Identification of Newcastle disease virus (NDV) genotype VIIi in wild birds in Turkey

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Abstract

Background: Newcastle disease viruses (NDVs) can spread across continents via migratory birds. Hence, we investigated the frequency of NDV in both nonmigratory and birds migrating on the South East European flyway, in Istanbul, Turkey. Birds were trapped using nets placed around the Kucukcekmece lake Avcilar, Istanbul, in spring seasons of 2016 and 2018. In total, 297 birds belonging to 42 different species were trapped, categorised according to species and sex, and oropharyngeal swabs were collected. Swabs from 115 mallards caught by hunters in Edirne and from 207 birds belonging to 31 species which had been treated in the Veterinary Faculty of Istanbul were also collected. Tissue samples were taken from dead wild birds brought by public to Veterinary Faculty of Istanbul. A total of 619 oropharyngeal swabs were pooled into 206 samples. RNA was extracted from the swabs and tissue samples. Real-time RT-PCR prob assay was used to detect NDV-RNA in samples. **Results:** There was no amplification in real time RT-PCR in samples taken from wild birds caught by traps. However, amplification of NDV-F gene was observed in oropharyngeal swabs taken from 2 waterfowls, and in tissue samples taken from 2 little owls and 1 common kestrel. Sequencing and phylogenetic analyses of these 5 samples for NDV-F gene showed great similarity with NDV lineage VIIi viruses. One (waterfowl) of the five NDV-F gene sequences obtained in this study was slightly different than previously isolated Turkish NDVs and NDVs from Bulgaria and Georgia. Remaining four NDV-F gene sequences were similar to NDVs that were previously isolated in Turkey, Bulgaria and Georgia. Although the strains found in this study are closely related, there is a relatively small degree of molecular divergence within F gene of the Turkish NDVs and strains from Iran Pakistan, Israel and Belgium. **Conclusions:** Our findings show that first time in Turkey, genotype VIIi of NDVs is predominant in wild birds in the Eurasia region and some degree of molecular evolution when compared to the earlier NDV-VIIIi isolates in Turkey. Also, NDV viruses prevailing in the migratory birds pose a serious risk to poultry in Turkey and other countries.

Background

Newcastle disease virus (NDV) can potentially infect all species of birds and widely circulates in poultry and wild birds [1]. NDV exists in different pathotypes (lentogenic, mesogenic, and velogenic), and carries distinguishing genetic markers in the virus surface glycoproteins hemagglutinin-neuraminidase (HN) and fusion (F). The different pathotypes can cause a range of morbidities in avian hosts; infection in chickens can vary from inapparent subclinical to severe disease with 100% mortality and in some cases genetic phenotype of NDV does not translate into its pathotype potential [2]. Therefore, in some cases, determining virus virulence has been equally important together with identification of genotypic virulence markers (presence of multi-basic amino acids) at the cleavage site of the F) protein for implementation of appropriate disease control measure.

Turkey is located in the palearctic bio-geography on the flyways of the main migratory bird's route [3]. Istanbul and Bosphorus areas are seasonally populated by birds migrating from eastern Europe [4]. In a recent study, 352 bird species were identified in the Istanbul area [5].

A range of bird species are reservoirs for the NDV strains with enormous genetic diversity [2; 6; 7; 8]. There is likely a dynamic population for NDV which is carried along the transcontinental flyways for transmission to domestic poultry [7; 9]. However, depending on virus genotypes and pathotypes NDV rarely causes severe disease in wild birds [2]. Therefore, it is critical to monitor the virus population diversity in wild birds. Knowledge on the extent of viral burden and their genotypic and pathotypic characteristics can provide real time risk assessment about the

emerging threats posed. This would allow development of appropriate disease control tools and implementation of informed disease control strategies. To achieve these goals, we analysed the presence, prevalence and molecular diversity of NDV within migratory and nonmigratory wild birds in Kucukcekmece lake as well as in ducks in Edirne which borders Greece and Bulgaria.

