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Virulent Newcastle disease viruses from chicken origin are more pathogenic and transmissible to chickens than viruses normally maintained in wild birds

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ABSTRACT

Five, class II, virulent Newcastle disease virus (vNDV) isolates of different genotypes from different host species were evaluated for their ability to infect, cause disease, and transmit to naïve chickens. Groups of five birds received a low, medium, or high dose, by the oculonasal route, of one of the following vNDV: three chicken-origin, one cormorant-origin, and one pigeon-origin. Three naïve birds were added to each group at two days post-inoculation (DPI) to evaluate transmission. Virus shedding was quantified from swabs (2/4/7 DPI), and seroconversion was evaluated at 14 DPI. All inoculated and contact birds in the chicken-origin vNDV groups succumbed to infection, displaying clinical signs typical of Newcastle disease and shed virus titers above 6 $\log_{10}$ EID_{50}/ml. Birds receiving a high and medium dose of the cormorant virus showed primarily neurological clinical signs with 80% and 60% mortality, respectively. The chickens showing clinical disease shed virus at titers below 4 $\log_{10}$ EID_{50}/ml, and the remaining bird in the high dose group seroconverted with a high HI titer. For the pigeon-origin virus, no clinical signs were observed in any of the birds, but all 5 chickens in the high challenge dose and one bird in the medium challenge group shed virus at mean titers of 3.1 and 2.2 $\log_{10}$ EID_{50}/ml, respectively. Overall, the chicken-origin viruses infected chickens and efficiently transmitted to naïve birds, while the cormorant- and pigeon-origin viruses infected chickens only at the higher doses and did not transmit to other birds.

1. Introduction

Newcastle disease (ND), caused by virulent Newcastle disease virus (vNDV), is a severe and often fatal infection in naïve chickens and is a threat to poultry worldwide (Miller and Koch, 2013). Newcastle disease viruses belong to the family *Paramyxoviridae*. The nomenclature of the genus has recently changed, and the International Committee on Taxonomy of Viruses (ICTV), the official organization for classification and naming of viruses, has created and renamed the subfamily, genus, and species of family *Paramyxoviridae* to subfamily *Avulavirinae,* genus *Orthoavulavirus,* species *Avian orthoavulavirus 1* (Kuhn et al., 2019). Newcastle disease viruses (used hereafter and also known as avian paramyxovirus 1, APMV-1) belong to species *Avian orthoavulavirus 1*. According to the World Organisation for Animal Health (OIE), Newcastle disease (ND) is defined as an infection of birds by vNDV that: i) has an intracerebral pathogenicity index (ICPI) in day-old chickens of 0.7 or greater; or ii) has multiple basic amino acids at the C-terminus of the F2 protein (at least three arginine or lysine residues between positions 113 and 116) and phenylalanine at the N-terminus (position 117) of the F1 protein (OIE, 2014). Member countries should immediately report to OIE the identification of vNDV in poultry.

Although all NDV isolates belong to a single serotype, the viruses are genetically and antigenically diverse. Based on genetic differences, NDV are currently classified in two major groups (class I and class II) (Czegledi et al., 2006), and the class II viruses have higher genetic and virulence variability and are known to infect a wide range of domestic and wild birds. They are divided into at least 18 genotypes (named I to XVIII), and vNDV belonging to multiple class II genotypes are identified in poultry outbreaks worldwide (Dimitrov et al., 2016). vNDV from different genotypes are often identified in wild birds, and the latter have been speculated to play a role in the spread of the virus (Snoeck et al., 2013). However, it is suggested that most of the wild bird isolations are a result

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of spillover of virus from infected poultry (Miller and Koch, 2013). Notably, there are two known exceptions where virulent viruses are endemic in wild birds. Birds of the family *Columbidae* are known to be a reservoir of the antigenic variants of vNDV, commonly referred to as pigeon paramyxoviruses 1 (PPMV-1). These pigeon-origin viruses are distributed worldwide, are commonly isolated from both wild and domestic pigeons, and are highly virulent for these species (Ujvari et al., 2003). The second group of viruses are endemic in some species of the genus *Phalacrocorax* (cormorant populations) in North America and are associated with periodic mortality events (Banerjee et al., 1994). Both groups of viruses have the potential to infect other avian species but appear to be highly adapted to pigeons and cormorants, respectively, and are rarely implicated with infections in poultry.

Chicken-origin vNDV cause high mortality (up to 100%) and severe clinical signs, mainly in naive gallinaceous birds (Miller and Koch, 2013). Although occasionally and/or under different experimental conditions, pigeon- and cormorant-origin viruses have been shown to infect chickens and can cause clinical disease (Alexander and Parsons, 1986; Diel et al., 2012b). In addition, chickens inoculated with isolates from pigeons and cormorants may have prolonged shedding of vNDV, usually in cloacal swabs (Miller and Koch, 2013). For these reasons and because these viruses are endemic in countries that are free of vNDV in poultry, the potential transmission of vNDV from wild pigeons and cormorants continues to be a concern for poultry producers. Additionally, experimental studies have demonstrated that serial passages in chickens increased the virulence of pigeon vNDV isolates (Kommers et al., 2001).

Understanding the potential risks of transmission of chicken- and wild bird-origin vNDV in poultry is a crucial element in outbreak response and control of ND. Low biosecurity measures could pose a risk to the poultry industry of an NDV-free country, with the possibility of transmission due to contacts at the wildlife-poultry interface (Wajid et al., 2017). Studies to evaluate the transmission of cormorant-origin viruses in chickens have not been performed and the reports for transmission of pigeon-origin viruses in chickens are limited (Shabbir et al., 2016). Therefore, the present study aimed to compare three vNDV isolates, corresponding to genotypes that are normally isolated from chickens, with two viruses from genotypes that are normally associated with cormorants and pigeons. The ability to cause infection in chickens as well as the transmissibility of each virus to naïve contact chickens was evaluated.

2. Material and methods

2.1. Viruses

Five vNDV were used in this study: chicken/Peru/1918-03/2008 (PE08), chicken/Egypt/Sohag/66/2011 (EG11), chicken/Tanzania/Tanga/N38/2012 (TZ12), cormorant/USA/A00874288/650/2010 (CO10), and pigeon/USA/Allegheny/PA/ND0007199/2013 (PI13). The C010 and PI13 had one and three passages in embryonating chicken eggs, respectively. All viruses were obtained from the Southeast Poultry Research Laboratory's (SEPRL) repository of the United States National Poultry Research Center, U.S. Department of Agriculture (USDA). The isolates were propagated in 9-to-11-day-old specific-pathogen-free (SPF) embryonating chicken eggs, the allantoic fluid was harvested for testing by the hemagglutination test (HA), and titered by the Reed and Muench test (Reed and Muench, 1938). Allantoic fluids from all viruses were diluted in brain heart infusion (BHI) medium (BD Bioscience, Sparks, MD) in order to prepare the inocula with a targeted 2, 4, or 6 $\log_{10}$ 50% egg infective doses (EID_{50}) per 0.1 ml. The inoculum was back titered to confirm the actual inoculum administered to the chickens. The intracerebral pathogenicity index (ICPI) assay for the viruses was conducted according to OIE recommendations (OIE, 2012).

2.2. Chickens

In total, 120, three-week-old SPF white Leghorn chickens (*Gallus gallus domesticus*) were obtained from SEPRL`s flock. Feed and water were provided with *ad libitum* access. Birds were kept in isolators, and the animal experiments were approved by and performed in accordance to the Institutional Animal Care and Use Committee (IACUC) in animal biosecurity level 3 enhanced (ABSL-3E) facilities at the SEPRL.

2.3. Experimental design

Seventy-five birds were split into 15 groups (three groups for each used virus) and directly-inoculated by oculonasal route using gavage needles ($n = 5$ per group) using three different virus doses (low - 2 $\log_{10}$ EID_{50}/ml, medium - 4 $\log_{10}$ EID_{50}/ml, and high dose - 6 $\log_{10}$ EID_{50}/ml) of each of the five viruses (PE08, EG11, TZ12, CO10, and PI13). Viral titers were confirmed by back titer of the inoculum. Virus transmission was assessed by exposing three naïve, uninfected, SPF chickens to each group of inoculated chickens at 2 days post-inoculation (DPI). The inoculated birds were observed daily for clinical signs and mortality. Humane endpoints were observed and utilized over the entire duration of the experimental study, and birds that were either unable or unwilling to eat and/or drink were euthanized.

Oropharyngeal (OP) and cloacal (CL) swabs were collected at 2, 4, and 7 DPI from directly-inoculated birds, and at 2- and 5-days post placement (DPP) from contact birds to determine virus shedding. All surviving birds were bled at 14 DPI/12 DPP for serology and were humanely euthanized. Euthanized moribund chickens were counted as dead on the next day. Necropsies were performed for gross lesion examination in directly-inoculated and contact birds. The overview of the experiment design is shown in Supplemental Figure S1.

