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Presence of Newcastle disease viruses of sub-genotypes Vc and VIn in backyard chickens and in apparently healthy wild birds from Mexico in 2017

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Abstract

Virulent Newcastle disease viruses (NDV) have been present in Mexico since 1946, and recently, multiple outbreaks have been reported in the country. Here, we characterized eleven NDV isolated from apparently healthy wild birds and backyard chickens in three different locations of Jalisco, Mexico in 2017. Total RNA from NDV was reverse-transcribed, and 1285 nucleotides, which includes 3/4 of the fusion gene, was amplified and sequenced using a long-read MinION sequencing method. The sequences were 99.99–100% identical to the corresponding region obtained using the Illumina MiSeq. Phylogenetic analysis using MinION sequences demonstrated that nine virulent NDV from wild birds belonged to sub-genotypes Vc and VIn, and two backyard chicken isolates were of sub-genotype Vc. The sub-genotype Vc viruses had nucleotide sequence identity that ranged from 97.7 to 98% to a virus of the same sub-genotype isolated from a chicken in Mexico in 2010. Three viruses from pigeons had 96.3–98.7% nucleotide identity to sub-genotype VIn pigeon viruses, commonly referred to as pigeon paramyxovirus, isolated in the USA during 2000–2016. This study demonstrates that viruses of sub-genotype Vc are still present in Mexico, and the detection of this sub-genotype in both chickens and wild birds suggests that transmission among these species may represent a biosecurity risk. This is the first detection and complete genome sequencing of genotype VI NDV from Mexico. In addition, the utilization of an optimized long-read sequencing method for rapid virulence and genotype identification using the Oxford nanopore MinION system is demonstrated.

Introduction

Avian avulaviruses 1 (AAvV-1) are enveloped viruses belonging to the *Paramyxoviridae* family, genus *Avulavirus* [1], and its isolates are commonly known as Newcastle disease viruses (NDV, used hereafter). The viruses have a negative-sense RNA genome, that varies in size from 15,186 to 15,198 nucleotides (nt) and encodes six genes in the following order: 3′-nucleoprotein (NP)-phosphoprotein (P)-matrix (M)-fusion (F)-hemagglutinin-neuraminidase (HN)-polymerase (L)-5′ [2]. Newcastle disease viruses have variable pathogenicity and have been shown to infect at least 250 bird species across the world [3]. NDV is distributed worldwide, and virulent strains often cause severe disease in poultry or wild birds [3].

The NDV isolates are genetically classified into two major groups (class I and class II) based on phylogenetic analysis [4]. Class II NDV isolates have higher genetic diversity and are divided into at least 18 genotypes (named I to XVIII) that generally have greater than 10% sequence differences between genotypes, and some genotypes are further sub-divided into sub-genotypes [5–7]. Virulent NDV strains have been detected in all class II genotypes, except genotype X [8]. Class I NDV isolates are primarily found in

wild birds, although poultry detections are not uncommon. There is only one class I virus that has been reported to be virulent for poultry [8].

Virulent NDV are endemic in many countries, and can cause a serious economic drag on poultry production, and when spread to NDV-free countries, can cause costly outbreaks. Such outbreaks result in significant economic losses due to imposed trade embargoes on live birds and poultry products and for the actions directly related to the outbreak such as, eradication, monitoring of the virus, and indemnities for certain countries [9]. In 2017, 58 countries on all continents, except Oceania and Antarctica, reported an occurrence of NDV to the World Organisation for Animal Health (OIE). Over 2400 outbreaks were reported to OIE in 2017, with reports mainly from Asia and in Africa [10].

