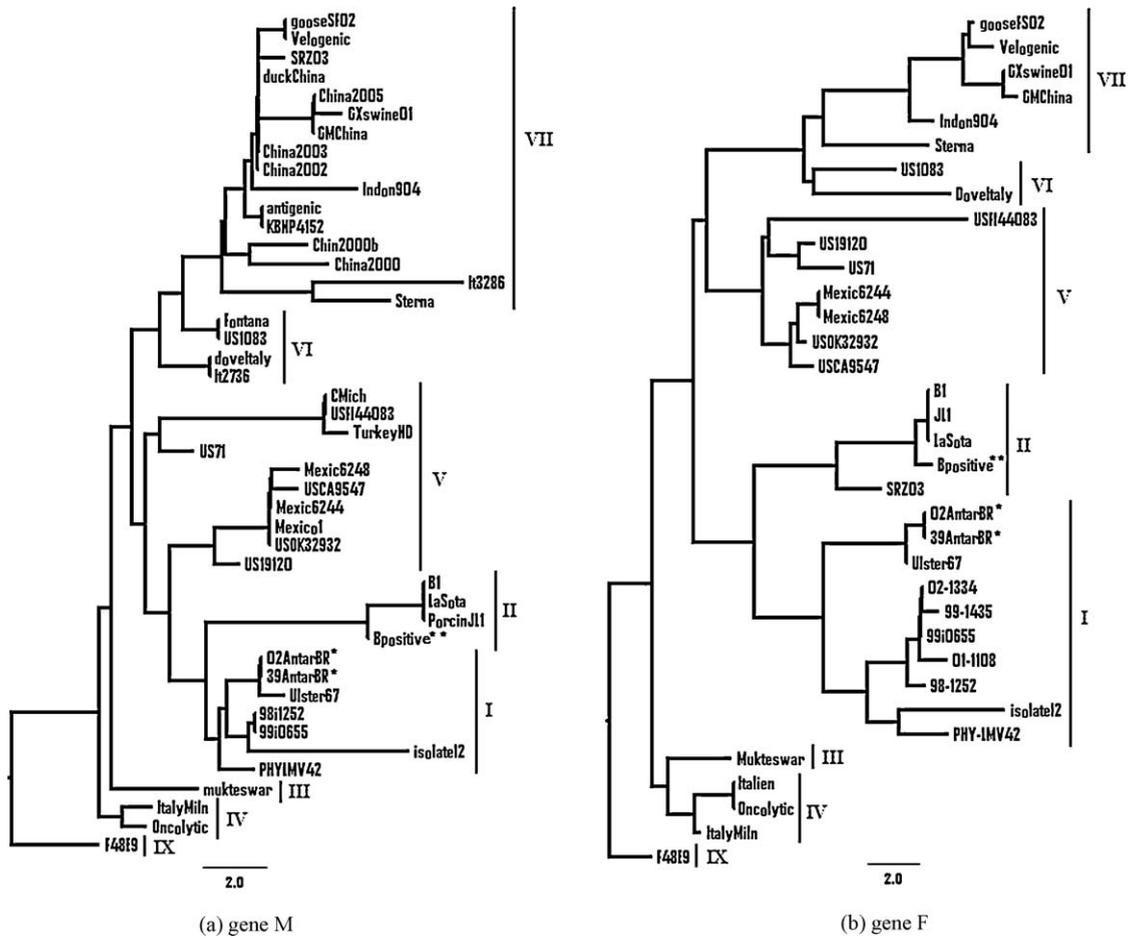


Fig. 2. Phylogenetic trees of the nucleotide sequences of the samples collected on the Antarctic based on a 121 bp region of the M protein gene (a) and a 172 bp region of the F protein gene (b) including the cleavage site. The bootstrap resampling of 100 replicates were obtained by PAUP* 4.0 (distance method), comparing the Antarctic* samples and the used positive control** with partial cds sequences obtained from the GenBank.

a low concentration (Ct between 35 and 39). Other authors have already described the difficulty of isolating the NDV from clinical specimens from healthy adult birds, especially the low-virulence strains (Sakai et al., 2006).

As the work of Lomniczi et al. (1998), even using a phylogenetic analysis based on smalls M gene fragment of 121 bp and F gene fragment of 172 bp, we produce the same basic topology of an expected tree using full

Table 1
Detection results of serum antibodies against NDV by HI in penguins from King George Island in November 2006.