2.4. Chicken infectious dose (CID_{50}/ml) and Mean death time (MDT) in chickens

The 50% chicken infectious dose (CID_{50}/ml) was calculated (Villegas, 2008) based on virus detection by RRT-PCR at 2 DPI in any swab type from the three infectious dose groups infected with the same virus. The calculation with the back titers for determination of the virus titers was based on the Reed and Muench method (Reed and Muench, 1938). The mean death time was calculated using the average death time (DPI) for the chickens that were either euthanized or found dead after inoculation with each virus dose.

2.5. Virus shedding

RNA was extracted from each sample using a MagMax-96 RNA isolation kit (Ambion Inc., USA) (Das et al., 2009) and an automated KingFisher Magnetic Particle Processor system (Thermofisher Scientific, USA). Virus shedding was quantified by using three different real-time RT-PCR (RRT-PCR) assays to measure the lowest limit of detection for each isolate. The RRT-PCR targeting the matrix (M) gene (M-4100 test) was used to quantify the virus in swabs from the PE08 groups (Wise et al., 2004). A new RRT-PCR test targeting the polymerase gene (L) (L-12200 test) was used to quantify the swabs collected from birds in groups inoculated with CO10, EG11, and TZ12 viruses. The following primers and probe for the L-12200 test were used: L + 12170 - ACA GCT GGG AAT CTC CAA CA, L-12282 - CTT TGA GAA TCA TTG GAT ATG TGA A, and probe L + 12212 - 5` -FAM /CAG ATG ACA /ZEN/ TTT ACC CCT GCA TCT CT/ IBFQ -3'). The samples collected from groups inoculated with the PI13 virus were tested by the RRT-PCR assay targeting the nucleoprotein (NP) gene (NP-2400 test). The following primers and probe were used for the NP-2400 test (NDV + 2429 - GAACACAGCAT ATCATGGAC, NDV-2587 – CTCCATCATAGACATCATCGC, and probe NDV + 2455 -5′-FAM/AGG AGT CAC /ZEN/ AAC TAT CAG CTG GTG /IBFQ -3′). The L-12200 and M-4100 tests used the AgPath ID one-step RT-PCR kit (Ambion Inc., USA) with the following reaction volumes: 12.5 µl of kit supplied buffer (2x), 1 µl of each primer, 0.5 µl of probe, 1 µl of kit supplied enzyme, and sterile nuclease-free water to bring individual reaction volumes to 25 µl containing 8 µL of RNA. Primers and

probes were used at a concentration of 20 and 6 pmol/µl, respectively, in all reactions. After an initial reverse transcription step at 45 °C for 10 min and an initial denaturation step at 95 °C for 10 min, 40 cycles (95 °C for 10 s, 52 °C for 30 s, and 72 °C for 10 s) were performed with fluorescence detection at the end of the annealing-extension step. Identical reaction volumes and cycle conditions were utilized for the NP-2400 test, with the exception that the 94 °C for 10 s and 50 °C for 30 s steps were used for 40 cycles. RRT-PCR reactions were carried out in an ABI7500 instrument (Applied Biosystems, USA)

For virus titration, a standard curve for each virus was established with RNA extracted and diluted from the same titrated stock of the viruses used to challenge the birds, and the results are reported as the 50% embryo infectious dose (EID_{50}/ml). The calculated lower detection limit of the assay varied between 1.5 and 1.7 log $_{10}EID_{50}$/ml.

2.6. Serology

Hemagglutination inhibition (HI) assays were used to evaluate the presence of antibodies in sera samples collected from all surviving birds at the end of the experiments. Following standard procedures (OIE, 2012), the respective challenge virus was used as an antigen for each group of sera. Titers were calculated as the reciprocal of the last HI-positive serum dilution, and samples with HI titers of 3 (log_2) and below were considered negative.

2.7. Phylogenetic and molecular analysis

Total RNA was extracted using Trizol LS (ThermoFisher Scientific, USA) from the five isolates from the SEPRL repository and were quantified by Qubit fluorimetry (ThermoFisher Scientific, USA). RNA was reverse-transcribed and DNA libraries for next-generation sequencing were prepared, sequenced and analyzed as described previously (Ababneh et al., 2018). Raw sequence data were analyzed and assembled using MIRA version 3.4.1 within a customized workflow on the Galaxy platform as described previously (Dimitrov et al., 2017).

Phylogenetic analyses were performed utilizing the fusion (F) gene coding sequence of NDV using MEGA7 (Kumar et al., 2016). Complete F gene coding sequences related to the viruses studied here and representative sequences from other genotypes were downloaded from GenBank ($n = 110$). The Maximum Likelihood method based on the General Time Reversible (GTR) model with a discrete gamma distribution (5 categories [+G]) was utilized for both trees (Nei and Kumar, 2000). There were a total of 1,656 nucleotide positions in the final analyses. Evolutionary distances were estimated between the nucleotide sequences obtained in this study and the most closely related sequences available in GenBank applying the Maximum Composite Likelihood model (Tamura et al., 2004) with 1,000 bootstrap replicates using MEGA7 (Kumar et al., 2016). For all analyses, complete deletion was used as the missing data treatment. The current NDV classification criteria for genotype and sub-genotype identification were followed in this study (Diel et al., 2012a). Amino acid changes shown to be associated with the virulence or antigenic sites in previous studies (Cho et al., 2008; Dortmans et al., 2011; Duan et al., 2014; Ji et al., 2018; Mast et al., 2006; Mayahi and Esmaelizad, 2017; Xu et al., 2016) were also investigated amongst the different studied viruses.

2.8. Statistical analysis

Data were analyzed using Prism (v.7.03) software and outliers were identified using the ROUT test (GraphPad Software Inc., USA). Survival curves were tested using the Log-rank (Mantel-Cox) test. The D'Agostino-Pearson normality test was performed to estimate if the values in each group come from a Gaussian distribution. Based on the normality distribution, parametric ANOVA test was used for multiple comparisons of mortality rates and viral titers in oropharyngeal and cloacal swab samples from the different viruses with the same infectious dose and same sampling point. Statistical significance was set at a P value of < 0.05.

2.9. GenBank numbers

The obtained complete genome sequences of TZ12, CO10, and EG11 NDV were submitted to GenBank and are available under the accession numbers MK673139 to MK673141. The sequences of the PE08 and PI13 viruses are available in GenBank under accession numbers JN800306 and KP780874, respectively.

3. Results

3.1. Chicken-origin viruses (PE08, EG11, and TZ12) cause characteristic vNDV infection and transmit efficiently to contact birds

3.1.1. Birds succumbed to death regardless of the used infectious dose with variable MDT

The mortality, MDT, chicken infectious dose, and HI titer from all experimental groups are presented in Table 1. Birds inoculated with the chicken origin viruses (PE08, EG11, and TZ12) had 100% mortality in all groups (Fig. 1), and had an MDT ranging from 3.2 to 8.8 DPI depending on the challenge dose. The PE08 virus had the lowest MDT among the chicken viruses, regardless of the infectious dose. The MDT using a low, medium and high dose was 6.0, 4.0, and 3.2 DPI, respectively. The EG11 virus had an intermediate pathogenic pattern with the MDT of 7.4, 4.8, and 3.2 DPI using a low, medium and high dose, respectively. Finally, the TZ12 virus had the highest MDT among the chicken-origin viruses with the MDT of 8.8, 5.0, and 4.6 DPI using a low, medium and high dose, respectively.

The chicken-origin viruses caused mild conjunctivitis, ruffled feathers, and mild lethargy, which was observed at 2 DPI in the directly-inoculated birds and progressed to severe conjunctivitis and severe lethargy by 3 to 5 DPI. Birds inoculated with the medium and high dose of PE08 ($n = 1$ and $n = 4$, respectively) and EG11 ($n = 1$ in each group) died or were euthanized due to severe clinical signs as early as 3 DPI. Other clinical signs such as labored breathing, periorbital edema, and ataxia were also observed in birds from groups inoculated with chicken viruses at 5 to 9 DPI (primarily in the low dose groups).

All contact birds in all three chicken-origin groups succumbed to NDV infection by 8 DPP. The MDT increased as the dose decreased for all chicken-virus groups, varying from 5 to 5.7 DPP and from 6.3 to 8.3 DPI in the high and low dose groups, respectively. The first clinical signs in contact birds were initially observed in the high dose EG11 (3DPP), PE08 (4 DPP), and TZ12 (5 DPP) groups, progressing to severe clinical signs, mainly severe lethargy, at 6 DPP to 8 DPP. Two TZ12 contact birds displayed neurological clinical signs, such as ataxia and head tremors, at 6 and 8 DPP in the medium and low dose groups, respectively.