NDV was first described in Mexico in 1946 [11]. The earliest Mexican virus to be sequenced was isolated in 1947 and was characterized as a member of genotype XVI (chicken/Mexico/Queretaro/452/1947), which seems to be the precursor of the viruses circulating in the Dominican Republic in 2008 [12]. In the early 1970's, a new group, sub-genotype Vb, was identified that was affecting the Mexican poultry industry, and the index strain is commonly referred to as the Chimalhuacan strain [13]. Related sub-genotype Vb NDV have caused many outbreaks in countries in the Americas in the 1970's [13–15]. Viruses of this sub-genotype also caused the outbreaks in California during 2002–2003 [16, 17]. Vaccination programs were implemented in the affected areas to help reduce the clinical disease. A related but distinct group of viruses, sub-genotype Vc was first detected in Mexico in 2004 and has continued to circulate in poultry from 2004 to 2011 with minor genetic changes [11, 17–19]. These sub-genotype Vc viruses, which have only been found in Mexico, were previously identified in free-living wild birds and in captive wild birds, in addition to chickens [11, 17]. Mexico was declared as an NDV-free country in 2016 [20], since virulent NDV had not been reported during 2014 and 2015. Nevertheless, three outbreaks were reported to OIE from the state of Jalisco in March of 2016 [10].

Most of the detections of virulent NDV in wild birds are likely a spillover from poultry into the free-ranging hosts [21, 22]. However, Columbiformes are recognized as the major natural reservoirs of genotype VI NDV, also referred to as pigeon paramyxoviruses 1 (PPMV-1). The genotype VI viruses can cause severe disease, including neurological signs in Columbiform birds [3]. Virulent NDV in feral pigeons was first detected in the Middle East in 1978 [23], and since then, genotype VI NDV has been reported in many countries worldwide with multiple sub-genotypes identified in pigeons or doves [24–26]. The sub-genotype VIh NDV has previously been detected in South America causing outbreaks in pigeons, and the sub-genotypes VIa and VIn have been reported in wild pigeons in the US [24, 27–29]. These

viruses do not always cause disease in chickens [30–32]. However, sporadic outbreaks in poultry, caused by NDV viruses from genotype VI, were reported in European countries, US, and Africa [33–36] but never in Latin America.

The present study aimed to improve the knowledge about the recurrence and circulation of NDV in Mexico, specifically by characterizing viruses isolated in Jalisco, Mexico, from samples collected from chickens and wild birds during 2017. Utilizing both MinION and Illumina sequencing technologies, we have rapidly identified the sub-genotypes of these new isolates, and provided the first report and complete genome sequence of genotype VI NDV isolated in Mexico. Furthermore, complete genome analyses show that viruses of sub-genotype Vc continue to circulate and evolve in Mexico despite the ongoing vaccination campaigns.

Materials and methods

Viruses

Nine isolates from the Southeast Poultry Research Laboratory (SEPRL) repository (Table 1), representing eight different genotypes from class II, were propagated in 9–11-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE), and the allantoic fluid was harvested for use in MinION optimization in this study. Eleven isolates from the Instituto Politecnico Nacional in Mexico were also used in this study. The isolates were obtained from apparently healthy wild birds and two from backyard chickens captured near three different chicken farms (A, B, and C) during three periods of 2017 (July, August, and October) in Jalisco, Mexico (Table 2).

RNA purification and RRT-PCR

Total RNA from the nine SEPRL repository NDV was extracted using Trizol LS reagent (Thermo Fisher Scientific, USA) following manufacturer's instructions. For the isolates from Mexico, total RNA was extracted from allantoic fluids using the QIAamp viral RNA kit (QIAGEN, Mexico.) and sent to SEPRL. Primers and probes targeting the matrix (M) gene were used for nucleic acid amplification and virus detection by RRT-PCR as described previously [37].

MinION assay optimization and sequencing of Mexican isolates

In order to obtain rapid identification and genotyping of field samples, we optimized a previously described assay for MinION sequencing [38] with the design of new primers binding to highly conserved regions. These NDV-specific primers (5′TAGAAAAAACACGGGTAGAAGAGT

Table 1 Nine class II NDV isolates from the SEPRL repository used for optimization of the MinION protocol. All viruses were identified by both MinION and MiSeq