Results

Clinical findings in birds caught in the field and birds submitted to Wildlife Rehabilitation Clinic

Neither respiratory signs nor evidence of diarrhoea were observed in the birds caught by traps. All trapped birds looked clinically healthy. In contrast, birds submitted to Veterinary Faculty of Istanbul exhibited a variety of clinical symptoms including exhaustion, diarrhea, emaciation and torticollis (Fig. 2). The common kestrel and 2 little owls submitted to the Wildlife Rehabilitation Clinics died after 2 days of clinical examination.

Necropsy

Tracheal hemorrhages which indicate tracheitis were observed in the dead common kestrel and little owls. Hemorrhages and necrosis were seen in the proventriculus of these birds (Fig. 3). The walls of the intestines were thickened and there was green mucoid diarrhoea. Patchy hemorrhages were present in the small intestine. The liver and kidneys were slightly congestive. Pale areas in the liver were noticed. Blood vessels at the surface of the cerebrum and cerebellum were conspicuous.

Histopathological findings

Nonsuppurative meningoencephalitis was observed in all birds. The histologic changes included mononuclear perivascular cuffing, edema, congestion, necrosis of purkinje cells and meningitis. Mononuclear perivascular cuffing was the most severe lesion both in the cerebrum and cerebellum (Fig. 4-A). Necrotic hepatitis and diffuse paranchyme degeneration was prominent in the liver (Fig. 4-B). The sinusoids were dilated due to congestion. There was mild infiltration of mononuclear cells in portal regions. Hyperplasia in the bile ducts was observed. The main findings in the kidneys were congestion, mild interstitial nephritis and tubular degeneration. There was necrosis, mononuclear cell infiltration and foci of vacuolation in the glandular acinar tissue of pancreas (Fig. 4-C). Alveolar vessels were congestive in the lungs. Interstitial pneumonia (Fig. 4-D), chronic myocarditis and chronic catarrhal enteritis were observed. Hemorrhages in the small intestine were prominent.

Real time RT-PCR probe assay

During optimization of the assay, optimal amplification signals were obtained when F and R primers were used in a concentration of 10 pmol/μl with 4 μl cDNA when using the positive controls. There was no positive amplification signal in samples taken from birds caught by traps as well as in the negative controls. However,

amplification of NDV F gene was observed in oropharyngeal swab samples taken from 2 waterfowl, and in tissue samples taken from 2 owls (*Athene noctua*) and 1 common kestrel (*Falco tinnunculus*)..

Sequencing and phylogenetic analysis

All 5 samples that were found positive for NDV by real time RT-PCR were subjected to NDV F gene-specific conventional RT-PCR which resulted in 534 bp amplicons (Fig. 5) that were sequenced by Sanger sequencing. Sequences were submitted to Genbank (MK210596.1, MK210597.1, MK210598.1, MK210599.1, MK210600.1). The phylogenetic analysis revealed that all analysed NDV F genes closely related to genotype VIII (Fig. 6). F sequences of the 5 NDV viruses clustered on the same branch on the phylogenetic tree with previously isolated Turkish NDV strains (KT585631.1, KP271974.1, KP271975.1, KP271976.1, KP271979.1). However, they were slightly different (0.028) than previously isolated Turkish strains (KT585617.1 and KT585629.1). In addition, one (waterfowl) of the 5 NDV-F gene sequences (MK210599.1) obtained in this study was slightly different than previously isolated Turkish NDV strains (KT585631.1, KP271974.1, KP271975.1, KP271976.1, KP271979.1) and strains isolated from Bulgaria (KP271973) and Georgia (KP271972.1). Remaining 4 NDV-F gene sequences (MK210596.1, MK210597.1, MK210598.1, MK210600.1) of NDV had a great similarity with NDV strains (KT585631.1, KP271974.1, KP271975.1, KP271976.1, KP271979.1) that were previously isolated in Turkey, Bulgaria (KP271973) and Georgia (KP271972.1) (Fig. 6). Turkish NDV viruses detected in this study showed some degree of molecular evolution when compared to the earlier isolates (15). Although strains detected in this study were very close to previously isolated Turkish strains, NDVs detected in a waterfowl, common kestrel and 2 little owls formed a different cluster from those NDVs isolated previously in Turkey, Georgia and Bulgaria (Fig. 6). Importantly, all recent Turkish NDV isolates carry an F gene that is closely related to genotype VIII of NDV class II viruses. Poultry outbreaks with this genotype VIII have been reported in Iran, Pakistan, Israel and Belgium. Although the strains found in this study are closely related, there is a relatively small degree of molecular divergence within 543 bp of F gene of the Turkish NDV isolate (KT585617.1 and KT585629.1) and strains from Iran (MG871466.1) Pakistan (KP776462.1), Israel (MH432252.1) and Belgium (MH432252.2) (Fig. 6). Suggesting that genotype VIII of NDV viruses is predominant in birds in the Eurasia Region and there is slight change in the NDV-F gene sequences.