3.1.2. Gross lesions found widespread throughout different tissues

Fifteen directly-inoculated birds, in the chicken-origin NDV low and medium dose groups, were euthanized due to severe clinical signs and were examined for gross lesions (Fig. 2). Gross lesions at 4 to 8 DPI in the chickens inoculated with PE08, EG11, and TZ12, were characteristic of infection with vNDV. In most of the euthanized birds, subcutaneous edema, splenomegaly associated with mottling and multifocal necrosis, atrophy of thymus with gelatinous edema and perithymic hemorrhages, atrophic bursa, swollen kidneys with lobular surface pattern, hemorrhages in the proventriculus and cecal tonsils, necrotic hemorrhages in the duodenum and in the ileum, eyelid hemorrhages, and necrosis and hemorrhages in the comb, were present. Interestingly, multifocal hemorrhages in the larynx were observed at varying levels in two chicken viruses: more frequent after infection with the PE08 virus, less evident after infection with the TZ12 virus, and absent in birds inoculated with EG11 virus. In total, nine contact birds were euthanized at 5 to 8 DPP for gross lesion observation. At least one bird from each of the EG11, TZ12, and PE08 low dose groups, two birds from the medium dose EG11 group, and three birds from the medium dose PE08 group were included in these 9 birds. Gross lesions were consistent to those observed in the directly-infected birds.

Table 1
ICPI, mortality, mean death time (MDT), 50% chicken infectious dose (CID50/ml), and HI titer for each NDV experimental group. All values were calculated for both the directly-inoculated and contact birds, except for the CID_{50}/ml, which was calculated only for the directly-inoculated birds considering birds positive by RRT-PCR at 2 DPI.

				Directly-inoculated birds				Contact birds		
Virus ID	Strain	ICPI	Virus dose* (log_{10} EID_{50}/ml)	Mortality	MDT (DPI)	log_{10} CID_{50}/ml	HI titer (log_2)	Mortality	MDT (DPP)	HI titer (log_2)
PI13	pigeon/USA/Allegheny/PA/ND0007199/2013	1.20	2.1	0/5	NA	>6.5	<2	0/3	NA	<2
			4.1	0/5	NA		3.5	0/3	NA	<2
			6.5	0/5	NA		6.4	0/3	NA	<2
CO10	cormorant/USA/A00874288/650/2010	1.55	2.7	0/5	NA	6.0	<2	0/3	NA	<2
			4.3	3/5	7		<2	0/3	NA	<2
			6.3	4/5	5.3		8	0/3	NA	<2
PE08	chicken/Peru/1918-03/2008	1.78	2.9	5/5	6.0	<2.9	NA	3/3	6.3	NA
			4.7	5/5	4		NA	3/3	5.7	NA
			6.7	5/5	3.2		NA	3/3	5	NA
TZ12	chicken/Tanzania/Tanga/N38/2012	1.88	2.7	5/5	8.8	3.1	NA	3/3	8.3	NA
			4.3	5/5	5		NA	3/3	6.7	NA
			6.5	5/5	4.6		NA	3/3	6.7	NA
EG11	chicken/Egypt/Sohag/66/2011	1.88	2.1	5/5	7.4	3.7	NA	3/3	8	NA
			4.9	5/5	4.8		NA	3/3	7	NA
			6.3	5/5	3.2		NA	3/3	6.3	NA

*Virus doses shown are the back titers and not the target dose. Low to high MDT, CID_{50}/ml, and HI titer values were highlighted in gradient color from light grey to dark grey, which corresponds the low to high values.

3.1.3. Birds shed high virus titers through oral and cloacal routes

All birds in the medium and high dose groups inoculated with the chicken-origin NDV shed virus by oropharyngeal and cloacal routes with no significant difference between the viruses and DPI (Fig. 3). All birds infected with a high dose of the chicken-origin NDV shed virus at 2 DPI by both the oral and cloacal routes with titers of 5.3 and 4.9 log_{10} EID_{50}/ml, respectively. Even after inoculation with 100× less virus (medium dose groups), all birds shed virus by both routes but with lower titers. At 2 DPI, virus titers in birds inoculated with the medium dose of chicken-origin viruses varied from 3.9 to 4.4 and 3.2 to 3.9 log_{10} EID_{50}/ml in oral and cloacal routes, respectively. Interestingly, at 4DPI, the virus shed by the surviving birds through the cloacal route had titers approximately 0.6 log_{10} EID_{50}/ml higher than virus shed through the oral route.

After the low dose challenge at 2 DPI, virus was detected in only up to three birds of the directly-infected birds and as low as one bird in some groups. Interestingly, a shedding transmission pattern was observed in these chicken-origin low dose groups (Table 2). For example, in the PE08 group at 2DPI, one bird shed virus by the oral route and three birds shed through the cloacal route with viral titers below 2.8 log_{10} EID_{50}/ml. However, the three birds that shed virus by the cloacal route at 2 DPI continued to shed virus above 6.3 log_{10} EID_{50}/ml two days later (at 4DPI) through both routes and succumbed to death shortly after. The remaining two birds in this group began to shed virus by the oral route with titers below 4 log_{10} EID_{50}/ml at 4 DPI. At 7 DPI, they shed virus by both routes at a higher titer (7.2 log_{10} EID_{50}/ml). The PE08 virus resulted in a lower CID_{50}/ml (< 2.9 log_{10}) due to the significant detection of virus shedding in more birds by the cloacal route at 2 DPI in the low dose group compared to other chicken-origin NDV viruses ($P < 0.05$). The endpoint of shedding in the low dose groups could not be established as the last sampling point was at 7 DPI and the remaining birds succumbed to death by 10 DPI.

Unlike the PE08 group, only one bird shed virus by the oral route after inoculation with the low dose of the EG11 virus at 2 DPI with a low titer (2.1 log_{10} EID_{50}/ml). At 4 DPI, three birds shed virus by both routes and succumbed to death later. One bird had detectable virus shedding by the oral route at 4 DPI with a low titer (2.0 log_{10} EID_{50}/ml). At 7 DPI, the two surviving birds in the group shed virus with titers above 5.7 log_{10} EID_{50}/ml and died two days later.

As for the TZ12 low dose group, a single bird had detectable virus shedding at 2 and 4 DPI by the oral route with titers at 2.8 and 5.2 log_{10} EID_{50}/ml, respectively. This bird succumbed to the infection at 5 DPI, and all the remaining birds in the group had detectable titers varying from below 2.9 to 7.0 log_{10} EID_{50}/ml at 7 DPI. The virus shedding by the oral and cloacal routes in the birds inoculated with the TZ12 low dose was significantly lower (2.3 log_{10} EID_{50}/ml) than the other chicken-origin viruses ($P < 0.05$) at 4 and 7 DPI, respectively.

Regarding the contacts, at the low challenge dose at 2 DPP, all birds shed virus by both routes in the PE08 group, two birds shed virus only by the oral route in the EG11 group, and no contact bird in the TZ12 group shed virus (Table 2). In contrast to the low dose group, all contacts inoculated with the high and medium dose of the chicken-origin NDV shed virus through the oral route at 2 DPP with mean titers of 3.4 log_{10} EID_{50}/ml and 3.2 log_{10} EID_{50}/ml, except one bird in each group inoculated with the EG11 virus. However, in the medium dose groups only one bird in the PE08 and EG11 groups and two birds in the TZ12 group shed virus by the cloacal route with mean virus titers at 2.2 log_{10} EID_{50}/ml. In the high dose groups, all contact birds, except one bird in the EG11 group, shed virus by the cloacal route with a mean titer of 2.8 log_{10} EID_{50}/ml. At 5 DPP, all surviving contact birds in all chicken-origin NDV groups shed virus by both routes.