SEPRL ID	GenBank number	Genotype	Isolate	NDV reads	Mean depth	% Pairwise nucleotide identity between MinION and MiSeq sequences
10	MH392213	I	Queensland V4	1401	730	100
LS	MH392212	II	USA/LaSota/1946	667	429	100
899	MH392215	IV	chicken/Nigeria/Kano/VRD09/025/1973	1443	741	100
250	MH392216	Va	cormorant/USA/MN/92-40140/1992	1166	775	99.92
1011	KX236101	VIk	pigeon/Pakistan/Lahore/25A/2015	531	399	100
996	KX496962	VIIi	pigeon/Pakistan/Lahore/20A/2015	1431	794	100
494	MK124761	VIId	goose/ChinaZJ/1/2000	1529	926	100
641	JQ697741	IX	China/03-45-641/2003	920	538	100
867	KY171995	XVII	chicken/Nigeria/VRD124/2006	1318	857	100

Table 2 Background data and RRT-PCR results of 11 Mexican NDV isolates

SEPRL ID	GenBank number	Sample Name	Bird Specie	Near farms (Jalisco, MX)	Collection date	RRT-PCR (Ct value)
1410		Ave1-01	Sparrow	A	08/11/2017	12.41
1264*	MK046916	Ave2-01	Dove	A	08/11/2017	14.56
1265*	MK046917	Ave3-01	Pigeon	A	08/11/2017	13.29
1266*	MK046918	Ave4-01	Pigeon	B	08/11/2017	14.58
1267*	MK046919	Ave5-01	Pigeon	B	08/11/2017	14.08
1268		Ave6-01	Pigeon	B	08/11/2017	14.24
1411		Ave7-01	Chicken	B	08/11/2017	24.74
1270*	MK046921	LV-7213	Pigeon	C	07/04/2017	15.33
1412		LV-1517	Pigeon	C	07/04/2017	25.99
1279		VX17144-2	Pigeon	B	10/10/2017	14.25
1280*	MK046920	LVVX17186-1	Chicken	C	10/10/2017	14.51

All samples were tested by MinION, and the six samples were tested by MiSeq are represented by asterisk

CTG and 5′TGCGATATGATACCBGGAGGGT) were designed using the complete genomes of 50 NDV isolates selected from each genotype and most sub-genotypes. The newly designed primers flanked a larger region of 1285 nt that includes 75% of the coding sequence of the fusion gene (it spans 1229 nt from the start of the coding sequence) and includes the fusion cleavage site.

To test the efficacy of the new primers, nine isolates representing class II genotypes I, II, IV, Va, VIk, VIId, VIIi, IX, and XVII (Table 1) were amplified as previously described [38] with slight modifications. Amplicon synthesis included using the OneStep RT-PCR kit (Qiagen, USA) and the new NDV-specific primers, tailed with the following universal adapter sequences (5′TTTCTGTTG GTGCTGATATTGC and 5′ACTTGCCTGTCGCTCTAT CTTC). The cycling conditions were as follows: 1 cycle of 50 °C for 30 min (min) and 95 °C for 3 min; 40 cycles of 94 °C for 15 s (sec), 58 °C for 30 s, and 68 °C for 90 s. The reaction amplified a 1285 bp product.

Following Oxford Nanopore's recommendations, MinION DNA libraries were created by pooling the bar-coded amplicons of the nine repository viruses, and the libraries were sequenced until approximately 60,000 reads that passed the quality filter (Q > 7) were collected. Data analysis of the Nanopore sequencing data was completed by using a previously described bioinformatics workflow for de novo assembly [38]. Next, the final consensus sequences of all sequenced isolates were BLAST (NCBI) searched in order to identify closely related, published sequences. To obtain the most accurate consensus sequence for each virus, the respective top BLAST sequence was downloaded and used for reference-based mapping of the MinION raw sequencing data.

Due to the successful sequencing of the repository isolates using the newly designed primers, the aforementioned protocol was then applied to sequence the 11 isolates from Mexico as described above. Sequencing of the pooled barcoded libraries and data analysis of the Mexico viruses resulted in 400,000 raw reads that passed the quality filter (Q > 7) and were used in subsequent data analyses as described above.