Discussion

Newcastle disease is a notifiable disease causing severe production losses and trade restriction with a significant economic impact on the poultry industry worldwide. The reservoirs of NDV are wild birds and domestic poultry and occasional asymptomatic zoonotic infections have also been reported in humans. NDV has a very wide host range of about 250 bird species [2; 6; 7; 8]. Migratory birds are the main reservoirs for NDV and play a key role in transmitting the virus in a transboundary fashion amongst regions and countries. Turkey is home to flyways of migratory birds which connect Europe and Asia, and thus could provide an early warning base for circulating NDV strains between the Eurasian countries. Therefore, we determine the dominance of NDV strains prevailing in both migratory and nonmigratory birds and ducks in the Marmara region of Turkey that could potentially pose disease risks to local commercial and a poultry as well as transnational dissemination risks to other countries *via* wild bird migration [6; 9; 16; 7]. Results of virus isolation has shown that AMPV-1 was prevalent between 0.5 and 2.5% in waterfowl including ducks [17; 18]. However, serological prevalence was reported up to 60% [18; 19]. At present,

there is no report on monitoring wild birds for NDV in Turkey. However, in Turkey, 4 and 81 domestic avian cases were reported to OIE in 2016 and 2017, respectively. There has been no report in 2018.

In a similar study in Sanjiang natural reserve of Heilongjiang Province of China, migratory waterfowls were monitored for NDV. NDVs were isolated from waterfowls (mallard, goose, common teal and mandarin duck) [20]. In the North Sea, 543 passerine birds were investigated and the lentogenic strain of AMPV-1 was detected in 1.1% of birds [21]. In the USA, virulent strains of NDV have been found in wild birds but more frequently in pigeons, doves and doublecrested Cormorants. Research on NDV in wild ducks, gulls, and shorebirds found novel viral diversity, but no fusion gene sequences associated with high pathogenicity in poultry [7; 8]. However, it has been reported that most prevalent virulent genotype VII causing the endemics in Asia are co circulating into the ducks and chicken [22; 23]. Different genotypes of NDV viruses are prevalent in both poultry and wild birds. For example, F gene of 47 NDV isolates analysed from poultry outbreaks in Bulgaria were belong to genotypes II, IV, V and VIIb [24]. The subgenotype VIIb was also found in the Middle East [25]. Later study revealed that genotype VIId is circulating in Bulgaria and Ukraine [16]. This subgenotype from Bulgaria and Ukraine may have been part of a broader epizootic process in Eastern Europe rather than separate introductions from Asia or Africa. Similarly, analysis of 2 velogenic strains of NDV from ducks in China showed closer identity with genotype VII [22]. In the last few decades, genotype VI and genotype VII of NDV have been causing sporadic disease outbreaks in many countries in Asia and Europe including Denmark, Sweden, Switzerland, Austria, Hungary, Greece Germany, Belgium, Netherlands, Spain, Italy, Middle East, the Indian subcontinent and Indonesia [26]. Isolates of velogenic NDVs from domestic and synanthropic birds (pigeons, crows, and jackdaws) in Kazakhstan, Kirghizia, Ukraine, and Russia in 1993 to 2007 was sequenced and they were clustered in genotype VII comprising VIIa, VIIb, VIId [27].