3.2. Pigeon and cormorant lineage viruses required a high challenge dose to infect chickens and did not transmit to contact birds

3.2.1. Variable mortality, clinical signs, and MDT in birds inoculated with cormorant and pigeon viruses

Birds inoculated with the CO10 had 80% and 60% mortality (Fig. 1) and an MDT of 5.3 and 7.0 DPI in the high and medium dose groups, respectively. Neither mortality, nor clinical signs were recorded in any of the PI13 groups. Neurological clinical signs, such as ataxia, recumbency with paralysis (Fig. 2) and head tremors were observed in the medium and high dose groups inoculated with the CO10 virus. At 4 DPI, one bird, in each of the medium and high dose groups, directly-inoculated with CO10 presented mild ataxia that progressed to paralysis. At 5 DPI, two birds, paralyzed and gasping, were euthanized in the CO10 high dose group. At 7 DPI, one bird in the high dose group had head tremors, which persisted until the end of the experiment. The two low dose groups inoculated with the PI13 and CO10 viruses had no evidence of virus infection, lacking both mortality and clinical signs. Additionally, both mortality and clinical signs were also absent in all contact birds at all

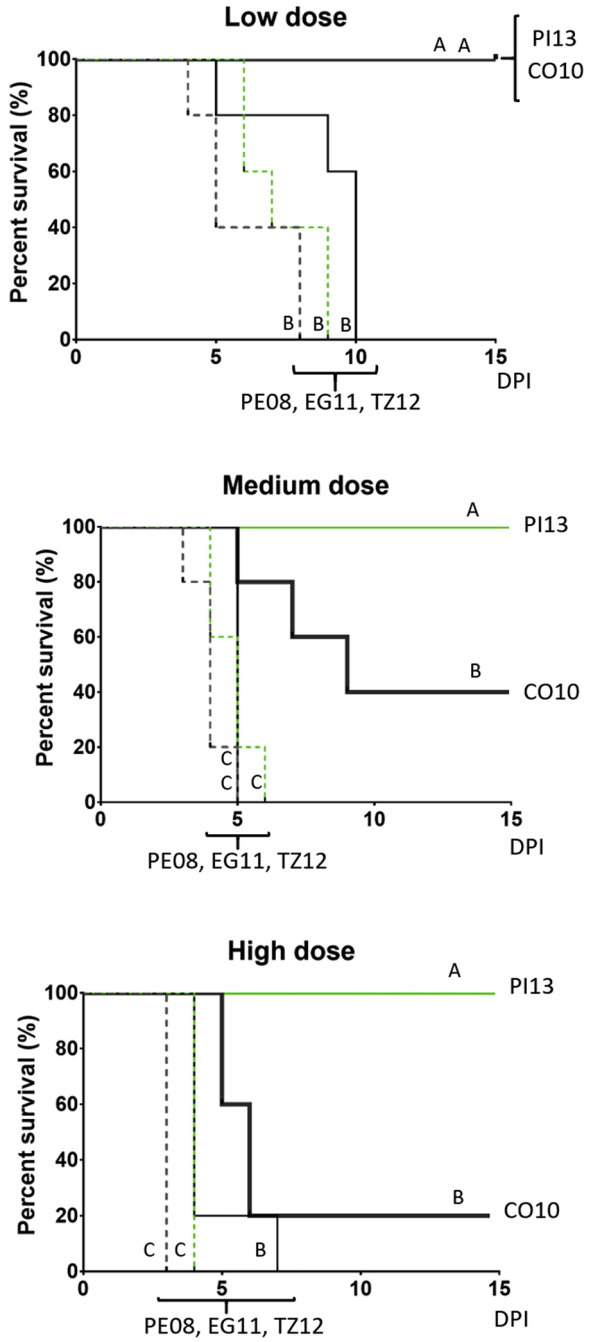

Fig. 1. Survival curves of directly-inoculated birds. Chickens were separated into 3 groups for each virus, and inoculated with a low, medium, and high dose of the five NDV strains (PE08, EG11, TZ12, CO10, PI13). Mortality in each experimental group was followed daily over 14 days. No common letters indicate significant differences ($P < 0.5$).

challenge doses from the CO10 and PI13 virus groups (Table 1).

Birds inoculated with the high dose of CO10 virus did not show a significant difference in survival rates when compared to the TZ12 virus, but there was a significant difference ($P < 0.05$) compared to the other two chicken-origin viruses (Fig. 1). However, in the low and medium dose groups, significant differences in survival rates were observed among the chicken-origin and CO10 NDV. The survival in all dose groups inoculated with PI13 NDV were significantly different compared to the chicken origin viruses. The survival rates of the two-wild bird NDV were significantly different ($P < 0.05$) from one another in the high and medium dose groups, but not in the low dose groups.

3.2.2. Gross lesions and severe clinical signs observed in birds inoculated with the cormorant virus

Five birds in the medium and high dose groups inoculated with the CO10 virus were evaluated for gross lesions after euthanasia due to severe clinical signs. Subcutaneous hemorrhages, splenomegaly associated with mottling and multifocal necrosis, swollen kidney, thymus with gelatinous edema were observed in euthanized birds at 4 to 5 DPI. Hyperemia and mild multifocal petechial hemorrhages in the meninges of the encephalon were present in birds from the medium dose group at 8 DPI (Fig. 3).

3.2.3. Virus shedding in directly-inoculated birds depends on virus infectious dose, without transmission

Three birds inoculated with the high dose of the CO10 NDV shed virus (mean of 2.6 $\log_{10}$ EID_{50}/ml) through the oral route at 2 DPI; while. all remaining birds in this group shed virus through the oral route in titers significantly higher than birds inoculated with the PI13 virus ($P < 0.05$, $P = 0.0039$) at 4 DPI. The viral shedding through the cloacal route was less, with no birds positive at 2 DPI, but four birds shed virus at 4 DPI with a low titer (2.6 $\log_{10}$ EID_{50}/ml) in the CO10 group. The only surviving bird in the CO10 high dose group shed virus with titers of 2.3 $\log_{10}$ EID_{50}/ml at 7 DPI. Despite the sporadic mortality in the CO10 medium dose group, only one bird shed virus at 4 DPI through both routes.

The virus shedding from birds in the PI13 high dose group was more pronounced through the cloacal route than through the oral route. At 2 DPI, only one bird shed virus with a titer of 2.6 $\log_{10}$ EID_{50}/ml. However, four and five birds shed virus at 4 and 7 DPI, respectively, with titers around 3.3 $\log_{10}$ EID_{50}/ml by the cloacal route, and in this same group, two and five birds shed 2.6 and 3.7 $\log_{10}$ EID_{50}/ml virus at 4 and 7 DPI, respectively, through the oral route. Although one other bird in the PI13 medium dose group shed through the oral route at 7 DPI, all other birds remained negative by both routes at 2, 4, and 7 DPI in the medium and low dose groups inoculated with the PI13 and CO10 viruses. Additionally, no contact bird shed virus in any group inoculated with the wild bird-origin NDV at different doses.

Birds inoculated with the chicken-origin NDV shed virus in significantly higher titers than birds inoculated with wild bird NDV at 2 DPI in the high dose groups ($P < 0.05$). At 4 DPI, birds inoculated with CO10 and TZ12 viruses shed significantly higher virus titers than those inoculated with the PI13 virus in the high dose group. Birds inoculated with the chicken origin viruses shed significantly higher titers than wild bird NDV after inoculation with the low and medium doses at all tested time points.

3.3. No specific antibody against NDV was detected in any of the contact birds inoculated with the wild-bird-origin viruses

All birds in the groups infected with the chicken-origin NDV succumbed to the virulent infection before blood collection on 14 DPI/12 DPP. However, some birds in the wild bird-origin NDV groups survived the infection (Table 1). The only directly-inoculated surviving bird in the CO10 high dose group had a titer of 8 ($\log_2$), but none of the surviving birds in the medium dose group had detectable NDV antibodies. The directly-inoculated birds in the high and medium dose of the PI13 groups had specific antibodies levels of 6.4 and 3.5 ($\log_2$). Birds directly-inoculated with the low dose of both PI13 and CO10 had no specific NDV antibody. In addition, no specific antibody against NDV was detected in any of the contact birds from the PI13 and CO10 groups (Table 1).

3.4. Major differences in the CID_{50}/ml were observed between the chicken-origin and wild bird-origin NDV

The chicken-origin viruses had an ICPI value above 1.75, while the wild bird viruses, CO10 and PI13, had ICPI values of 1.55 and 1.20, respectively (Table 1). Three different doses (2 $\log_{10}$ EID_{50}/ml -low, 4 $\log_{10}$ EID_{50}/ml -medium and 6 $\log_{10}$ EID_{50}/ml –high) were used for each virus, but based on the back titers of the inoculum, some differences were observed from the targeted challenge and the measured