MiSeq sequencing

Total RNAs from six isolates from Mexico (Table 3) and one isolate from the SEPRL repository (SEPRL ID: 494) were quantified by Qubit fluorimetry (Thermo Fisher Scientific, USA). RNA was reverse-transcribed and DNA libraries for next-generation sequencing were prepared as described previously [24]. Paired-end sequencing (2 × 250 base pairs) of the generated libraries was performed on an Illumina MiSeq instrument using the 500 cycle MiSeq Reagent Kit version 2 (Illumina, USA). Raw sequence data were analyzed and assembled using MIRA version 3.4.1 within a customized workflow on the Galaxy platform as described previously [39].

Comparison between MinION and MiSeq consensus sequences

To evaluate the accuracy of the results obtained with the newly designed primers, the pairwise nucleotide identity between the consensus sequences of the nine repository viruses obtained by MinION were also compared to the MiSeq sequences of these viruses already available in GenBank, except for NDV SEPRL ID: 494 isolate, which was sequenced by MiSeq in this study. Similarly, a pairwise identity analysis was done using the consensus sequences of six Mexican viruses sequenced by both MinION and MiSeq. The pairwise distances were estimated using the Maximum Likelihood model [40] as implemented in MEGA7 [41] from an alignment containing a total of 1229 positions.

Phylogenetic analysis of the newly characterized Mexican viruses

Phylogenetic analyses were performed utilizing the fusion gene coding sequences and complete genome concatenated coding sequences of NDV obtained by MinION and MiSeq using MEGA7 [41]. Complete fusion gene coding sequences and complete genomes closely related to the viruses studied here were downloaded from GenBank, resulting in two datasets (n = 85 and n = 51, respectively). 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 [42]. There were a

total of 1229 and 13,746 nucleotide positions, respectively, in the final analyses. Evolutionary distances were estimated between the nucleotide sequences obtained from Mexican viruses and the most closely related sequences available in GenBank applying the Maximum Composite Likelihood model [40] with 1000 bootstrap replicates using MEGA7 [41]. 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 [5].

GenBank numbers

The obtained complete genome sequences of NDV were submitted to GenBank and are available under the accession numbers MK046916 to MK046921, and MK124761.

Results

RRT-PCR

All of the isolates obtained from Mexico were NDV positive by the RRT-PCR targeting the M gene (Table 2). The Ct values were low and ranged from 12.41 to 25.99.

MinION assay optimization and MinION sequencing of Mexican isolates

The nine isolates from class II genotypes I, II, IV, Va, VIk, VIId, VIIi, IX, and XVII were sequenced successfully, with number of reads varying from 531 to 1529 and mean depths ranging from 399 to 926. The final consensus sequences allowed the correct determination of the respective sub/genotype of each isolate. Furthermore, pairwise nucleotide identities between the MinION sequences and sequences of these same viruses obtained in Miseq were 100% (except the sequence of cormorant/USA/MN/92-40140/1992, where a single nucleotide substitution resulted in 99.92% identity) (Table 1).

Eleven Mexican NDV isolates were successfully sequenced in the Nanopore MinION device using this new primer set. This amplicon sequencing technique produced raw reads varying from 2072 to 39,709 reads per sample, with an average read length of 1277 nucleotides and an overall high mean depth of ×6902 (Table 3). Artifact deletions of one base (after homopolymeric sequences) were observed in the MinION sequences of the LV-7213, LV1517, and VX17144-2 viruses (deletion of C at position 282 or deletion of G at positions 123 and 768) and these were manually corrected.