In the past, NDV-II, VI and VII lineages were found in domestic poultry. This is the first study indicating the NDV lineage VIIi is circulating amongst wild birds and can spread virus in and amongst countries. Lately virulent strains of NDV belonging to genotype VII have been causing severe diseases outbreaks in poultry in many neighbouring countries of Turkey. Genotype VIId has been isolated from Bulgaria and Ukraine between 2002 and 2013. Iran has reported poultry outbreaks with genotype VIIb [25] and VIId [27] and VIIi [28; 29]. These studies conclude that genotype VII is a dominant strain in poultry and wild migratory birds and gradually undergoing adaptive changes, retaining fitness to survive in both immune and naturally exposed birds and having the ability to spread via migratory birds.

Our study validates these findings, 4 out of the 5 F gene sequences (MK210596.1, MK210597.1, MK210598.1, MK210600.1) of NDV had a greater similarity with NDV strains (KT585631.1, KP271974.1, KP271975.1, KP271976.1, KP271979.1) that were previously isolated in Turkey, Bulgaria (KP271973) and Georgia (KP271972.1) suggesting that this genotype remains endemic. However, they were slightly different than previously isolated Turkish strains (KT585617.1 and KT585629.1). Our data indicate that recent Turkish isolates of this study showed some degree of molecular evolution when compared to the earlier isolates [15]. Interestingly, one (waterfowl) of the 5 NDV-F gene sequences (MK210599.1) obtained in this study was slightly different than previously isolated Turkish NDV strains (KT585631.1, KP271974.1, KP271975.1, KP271976.1, KP271979.1) and strains isolated from Bulgaria (KP271973) and Georgia (KP271972.1). This indicates, multiple variants of genotype VIIi are co-circulating in birds. The data may also predict an introduction of isolates from neighbouring countries. Importantly, although the strains found in this study are closely related, there is a relatively small degree of molecular divergence within F gene of the Turkish NDV isolate (KT585617.1 and KT585629.1) and strains from

Iran (MG871466.1) Pakistan (KP776462.1), Israel (MH432252.1) and Belgium (MH432252.2). The currently used modified live viruses, LaSota and Hitchner-B1 vaccine strains are clustering on a different branch of the phylogenetic tree than the NDV isolates obtained in this study. As suggested recently by Dimitrov and others [2017], efficacy of the above mentioned modified live vaccines against the presently circulating NDV strains needs to be taken into consideration. Therefore, new vaccines and vaccination strategies may be required after searching the efficacy of current vaccines and application failures for NDV in chickens in the field.

Conclusions

Results of this study indicate that for the first time, NDV-VIII is circulating amongst wild birds and risk of spreading to different countries and continents. This threatens commercial and backyard poultry throughout the world, including Turkey, and as a result poultry production in many countries are experiencing economic loss. Therefore, continued surveillance of NDV in both migratory birds and poultry is critical for assessment of genetic traits of these viruses and their potential threats to poultry and wild birds. This can only be achieved through establishment of stronger national and international collaborations performing regional surveillance and improving disease control strategies.

Methods

Description of the wild bird trapping area

In the present study, field work was performed on the South East European migratory route of wild birds in the Marmara region of Turkey. Traps were placed around the Kucukcekmece Lake in Avcilar, Istanbul, in order to catch migratory and nonmigratory wild birds (Fig. 1). The lake contains brackish water and is about 16 km² in size, surrounded by villages, agricultural areas and forests with oaks, ash trees, shrubbery, and turpentine trees [5].

Bird traps, wild bird population and collection of samples

Mist nets were placed around Kucukcekmece lake (Fig. 1) in spring 2016 and 2018 for 50 days. The targeted bird population was migratory and nonmigratory wild birds. The traps were nylon nets, black in colour and 4x12 meter in diameter. They were left open from sunrise to sunset and checked hourly according to instructions established by the South East European Network (SEEN) for researchers [10]. *For sample collection, approval and permission were taken from the Ethics Committee of the Istanbul University-Cerrahpasa (Ethics No: 2016/39). Moreover, a legal permission to do field studies was taken from the Ministry of Forestry of Turkey and local legislation rules were strictly followed as well as international guidelines.* The birds caught were categorised according to species and sex (in species having sexual dimorphism) as described previously [11]. A total of 297 birds belonging to 42 species were trapped (Supplementary document Table 1). To avoid duplicate sampling, they were ringed under the rules and the licenses given by the General Directorate of Nature Conservation and National Parks of the Ministry of Agriculture and Forestry in Turkey. *Birds were immediately released after taking oropharyngeal swabs (Copan flocced swabs; 503CS01).*