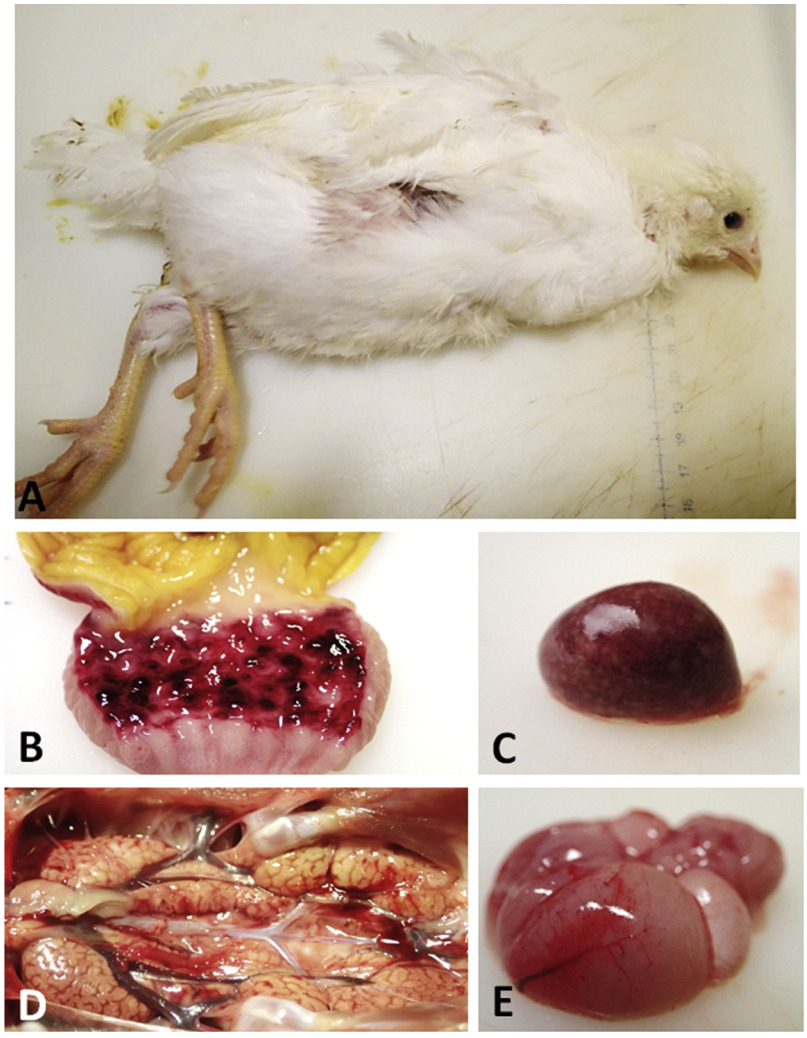

Fig. 2. Clinical signs and gross lesions after infection with different NDV strains at 3 infectious doses. A) Paralysis in a bird inoculated with a medium dose of the CO10 virus; B) Multifocal hemorrhages in the proventriculus of a bird inoculated with a medium dose of the EG11 virus; C) Enlarged spleen with mottling and multifocal necrosis in a bird inoculated with a high dose of the CO10 virus; D) Swollen kidneys in a bird inoculated with a medium dose of the EG11 virus; E) Hyperemia and mild multifocal petechial hemorrhages in the meninges of the encephalon after inoculation with a medium dose of the CO10 virus at 8 DPI.

challenge doses (Table 1). The chicken viruses had a CID_{50}/ml varying from below 2.9 to 3.7 $\log_{10}$ CID_{50}/ml, the CO10 had 6.0 $\log_{10}$ CID_{50}/ml, and PI13 had $\log_{10}$ CID_{50}/ml values above 6.5.

3.5. The virus viruses belong to different NDV genotypes

The phylogenetic analysis performed with the coding sequence of the fusion gene showed that the five NDV isolates cluster into five different class II genotypes (Fig. 4). The EG11 clustered with other viruses from Egypt isolated between 2012–2017 and belongs to sub-genotype VIIj. The PE08 clustered with a peacock virus isolated in Peru in 2011 and poultry isolates from Columbia from 2009 and is classified as a member of sub-genotype XIIa. The TZ12 virus clustered with isolates from several African countries isolated during 2008–2015 and belongs to sub-genotype XIIIa. The CO10 clustered with cormorant isolates from the USA from 2010 to 2012 and is classified as sub-genotype Va. The PI13 virus clustered with samples from pigeons and doves in Pennsylvania and Massachusetts from 2012 and 2014 and belongs to sub-genotype *VIa*.

The sequences of all five viruses had three basic amino acids between residues 113 and 116 in the C-terminus of the F2 protein and a phenylalanine at residue 117 in the N-terminus of the F1 protein (^{113}RQKR↓F^{117}), and the PI13 had four basic amino acids (^{113}RKKR*F^{117}) between residues 113 and 116 in the C-terminus of the F2. Two amino acid residues associated with virulence in the F protein and two amino acid residues located in the antigenic site in the hemagluting-neuraminidase (HN) protein, respectively, were unique either to the sequences of chicken- or wild bird-origin viruses (Table 3). Other amino acid changes were observed in these two proteins, but with different amino acid residues from those reported previously. The position 347, which is known as an important amino acid in the antigenic site of the HN protein, had the residues G, E, K in the different isolates studied here. All amino acids residues in the matrix (M) and large polymerase (L) protein, previously reported to be associated with an increase or attenuation of virulence, were observed in all tested viruses (Cho et al., 2008; Dortmans et al., 2011; Duan et al., 2014; Ji et al., 2018; Mast et al., 2006; Mayahi and Esmaelizad, 2017; Xu et al., 2016). No residue previously associated with an increase in virulence in the phosphoprotein (P) protein was found in the studied viruses.

4. Discussion

Newcastle disease viruses belonging to five different genotypes of NDV, isolated from both chickens and wild bird species, were inoculated into SPF chickens to compare susceptibility and transmissibility. All five viruses meet the OIE criteria for virulent Newcastle disease virus, as both their ICPI values exceed the 0.7 index and their F

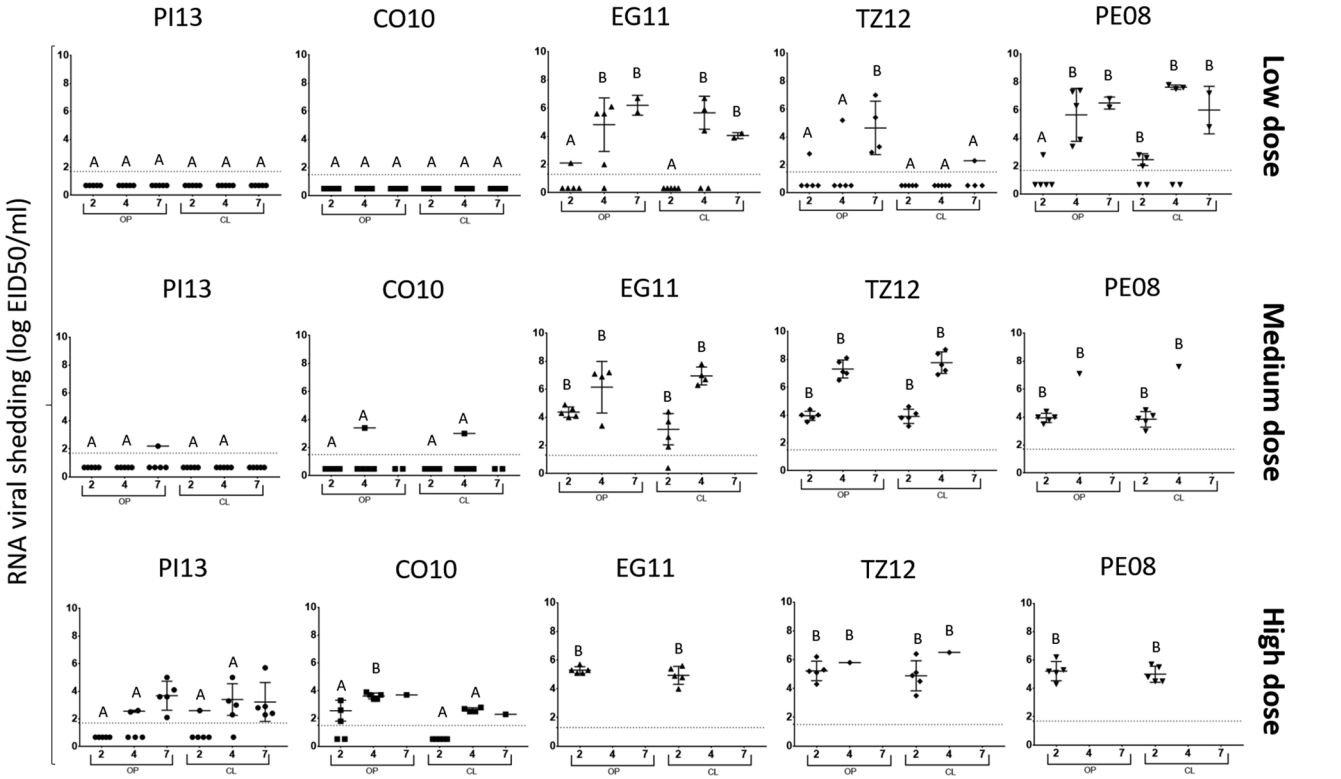

Fig. 3. Virus shedding in directly-inoculated birds after inoculation with chicken- and wild bird-origin NDV. NDV titers were estimated in both OP and CL swabs of directly-inoculated birds with three different doses of NDV strains at 2, 4, and 7 DPI. The detection limit of the different RRT-PCR assays targeting the NDV strains varied between 1.5 and 1.7 $\log_{10}EID_{50}$/ml and are shown as dotted lines on the Y axis. Mean and standard deviation of the mean for positive swabs at each time point are shown as bars. No common letters (A or B) differ significantly ($P < 0.05$), when comparing oropharyngeal or cloacal swab samples from the different viruses with the same infectious dose and same sampling point. Non-detected swabs were added below the limit of detection for each virus.