The cleavage site of all of the sequences that were classified into sub-genotype Vc had three arginines between

Table 3 Results from sequencing 11 Newcastle disease viruses isolated from chickens and wild birds in Mexico by MinION and MiSeq

| SEPRL ID | Isolate Name | MinION | | | | | MiSeq | | | | | MinION vs MiSeq |
		NDV Genotype	% Identity	Consensus length	NDV reads	Mean depth	NDV genotype	% Identity	Alignment length	% Coverage	Mean depth	% Pairwise Identity
1410	Ave1-01	Vc	97.9	1285	3303	1808	ND					NA
1264	Ave2-01	Vc	98.2	1254	15,651	12,320	Vc	97.5	13,439	88.5	15	99.99
1265	Ave3-01	Vc	97.9	1285	2072	1301	Vc	97.6	15,172	99.9	2433	99.99
1266	Ave4-01	Vc	97.9	1285	9082	5567	Vc	97.6	15,168	99.9	192	99.99
1267	Ave5-01	Vc	98.1	1285	39,709	26,349	Vc	97.6	15,175	99.9	9428	99.99
1268	Ave6-01	Vc	97.9	1285	21,030	13,898	ND					NA
1411	Ave7-01	Vc	97.9	1285	3576	2679	ND					NA
1270	LV-7213	VIn	98.5	1285	4084	3363	VIn	98.1	15,168	99.9	1135	99.99
1412	LV-1517	VIn	97.6	1222	6196	5317	ND					NA
1279	VX17144-2	VIn	98.4	1285	267	242	ND					NA
1280	LVVX17186-1	Vc	97.7	1285	4197	3078	Vc	97.4	15,171	99.9	885	99.99

The alignment length, nucleotide identity (%) obtained using BLAST, coverage of NDV genome (%), and sequencing depth are shown. The nucleotide identities that are shown are to the closest publicly available sequences in GenBank (JX974435.1 and JN872179.1 for sub-genotypes Vc and Vin, respectively)

ND not done, *NA* not applicable

The nucleotide identity between the consensus sequences obtained by MinION and MiSeq for each of the studied viruses is also presented

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]RQRR↓F[117]); whereas, the cleavage site of the sequences from sub-genotype VIn had four basic amino acids (two arginines and two lysines) 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]RKKR↓F[117]). Such cleavage sites are specific for virulent viruses based on criteria utilized by OIE to assess virulence of NDV isolates [43].

MiSeq sequencing

One virus from chickens and five viruses from wild birds were selected for complete genome random MiSeq sequencing. These six isolates were chosen to represent all species, locations, and time periods (Table 3). Of all isolates, the results of the Ave2 had the lowest mean depth of ×15 with a coverage of 88.5%. The other five consensus sequences had a coverage of 99.9% with the number of sequencing reads to produce them ranged from 17,579 to 836,012 with a mean depth varying from ×192 to ×9428.

Comparison between MinION and MiSeq sequences

Pairwise nucleotide distances between the MinION and MiSeq sequences of the Mexican isolates were 99.99% for all sequences when comparing the 1229 nucleotide region of the fusion gene (Table 3). The cleavage sites of all sequences obtained by MinION were identical to those obtained by MiSeq sequencing.

Phylogenetic analysis

The performed phylogenetic analyses of the 11 isolates from Mexico using the sequences obtained from MinION allowed the identification of NDV from two sub-genotypes (Fig. 1, Table 3). Eight isolates from both wild birds and backyard chickens were classified as members of sub-genotype Vc (Fig. 1). Not only did all sub-genotype Vc viruses cluster together, but they also clustered with viruses from Mexico isolated from 2008 to 2010. The sequences of one chicken virus (Ave07) and the six sub-genotype Vc viruses isolated from wild birds in August 2017 from farm A and B were identical, except one synonymous nucleotide substitution (T1218C) of the fusion coding sequence in isolates Ave3 and Ave5 (Supplementary Table 1). These seven isolates had 99.7–99.8% nucleotide identity to the chicken virus LVVX17186-1 isolated near farm C in October 2017. The seven isolates from wild birds and a chicken had 97.5–98.5% nucleotide identity to viruses detected in Mexico during 2008-2010 (Table 3), while the chicken isolate LVVX17186-1 had 97.2 and 98.2% nucleotide identity to the same sequences from Mexico.