In addition, oropharyngeal swabs (Copan flocked swabs; 503CS01) were also taken from 115 mallards (*Anas platyrhynchos*) which were caught by hunters in Edirne area of Turkey and *released after sampling*.

Wild birds submitted to Wildlife Rehabilitation Clinic

Wild birds (31 species, a total of 207) brought by citizens to Wildlife Rehabilitation Clinic at the Veterinary Faculty of Istanbul were also included in the study. After examination and recording of species and sex (in species having sexual dimorphism), oropharyngeal swabs (Copan flocked swabs; 503CS01) were taken. *These birds were kept for the rehabilitation in an isolated room till they got cured and released to the appropriate environment. Some of the birds were taken back by the same citizens who brought the birds to the Wildlife Rehabilitation Clinic.*

In addition, tissues (brain, trachea, lung, liver, pancreas and intestine) at necropsies were taken from dead birds including falcons, common kestrels submitted to Istanbul Veterinary Faculty of Istanbul (originally examined in the Wildlife Rehabilitation Clinic) and wild birds submitted to Ceyhan Veterinary Faculty.

Histopathology

Tissue samples (brain, lung, liver, pancreas and intestine) from the dead birds were analysed histopathologically. For this, samples were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, cut into 4–5 µm sections, stained with hematoxylin and eosin (HE), and blindly examined in the Department of Pathology.

RNA extraction and reverse transcription

Tubes containing oropharyngeal swabs were vortexed individually after adding 500µl of nuclease free water. A total of 619 oropharyngeal swabs were pooled into 206 samples (619:3) pools by mixing 100 µl of each swab sample to make a 300 µl pool, then 140 µl were taken for RNA extraction using QIAamp viral RNA mini kit (Qiagen, Catalog No: 52906) as per manufacturer instructions. Tissue samples taken from dead birds were homogenised using the ribolyser (Hybaid, UK) and RNA was extracted using RNeasy mini kit (Qiagen, Catalog No: 74106) as per manufacturer instructions.

The amount of RNA in the eluted samples (50 µl) was measured using a NanoDrop spectrophotometer (NanoDrop 1000c, Thermo Scientific, Waltham, USA). RNA (about 100ng) was subjected to reverse transcription for generation of cDNA using reverse transcription kit (Applied Biosystems, Cat No: 4368814) as described by the manufacturer.

Real time RT-PCR probe assay for NDV

All samples were analysed by real time RT-PCR assays for the matrix gene of NDV. Primers and probes used to detect NDV-RNA were described previously [12]. An optimized real-time RT-PCR reaction consisted of a 25 µl mixture containing of 12.5 µl Maxima/ROX qPCR Master Mix (Thermo Scientific, Catalog No: K0232), 1.25 µl forward primer (10 pMol / µl), 1.25 µl reverse primer (10 pMol / µl), 0.4 µl probe (10 pMol / µl), 2.5 µl cDNA and 7.1 µl nuclease free water. The mixture was placed in a thermal cycler (Stratagene Mx3000P, Agilent

Technologies) and the polymerase activated by incubation at 95°C for 10 minutes. Cycling conditions were 95°C for 15 seconds, 52°C for 30 seconds and 72°C for 10 seconds over 40 cycles. For all PCR reactions, nuclease-free water was used as negative control in place of the template and NDV specific RNA as positive control.