Table 2
Shedding transmission pattern by the oral route from birds inoculated with low dose of EG11, TZ12, and PE08. The virus titers (expressed in $\log_{10}$ EID_{50}/ml) at 2, 4, and 7 DPI or 2 and 5DPP.

	EG11			TZ12			PE08		
	Virus titers ($\log_{10}EID_{50}$/ml)			Virus titers ($\log_{10}EID_{50}$/ml)			Virus titers ($\log_{10}EID_{50}$/ml)		
	2DPI	4DPI	7DPI	2DPI	4DPI	7DPI	2DPI	4DPI	7DPI
Directly-infected (n = 5)	ND	6.1	NS	2.8	5.2	NS	2.8	6.3	NS
	ND	2.0	5.7	ND	ND	3.3	ND	7.3	NS
	2.1	5.6	NS	ND	ND	2.9	ND	3.9	6.8
	ND	5.6	NS	ND	ND	7.0	ND	7.4	NS
	ND	ND	6.7	ND	ND	5.4	ND	3.4	6.2
Mean ($\log_{10}$ EID_{50}/ml)	2.1	4.8	6.2	2.8	5.2	4.7	2.8	5.7	6.5
	2DPP	5DPP		2DPP	5DPP		2DPP	5DPP	
Contacts (n = 3)	2.4	5.5		ND	5.9		2.9	7.2	
	1.9	5.4		ND	6.3		3.5	7.0	
	ND	5.0		ND	2.5		3.8	6.6	
Mean ($\log_{10}$ EID_{50}/ml)	2.2	5.3			4.9		3.4	6.9	

ND: not detected by the RRT-PCR; NS: no survival. Virus titers were highlighted in gradient color from light grey to dark grey, which corresponds to low to high values. NS and ND birds are highlighted in black and green, respectively.

protein cleavage site contains the amino acid motif characteristic for virulence in chickens (OIE, 2014). Despite all these viruses being classified as virulent, there was a clear difference in pathogenicity and transmissibility in chickens between the chicken-origin viruses and the pigeon and cormorant lineage viruses. One of the objectives of this study was to determine if the chicken- and- wild bird- origin viruses have demonstrable differences in infectivity and transmissibility in chickens which would indicate how chicken adapted the viruses were.

Expectedly, the chicken-origin viruses were able to infect chickens at lower doses, efficiently transmit to direct contact controls, and had a much lower CID_{50}/ml. Mortality rates, clinical signs, and gross lesions observed in birds infected with the chicken-origin NDV were similar to those reported previously (Courtney et al., 2013; Diel et al., 2012c). The virus titers shed by birds inoculated with the chicken-origin NDV at 2 DPI were directly proportional to inoculum titers. As previously reported (Dimitrov et al., 2019; Miller et al., 2013), chicken-origin NDV with sustained replication in chickens usually have an increase in virus titers through 4 DPI, typically shortly before death. However, not all birds directly-inoculated with the low dose of chicken-origin NDV shed virus at 2 DPI, but at least one bird did appear to be infected and likely transmitted virus to the rest of the birds, eventually infecting all birds in the group. In the present study, the difference of the MDT in directly-inoculated birds and contact birds with chicken-origin NDV decreased when the infectious dose decreased. The mean of this difference (MDT of contact birds minus MDT of directly-inoculated) is 0.1, 1.9, and 2.3 days in the low, medium, and high groups, respectively. The low difference of MDT between directly-inoculated and contact birds in the low dose groups could be explained as few birds are indeed infected directly by the inoculum, and the transmission occurs after the few infected chickens starts shedding virus at high titer and exposes the originally uninfected and contact controls at the same time. Consequently, the low dose groups had the lowest numbers of birds shedding virus at 2 DPI but with all birds eventually becoming infected, resulting in a longer MDT. Finally, the low CID_{50}/ml, efficient transmission, and fast mortality for all three viruses support that these viruses are highly adapted to chickens.

Major differences in the CID_{50}/ml were observed between the chicken-origin and wild bird-origin NDV, showing that a higher dose is needed for wild bird NDV to infect chickens. The cormorant and pigeon isolates had high CID_{50}/ml based on the conservative approach of detection of shed virus on 2 DPI. However, the virus shedding at days 4 and 7 and the serology data support that more birds were infected than identified on day 2. The slower replication pattern of these birds relative to the chicken adapted strains support that they are less chicken

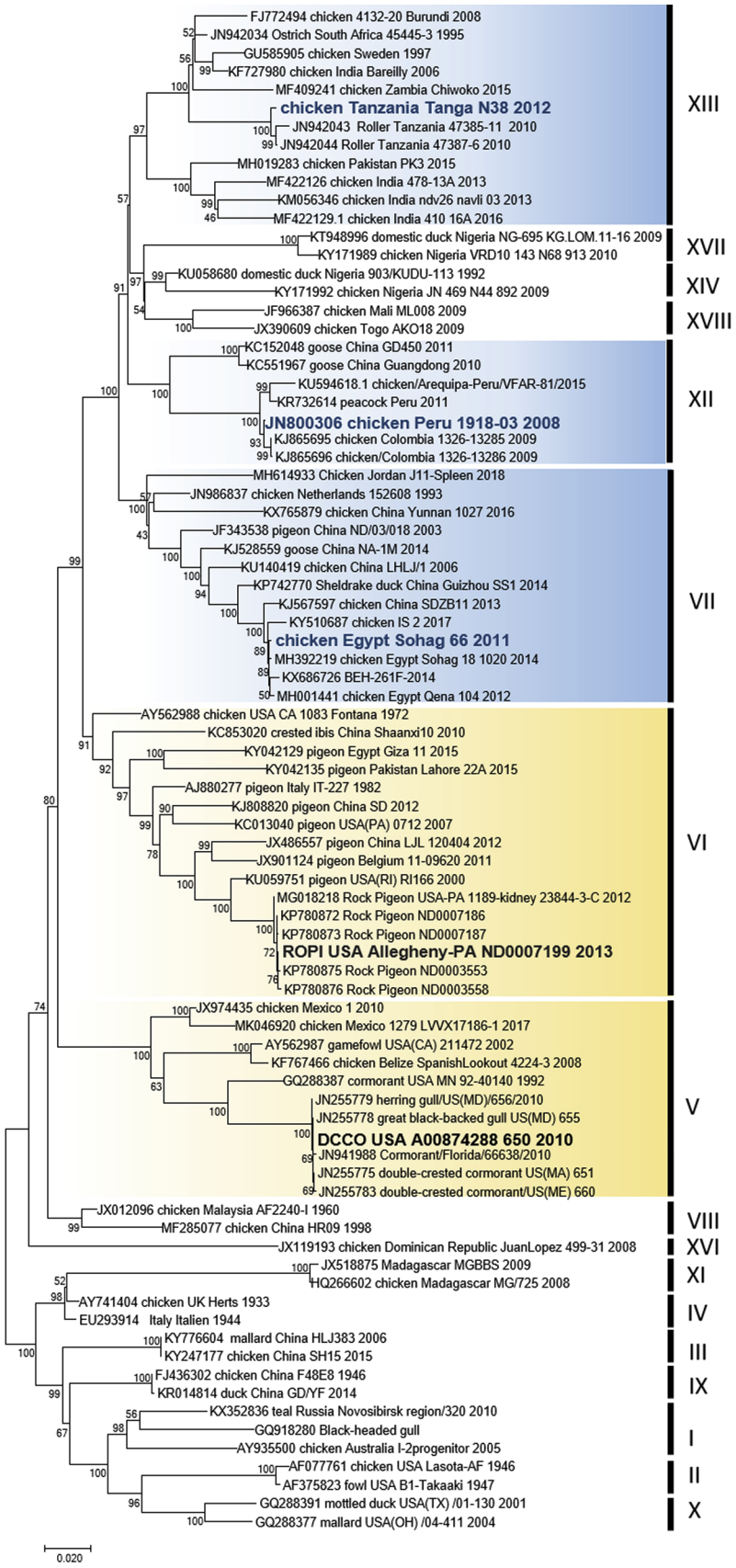

Fig. 4. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei and Kumar, 2000). The tree with the highest **log** likelihood (-20090.41) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior **log** likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+ *G*, parameter = 0.4152)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 83 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1651 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). The three chicken-origin NDV strains, TZ12, PE08, and EG11 that belong to the genotypes XIII, XII, and VII, respectively, were highlighted in blue. The wild bird-origin strains, PI13 and CO10 that belong to genotypes VI, V, respectively, were highlighted in yellow (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

adapted. The methods used for calculation of the CID_{50}/ml between the chicken adapted and wild-bird origin will need to differ to measure the susceptibility of chickens to these viruses accurately. Even when using a high infectious dose, transmission of both wild bird viruses from inoculated to contact chickens still did not occur. The pigeon virus, even at the high dose, did not cause any clinical signs in chickens, consistent

Table 3
Amino acid residues differences among the studied strains in the F and HN NDV proteins. Two residues in each of the F and HN proteins were unique in one of the tested viruses (grey). Changes in other amino acid residues in the F and HN did also occur (Gu et al., 2011; Samal et al., 2011).