Three pigeon isolates (LV-7213, LV1517, and VX17144-2) were classified as sub-genotype VIn members. Isolates LV-7213 and VX17144-2 were almost identical with 99.8% nucleotide identity. These two isolates had 98.2–98.7% nucleotide identity, respectively, to viruses isolated from pigeon and dove in Texas and Nevada from 2000 to 2005 (Supplementary Table 1), and 97.2–97.9% identity when compared to more recent viruses detected in the USA in 2014–2016. Isolate LV-1517 was more distant to the other two studied viruses with 96.5 and 96.3% nucleotide identities when compared to LV-7213 and VX17144-2, respectively. LV-1517 showed 97.5 and 96.6% nucleotide identity to viruses isolated from pigeons in Nevada from 2003 and in Minnesota from 2007, respectively.

Phylogenetic analysis using NDV complete genome sequences confirmed that one chicken and four wild bird isolates are members of sub-genotype Vc and one pigeon isolate (LV-7213) belongs to sub-genotype VIn (Supplementary Fig. 1).

Discussion

NDV has been present in Mexico as early as 1946 [11] and since then, over 510 outbreaks were reported to OIE, causing economic losses to the poultry industry of the country [10, 17–19, 44]. Viruses of sub-genotypes Vb and Vc were identified and characterized in some of these outbreaks [19, 44]. Our study shows that viruses of sub-genotype Vc are still present in Mexican chickens. Additionally, the presence of sub-genotype Vc viruses, commonly isolated from chickens, in wild birds suggests spillover between poultry and wild bird populations. The continuous presence of sub-genotype Vc viruses in domestic and wild birds over the years indicates that this NDV sub-genotype, which has only been reported in Mexico, may be endemic in this country.

The reported spillover of NDV from domestic to wild birds most often involves vaccine viruses, and the spillover of virulent viruses is not well documented [8, 11]. Because pigeons, doves, and sparrows are peridomestic birds, these birds could act as a biological vector of spread of NDV between poultry farms and other wild bird species. Indeed, virulent NDV genotypes have been detected in free-living wild birds [8]. However, the impact of these viruses on wild bird populations, including on endangered species, needs to be further elucidated. This underlines the continuous need to characterize isolates from free-living wild birds and increase the knowledge of NDV ecology in wild bird species.

Our study reports the first occurrence of genotype VI in Mexico and surprisingly in apparently healthy pigeons. The Mexican viruses of sub-genotype VIn are closely related to