RT-PCR for sequencing partial NDV-F gene

Primers used for sequencing parts of the NDV-F gene were designed based on a previous study [13]. Samples found to be positive for NDV by real time RT-PCR were subjected to RT-PCR as described previously [13]. An optimised RT-PCR reaction consisted of a 25 µl mixture containing of 12.5 µl Maxima/ROX qPCR Master Mix (Thermo Scientific, Catalog No:K0232), 2 µl forward primer (20 pMol / µl), 2 µl reverse primer (20 pMol / µl), 0.5 µl MgCl₂ (25nM), 1 µl 2% DMSO, 5 µl nuclease free water and 2 µl cDNA. The mixture was placed in a thermal cycler (Stratagene Mx3000P, Agilent Technologies) and the polymerase activated by incubation at 95°C for 10 minutes. Cycling conditions were 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds over 30 cycles. 72°C for 5 minutes of final extension step was added at the end of the reaction. For all PCR reactions, nuclease-free water was used as negative control in place of template as well as NDV specific RNA as positive control. After the PCR, the presence of the 534 bp product for NDV-F gene was confirmed by agarose gel (1.5%) electrophoresis. Products obtained by RT-PCR using the primers specific for the partial NDV-F gene were sequenced by a commercial company (MedSanTek, Istanbul, Turkey).

Phylogenetic analysis

Multiple alignments of the NDV F gene region sequences were made using the MEGA-7 software. Phylogenetic analyses were carried out using the criterion of neighbor-joining trees based on genetic distance model by Tamura and others [14]. The reference sequences from the GenBank for NDV classification were used to reconstruct the topology of the F gene sequences generated in this study. The partial NDV F gene sequences obtained in this study were submitted to GenBank (MK210596, MK210597, MK210598, MK210599, MK210600).

Abbreviations

ND: Newcastle disease; NDV: Newcastle disease virus; HN: Hemagglutinin-neuraminidase; F: Fusion; OIE: Office Internationale des Epizootics; SEEN: South East European Network

Declarations

Ethics approval and consent to participate

International, national and institutional guidelines (University of Istanbul-Cerrahpasa Ethical Committee instructions) for the care and use of animals were followed. Ethics approval was taken from the University of Istanbul-Cerrahpasa Ethical Committee (Ethics No: 2016/39).

Consent was taken from all authors to participate in the study.

Consent for publication

Consent was taken from all authors for publication.

Availability of data and material

The data generated and/or analyzed during this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the study: NT, CO, AY, JR, MI, HY

Field work: CO, AK, KO, OA, ET, HT, SY, MY

Clinical work: SO, KO, KT, AK

Laboratory analyses: AG, AY, UC, OA, OEB, ET, HT, SY, MY

Performed the data collection: AY, UC, OA, OEB, KT, ET, HT, SY, MY, KO

Analyzed the data: AY, UC, CO, NT, HY

Wrote and/or revised the manuscripts: NT, CO, AY, AG, SO, JR, MI, HY

All authors read and approved the final version of the manuscript.

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Figures

Figure 1

Area of bird traps around Kucukcekmece lake in Avcilar, Istanbul. Bird traps were placed in this area and left for observation. Trapped birds were sampled and released. The figure was taken from the Google Earth (<https://www.google.com.tr/intl/tr/earth/>).

Figure 2

Picture of wild birds which were found to be positive for NDV-RNA by real time RT-PCR. A common kestrel (A) and 2 little owls (B).

Figure 3

Necropsy findings of the dead common kestrel. Hemorrhages and necrosis seen in the proventriculus.

Figure 4

Histopathological findings in the internal organs and brain of NDV positive birds. A: Perivascular cuffing with mononuclear cells in the cerebellum (arrow); B: Necrosis in the liver (star); C: Foci of vacuolation in the glandular acinar tissue of pancreas; D: Congestion and interstitial pneumonia in lungs.

Figure 5

Sequencing PCR for NDV. A: 100 bp Marker; B: Positive control; D: Negative control; C and E: Positive samples; Other wells: Negative samples.

Figure 6

Neighbour-joining phylogenetic tree based on partial NDV-F gene (534bp) sequences. Strain classification has been performed using the reference sequences submitted to GenBank. Red marks indicate strains detected in this study. Bootstrap supports reported near to the corresponding tree node.

Supplementary Files

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- [SupplementarydocumentTable1.docx](#)