Blood serum samples																				
	1	2	3	6	7	8	10	11	12	13	15	16	17	18	21	22	26	28	29	31
NDV	40	40	40	40	-	-	-	-	-	80	40	-	40	-	-	-	-	80	-	-
Blood serum samples																				
	33	34	35	38	39	40	42	43	44	45	47	49	50	51	52	53	55	56	59	60
NDV	-	40	-	80	40	80	-	-	-	-	-	-	-	-	-	-	80	-	40	-
Blood serum samples																				
	61	62	64	69	70	71	72	76	77	78	79	81	82	83	84	85	86	87	88	90
NDV	-	320	-	40	-	80	320	-	-	-	40	-	-	40	-	320	-	-	-	640
Blood serum samples																				
	92	93	94	95	96	97	98	99	100											
NDV	-	-	-	-	-	-	80	-	-											

On shaded, positive samples by real-time PCR.

sequences of the main genes studied (F or HN), including the most recognized genotyping system suggested by Aldous et al. (2003), inferring that the use of relatively short sequences of different subgenetic regions are suitable for the identification of genotypes and did not significantly affect the genetic distances values. The unique exception was the strain designated as SRZ03 which the phylogenetic tree based on M gene showed that SRZ03 fell into the cluster of genotype VII NDV strains, while the tree based on the F gene showed that SRZ03 fell into the cluster of genotype II NDV strains, but this find was previously described by Qin et al. (2008) that reported a recombination in this strain whose the N-terminal of SRZ03 F gene originated from a genotype II NDV strain, whereas the C-terminal of F gene and the rest of the genes originated from a prevalent velogenic genotype VII NDV strain, by complete genome sequences analysis.

The analyses indicated that Antarctic isolates are more closely related to the genotype group I, same of the Ulster strain (broadly used as vaccine all around the world) and share the same cleavage site of low pathogen strains.

Alexander et al. (1989) already had isolated nine paramyxovirus isolates obtained from penguins in Antarctica and sub-Antarctica during 1976–1979. By serological tests and analysis of structural polypeptides six of the penguin viruses, showed reaction with a monoclonal antibody raised against NDV Ulster 2C and the other three isolates also reacted with another APMV-1 directed monoclonal antibody. In addition, non-pathogenic paramyxovirus strains have been isolated from royal and king penguins (Morgan and Westbury, 1981; Morgan et al., 1981).

We detected antibodies in 33.3% of the sera of penguins, in contrast isolate only 2 positive samples. May be losing some NDV strains since the primers used are not able to detect some of class I type (Kim et al., 2008). This was not surprising since antibodies for NDV have been detected frequently in serum of penguins in the Antarctic region in the absence of an epizootic (Morgan and Westbury, 1981; Morgan et al., 1981; Alexander et al., 1989). However, the significance of these antibodies is uncertain; even, it is known that penguins are susceptible to pathogenic virus strains since disease has occurred in captive Adelie penguins believed to have become infected in the wild (Pierson and Pflow, 1975) and in a captive king penguin *Aptenodytes patagonicus* (Krauss et al., 1963).

The evidence of infection with NDV in penguins increases the questions about which other species of birds may be involved in the transmission of the virus. Is it possible the penguins transmit the virus to other species, and what are the potential pathological effects of NDV on penguins?

Antarctica is not as remote as it may appear; a large proportion of a variety of bird species migrates from northerly latitudes to breed there, including procellariids, terns and cormorants. In a study of sera collected from migratory cormorants in Alabama and Mississippi, 47% were positive for antibodies to NDV; 78% of egg yolks collected during breeding in these same colonies were also positive with titers ranging between 4 and 256 (Farley et al., 2001). Other birds that could be involved in the

spread of the viruses between Antarctic and temperate regions are the skuas, due to their feeding behavior and movements. Skuas feed on penguin eggs, chicks and dead birds and may acquire and spread infections in this way (Davis and Miller, 1992).

The involvement of other species in the transmission of NDV and attempts to characterize the role of penguins in the epidemiology of Newcastle disease in the Antarctic region should be the focus of further study.

The speed of modern transport to Antarctica and the increase in the numbers of people visiting south polar regions may increase the possibility of unwitting introduction of diseases. Changes in global climate may also facilitate the spread of disease through altered migratory habits of animals and birds and the introduction of vectors. A great example is the discovery of two little colonies of gentoo penguins (*Pygoscelis papua*) on south coast of Argentina, breaking the paradigm that species of penguins founding on Antarctic region are not presence on others continents, thus making the penguins possible carrier agents of the NDV, like anywise migratory bird.