			AA residue					
Gene	Function	Position	PI13	CO10	PE08	EG11	TZ12	Reference
F	Virulence	D72Y	K	K	K	R	K	(Mast et al., 2006)
		R101M	R	R	R	K	R	
		Q114R	K	Q	Q	K	Q	(Samal et al., 2011)
HN	Antigenic site	D115S	S	N	N	N	N	(Mast et al., 2006)
		G362K	G	R	G	A	G	(Mayahi and Esmaelizad, 2017)
		V266A	T	T	T	A	T	(Gu et al., 2011)
		E347Q	G	E	E	K	G	(Cho et al., 2008; Gu et al., 2011; Mayahi and Esmaelizad, 2017)

with previous reports (Guo et al., 2014). Indeed, adaptation is required of PPMV-1 isolates to transmit and cause disease in chickens. A change of the cleavage fusion site, due to serial passage, has been reported as being sufficient to increase the frequency of clinical signs, mortality, and the level of neuro-invasiveness (Kommers et al., 2001; Moura et al., 2016), but serial passage was not part of our study design.

As previously mentioned, the pigeon and cormorant viruses studied here meet the definition of virulent NDV, and they are potentially reportable to OIE. If the viruses are found in wild birds, they should not have an impact on trade, but pigeons are commercially raised in some countries and could meet the definition for poultry (OIE, 2014). The PPMV-1 viruses from wild birds, which are found throughout the world, have not been shown to be a serious risk to poultry production. This study provides data that supports the lower risk that pigeon origin NDV pose compared to chicken-adapted viruses, and provides an objective measure that supports the recent removal of PPMV-1 from the U.S. select agent list (FSAP, 2019). Because pigeons have been reported to be infected with other vNDV genotypes, it is necessary to have diagnostic tools to allow differentiation (Kim et al., 2006). For example, PPMV-1 can be distinguished from other NDV by a specific panel of monoclonal antibodies (Collins et al., 1989). However, the monoclonal panel is neither widely available nor often used by most reference laboratories. Sequence analysis appears to be a widely available and practical tool, but it can only distinguish the genotype of NDV. As not all genotype VI viruses have been shown to be PPMV-1, sequencing alone cannot currently be used as regulatory tool to discriminate PPMV-1 from NDV, and therefore, further research for diagnostic differentiation is still needed.

The current OIE definition, which relies on the ICPI index, suffers from its requirement for an abnormal inoculation route of virus into the brain. Historically, the ICPI provided a unique and repeatable tool to evaluate virulence of NDV isolates. In recent years, the ICPI values of most virulent NDV viruses are higher than 1.7, and little distinction of virulence between strains can be inferred from such high values (Courtney et al., 2013; Diel et al., 2012c). For example, in this study, all 3 chicken-origin viruses had similar ICPI values and were also highly transmissible to chickens but had measurable differences in MDT. Ideally, an alternative measure that does not require animal challenge can be used to differentiate between virulent and non-virulent viruses. Fusion cleavage site sequence is clearly one tool that can be used, but the fusion cleavage site sequence alone would have suggested both the pigeon and cormorant viruses would have been virulent to chickens. Phylogenetic analysis can be used to improve virulence prediction because of our growing databases of sequence and pathotyping information. Continued research is needed to improve our predictive ability for NDV infectivity and pathogenicity in chickens and other species.

The cleavage site of the fusion protein is the main determinant for viral virulence, but other proteins such as HN (de Leeuw et al., 2005), the polymerase complex (P and L) and M can impact the virulence and the virus replication in chickens (Dortmans et al., 2011; Duan et al., 2014; Ji et al., 2018; Xu et al., 2016). Nevertheless, we did not identify a clear molecular marker that could differentiate the wild bird and chicken NDV as all amino acid residues in those proteins described previously were common in all strains. The only unique amino acid residues associated with the antigenic sites were found in the F and HN proteins in one of the tested isolates. These findings reiterate the complexity of NDV pathogenicity based on molecular features, and the lack of understanding about what factors may affect replication, pathogenicity, and transmissibility.

5. Conclusion

This study evaluated the transmissibility, in chickens, of viruses normally maintained in wild birds, and here we showed that the pigeon and cormorant lineage viruses required a high challenge dose to infect chickens and did not transmit efficiently to contact birds, even when a high infectious dose was used. This result may provide an explanation of why pigeon and cormorant lineage viruses are rarely found in poultry. Despite the viruses having characteristics of being virulent viruses, including an ICPI in chickens > 0.7 and a cleavage site compatible with virulent NDV, these viruses were less virulent for chickens when the virus was given by a natural route of infection. Without some adaptation of the virus to chickens, these viruses appear unlikely to cause a serious disease outbreak in chickens. Unfortunately, molecular markers related to virulence are not currently known that can identify pigeon and cormorant lineages, although the viruses do uniquely cluster in phylogenetic trees. A standardized model to measure infectious dose and transmissibility will provide a more objective measure to evaluate the pathogenic potential of NDV viruses to chickens.

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Ethical approval

All procedures performed in the present study involving animal experiments followed the applicable international, national, and/or institutional guidelines for the care and use of animals. The Southeast Poultry Research Laboratory IACUC uses the procedures as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Conflict of interest

All authors declare that they have no conflict of interest regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.06.004.

References

Ababneh, M., Ferreira, H.L., Khalifeh, M., Suarez, D.L., Afonso, C.L., 2018. First genome sequence of Newcastle disease virus of genotype VIIi from Jordan. Microbiol. Resour. Announc. 7. https://doi.org/10.1128/MRA.01136-18.

Alexander, D.J., Parsons, G., 1986. Pathogenicity for chickens of avian paramyxovirus type 1 isolates obtained from pigeons in Great Britain during 1983-85. Avian Pathol. 15, 487–493. https://doi.org/10.1080/03079458608436309.

Banerjee, M., Reed, W.M., Fitzgerald, S.D., Panigrahy, B., 1994. Neurotropic Velogenic Newcastle-Disease in cormorants in Michigan - pathology and virus characterization. Avian Dis. 38, 873–878. https://doi.org/10.2307/1592127.

Cho, S.H., Kwon, H.J., Kim, T.E., Kim, J.H., Yoo, H.S., Kim, S.J., 2008. Variation of a newcastle disease virus hemagglutinin-neuraminidase linear epitope. J. Clin. Microbiol. 46, 1541–1544. https://doi.org/10.1128/JCM.00187-08.

Collins, M.S., Alexander, D.J., Brockman, S., Kemp, P.A., Manvell, R.J., 1989. Evaluation of mouse monoclonal antibodies raised against an isolate of the variant avian paramyxovirus type 1 responsible for the current panzootic in pigeons. Arch. Virol. 104, 53–61.

Courtney, S.C., Susta, L., Gomez, D., Hines, N.L., Pedersen, J.C., Brown, C.C., Miller, P.J., Afonso, C.L., 2013. Highly divergent virulent isolates of Newcastle disease virus from the Dominican Republic are members of a new genotype that may have evolved unnoticed for over 2 decades. J. Clin. Microbiol. 51, 508–517. https://doi.org/10.1128/JCM.02393-12.

Czegledi, A., Ujvari, D., Somogyi, E., Wehmann, E., Werner, O., Lomniczi, B., 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 120, 36–48. https://doi.org/10.1016/j.virusres.2005.11.009.

Das, A., Spackman, E., Pantin-Jackwood, M.J., Suarez, D.L., 2009. Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of Avian influenza virus by RT-PCR. J. Vet. Diagn. Invest. 21, 771–778. https://doi.org/10.1177/104063870902100603.

de Leeuw, O.S., Koch, G., Hartog, L., Ravenshorst, N., Peeters, B.P., 2005. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. J. Gen. Virol. 86, 1759–1769. https://doi.org/10.1099/vir.0.80822-0.

Diel, D.G., da Silva, L.H., Liu, H., Wang, Z., Miller, P.J., Afonso, C.L., 2012a. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect. Genet. Evol. 12, 1770–1779. https://doi.org/10.1016/j.meegid.2012.07.012.

Diel, D.G., Miller, P.J., Wolf, P.C., Mickley, R.M., Musante, A.R., Emanueli, D.C., Shively, K.J., Pedersen, K., Afonso, C.L., 2012b. Characterization of Newcastle disease viruses isolated from cormorant and gull species in the United States in 2010. Avian Dis. 56, 128–133. https://doi.org/10.1637/9886-081111-Reg.1.

Diel, D.G., Susta, L., Cardenas Garcia, S., Killian, M.L., Brown, C.C., Miller, P.J., Afonso, C.L., 2012c. Complete genome and clinicopathological characterization of a virulent Newcastle disease virus isolate from South America. J. Clin. Microbiol. 50, 378–387. https://doi.org/10.1128/JCM.06018-11.

Dimitrov, K.M., Ferreira, H.L., Pantin-Jackwood, M., Taylor, T., Goraichuk, I.V., Crossley, B.M., Killian, M.L., Bergeson, N.H., Torchetti, M.K., Afonso, C.L., Suarez, D.L., 2019. Pathogenicity and transmission of virulent Newcastle disease virus from the 2018–2019 California outbreak and related viruses in young and adult chickens. Virology March. https://doi.org/10.1016/j.virol.2019.03.010.

Dimitrov, K.M., Ramey, A.M., Qiu, X., Bahl, J., Afonso, C.L., 2016. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infect. Genet. Evol. 39, 22–34. https://doi.org/10.1016/j.meegid.2016.01.008.

Dimitrov, K.M., Sharma, P., Volkening, J.D., Goraichuk, I.V., Wajid, A., Rehmani, S.F., Basharat, A., Shittu, I., Joannis, T.M., Miller, P.J., Afonso, C.L., 2017. A robust and cost-effective approach to sequence and analyze complete genomes of small RNA viruses. Virol. J. 14, 72. https://doi.org/10.1186/s12985-017-0741-5.

Dortmans, J.C., Rottier, P.J., Koch, G., Peeters, B.P., 2011. Passaging of a Newcastle disease virus pigeon variant in chickens results in selection of viruses with mutations in the polymerase complex enhancing virus replication and virulence. J. Gen. Virol. 92, 336–345. https://doi.org/10.1099/vir.0.026344-0.

Duan, Z., Li, J., Zhu, J., Chen, J., Xu, H., Wang, Y., Liu, H., Hu, S., Liu, X., 2014. A single amino acid mutation, R42A, in the Newcastle disease virus matrix protein abrogates its nuclear localization and attenuates viral replication and pathogenicity. J. Gen. Virol. 95, 1067–1073. https://doi.org/10.1099/vir.0.062992-0.

FSAP, F.S.A.P., 2019. animals and animal products. In: E.C.o.F (Ed.), Title 9 Regulations, pp. 7631 Washington, DC.

Gu, M., Liu, W., Xu, L., Cao, Y., Yao, C., Hu, S., Liu, X., 2011. Positive selection in the hemagglutinin-neuraminidase gene of Newcastle disease virus and its effect on vaccine efficacy. Virol. J. 8, 150. https://doi.org/10.1186/1743-422X-8-150.

Guo, H., Liu, X., Xu, Y., Han, Z., Shao, Y., Kong, X., Liu, S., 2014. A comparative study of pigeons and chickens experimentally infected with PPMV-1 to determine antigenic relationships between PPMV-1 and NDV strains. Vet. Microbiol. 168, 88–97. https://doi.org/10.1016/j.vetmic.2013.11.002.

Ji, Y.H., Liu, T., Du, Y.Y., Cui, X.L., Yu, Q.Z., Wang, Z.X., Zhang, J.J., Li, Y., Zhu, Q.Y., 2018. A novel genotype VII Newcastle disease virus vaccine candidate generated by mutation in the L and F genes confers improved protection in chickens. Vet. Microbiol. 216, 99–106. https://doi.org/10.1016/j.vetmic.2018.01.021.

Kim, L.M., Afonso, C.L., Suarez, D.L., 2006. Effect of probe-site mismatches on detection of virulent Newcastle disease viruses using a fusion-gene real-time reverse transcription polymerase chain reaction test. J. Vet. Diagn. Invest. 18, 519–528.

Kommers, G.D., King, D.J., Seal, B.S., Brown, C.C., 2001. Virulence of pigeon-origin Newcastle disease virus isolates for domestic chickens. Avian Dis. 45, 906–921.

Kuhn, J.H., Wolf, Y.I., Krupovic, M., Zhang, Y.Z., Maes, P., Dolja, V.V., Koonin, E.V., 2019. Classify viruses - the gain is worth the pain. Nature 566, 318–320. https://doi.org/10.1038/d41586-019-00599-8.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054.

Mast, J., Nanbru, C., Decaesstecker, M., Lambrecht, B., Couvreur, B., Meulemans, G., van den Berg, T., 2006. Vaccination of chicken embryos with escape mutants of La Sota Newcastle disease virus induces a protective immune response. Vaccine 24, 1756–1765.

Mayahi, V., Esmaelizad, M., 2017. Identification of novel E347Q and G362K amino acid substitutions in HN neutralization epitope and major antigenic difference in novel sub-genotype VIIj isolates. Acta Virol. 61, 438–444. https://doi.org/10.4149/av_2017_405.

Miller, P.J., Afonso, C.L., El Attrache, J., Dorsey, K.M., Courtney, S.C., Guo, Z.J., Kapczynski, D.R., 2013. Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. Dev. Comp. Immunol. 41, 505–513. https://doi.org/10.1016/j.dci.2013.06.007.

Miller, P.J., Koch, G., 2013. Newcastle disease. In: Swayne, D. (Ed.), Diseases of Poultry. John Wiley & Sons, Inc., Ames, IA, pp. 120–130 89-107.

Moura, V.M., Susta, L., Cardenas-Garcia, S., Stanton, J.B., Miller, P.J., Afonso, C.L., Brown, C.C., 2016. Neuropathogenic capacity of lentogenic, mesogenic, and velogenic Newcastle disease virus strains in day-old chickens. Vet. Pathol. 53, 53–64. https://doi.org/10.1177/0300985815600504.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

OIE, 2012. Newcastle Disease (infection With Newcastle Disease Virus), in: OIE (Ed.) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Mammals, Birds and Bees. Biological Standards Commission, World Organization for Animal Health, Paris, France, pp. 555–574.

OIE, 2014. CHAPTER 10.9. Newcastle disease. In: OIE (Ed.), Terrestrial Animal Health Code 2014.

Reed, J.I., Muench, H., 1938. A simple method for estimating fifth percent endpoints. Am. J. Hyg. 27.

Samal, S., Kumar, S., Khattar, S.K., Samal, S.K., 2011. A single amino acid change, Q114R, in the cleavage-site sequence of Newcastle disease virus fusion protein attenuates viral replication and pathogenicity. J. Gen. Virol. 92, 2333–2338. https://doi.org/10.1099/vir.0.033399-0.

Shabbir, M.Z., Akhtar, S., Tang, Y., Yaqub, T., Ahmad, A., Mustafa, G., Alam, M.A., Santhakumar, D., Nair, V., Munir, M., 2016. Infectivity of wild bird-origin avian paramyxovirus serotype 1 and vaccine effectiveness in chickens. J. Gen. Virol. 97, 3161–3173. https://doi.org/10.1099/jgv.0.000618.

Snoeck, C.J., Marinelli, M., Charpentier, E., Sausy, A., Conzemius, T., Losch, S., Muller, C.P., 2013. Characterization of newcastle disease viruses in wild and domestic birds in Luxembourg from 2006 to 2008. Appl. Environ. Microbiol. 79, 639–645. https://doi.org/10.1128/AEM.02437-12.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. 101, 11030–11035. https://doi.org/10.1073/pnas.0404206101.

Ujvari, D., Wehmann, E., Kaleta, E.F., Werner, O., Savic, V., Nagy, E., Czifra, G., Lomniczi, B., 2003. Phylogenetic analysis reveals extensive evolution of avian paramyxovirus type 1 strains of pigeons (Columba livia) and suggests multiple species transmission. Virus Res. 96, 63–73.

Villegas, P., 2008. Titration of biological suspensions. In: Dufour-Zavala, L., Swayne, D.E., Glisson, J.R., Pearson, J.E., Reed, W.M., Jackwood, M.W., Woolcock, P. (Eds.), A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens. AAAP, Athens, GA, pp. 217–221.

Wajid, A., Dimitrov, K.M., Wasim, M., Rehmani, S.F., Basharat, A., Bibi, T., Arif, S., Yaqub, T., Tayyab, M., Ababneh, M., Sharma, P., Miller, P.J., Afonso, C.L., 2017. Repeated isolation of virulent Newcastle disease viruses in poultry and captive non-poultry avian species in Pakistan from 2011 to 2016. Prev. Vet. Med. 142, 1–6. https://doi.org/10.1016/j.prevetmed.2017.04.010.

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, 329–338. https://doi.org/10.1128/JCM.42.1.329-338.2004.

Xu, H., Duan, Z., Chen, Y., Liu, J., Cheng, X., Liu, J., Zhu, J., Wang, X., Liu, X., Hu, S., Liu, X., 2016. Simultaneous mutation of G275A and P276A in the matrix protein of Newcastle disease virus decreases virus replication and budding. Arch. Virol. 161, 3527–3533. https://doi.org/10.1007/s00705-016-3046-x.