Acknowledgements

We thank the Brazilian navy for the logistical support (Program PROANTAR), National Counsel of Technological and Scientific Development (CNPq – process 141981/2006–7) and The State of São Paulo Research Foundation (FAPESP – VGDN project) for the financial support. We are grateful to Adriano Carrasco for the positive controls provided.

References

- Aldous, E.W., Mynn, J.K., Banks, J., Alexander, D.J., 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Pathol.* 32 (3), 239–256.
- Alexander, D.J., 1989. Newcastle disease. In: Purchase, H., Arp, L., Dommert, C., Pearson, J. (Eds.), *A Laboratory Manual for Isolation and Identification of Avian Pathogens*. 3rd ed. American Association of Avian Pathologists, Inc., Kennet Square, PA, pp. 114–120.
- Alexander, D.J., Manvell, R.J., Collins, M.S., Brockman, S.J., Westbury, H.A., Morgan, I., Austin, F.J., 1989. Characterization of paramyxoviruses isolated from penguins in Antarctica and sub-Antarctica during 1976–1979. *Arch. Virol.* 109, 135–143.
- Davis, L.S., Miller, G.D., 1992. Satellite tracking of Adelie penguins. *Polar Biol.* 12, 503–506.
- Farley, J.M., Romero, C.H., Spalding, M.G., Avery, M.L., Forrester, D.J., 2001. Newcastle disease virus in double-crested cormorants in Alabama, Florida, and Mississippi. *J. Wildl. Diseases* 37 (4), 808–812.
- Kim, L.M., Suarez, D.L., Afonso, C.L., 2008. Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. *J. Vet. Diagn. Invest.* 20, 414–425.
- Krauss, H., Paulick, C., Huchzermeyer, F., Gylstorff, I., 1963. Newcastle disease in a king penguin. *Dtsch. tierarts. Wschr.* 70, 307–309.
- Lomniczi, B., Wehmann, E., Herczeg, J., Ballagi-Pordany, A., Kaleta, E.F., Werner, O., et al., 1998. Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). *Arch. Virol.* 143, 49–64.
- Morgan, L.R., Westbury, H.A., 1981. Virological studies of Adelie penguins (*Pygoscelis adeliae*) in Antarctica. *Avian Dis.* 25, 1019–1026.
- Morgan, I.R., Westbury, H.A., Caple, I.W., Campbell, J., 1981. A survey of virus infection in sub-Antarctic penguins on Macquarie Island, Southern Ocean. *Aust. Vet. J.* 57 (7), 333–335.

- Morgan, L.R., Westbury, H.A., 1988. Studies of viruses in penguins in the Vestfold Hills. *Hydrobiologia* 165, 263–269.
- OIE (Office International des Epizooties) Newcastle disease, 2004. OIE Manual of Standards for Diagnostic Tests and Vaccines, chapter 2.1.15.
- Pierson, G.P., Pfof, C.J., 1975. Newcastle Disease Surveillance in the United States. *J. Am. Vet. Med. Assoc.* 801–803.
- Qin, Z., Sunb, L., Mac, B., Cui, Z., Zhub, Y., Kitamura, Y., Liu, W., 2008. F gene recombination between genotype II and VII Newcastle disease virus. *Virus Res.* 131, 299–303.
- Sakai, K., Yada, K., Sakabe, G., Tani, O., Miyaji, K., Nakamura, M., et al., 2006. Serological and virological studies of Newcastle disease and avian influenza in slaughter-age ostriches (*Struthio camelus*) in Japan. *J. Vet. Med. Sci.* 68 (5), 491–494.
- Seal, B.S., King, D.J., Bennett, J.D., 1995. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. *J. Clin. Microbiol.* 33 (10), 2624–2630.
- Wallensten, A., Munster, V.J., Osterhaus, A.D.M.E., Waldenström, J., Bonnedahl, J., Broman, T., Fouchier, R.A.M., Olsen, B., 2006. Mounting evidence for the presence of influenza A virus in the avifauna of the Antarctic region. *Antarct. Sci.* 18 (3), 353–356.
- Wise, M.G., Suarez, D.L., Seal, B.S., Pedersen, J.C., Senne, D.A., King, D.J., Kapczynski, D.R., Spackman, E., 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. *J. Clin. Microbiol.* 42 (1), 329–338.