Fig. 1 Phylogenetic analysis of the fusion protein gene sequences obtained with Min-ION. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (− 10575.43) 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 a superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.4498)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 85 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1229 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The sequences from Mexico from 2017 are highlighted in red and blue and are classified into sub-genotypes VIn and Vc, respectively
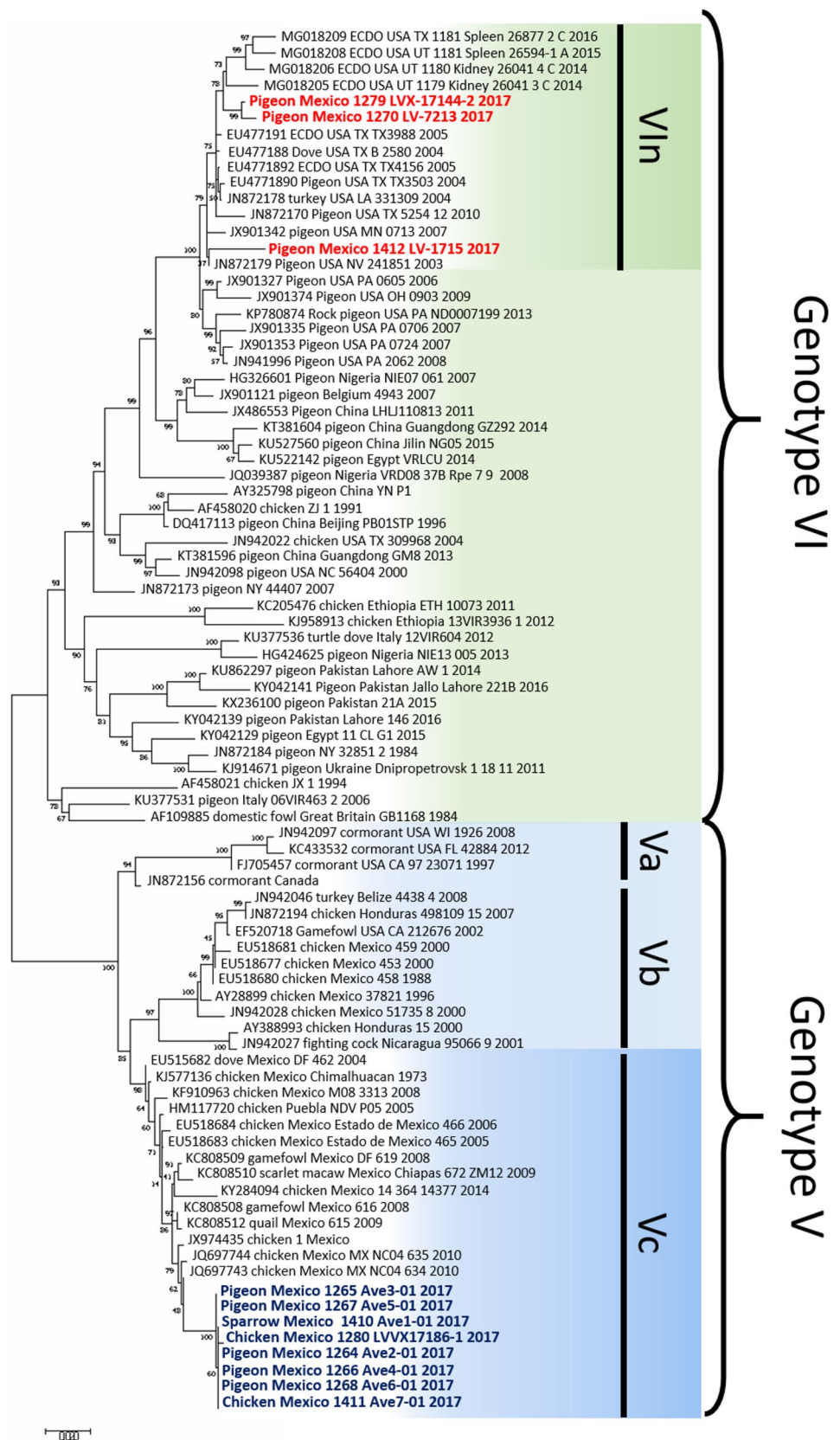
viruses identified in three states of the USA [24]. From 2003 to 2010, the sub-genotypes VIa and VIn caused mortality in pigeons in the USA [24]. The present work also demonstrates the epidemiological connection between US and Mexican pigeon-adapted NDV. However, pigeons are non-migratory birds, and the epidemiological connection and introduction of the viruses to one or the other place remains unclear. Interestingly, genotype VI viruses from other sub-genotypes that are closely related to each other have been detected in distant regions, such as Western Europe, Asia, and North Africa [45]. It has been speculated that the spread of viruses of this genotype could be caused by movement of birds during pigeon competitions and exhibitions or trade [46]. The illegal movement of these birds is also a viable possibility. Although genotype VI is endemic in many countries, this genotype is rarely reported from poultry farms [25, 35, 47, 48].

During outbreaks, it is important to identify the viral pathogens, and to sequence their partial or complete genomes to quickly characterize them [49]. Currently, this is achieved through a combination of methods that are sometimes laborious and often require several days to conduct. The MinION sequencing provides the ability to not only quickly identify the virus causing an outbreak but also to obtain genetic information that allows the patho- and genotyping of the virus [38]. Here, a previously described MinION protocol was further optimized with the design of new primers that amplify 1229 nucleotides of the fusion gene coding sequence. The accuracy of the results obtained using the newly designed primers was demonstrated by the 99.92–100% nucleotide identity between the sequences obtained by MinION and MiSeq. These new primers amplify a larger part of the fusion gene coding sequence, allowing the utilization of more genetic information in the phylogenetic inferences. This protocol was used to successfully sequence 11 viruses isolated in Mexico and to identify the sub-genotype to which these viruses belong to. The results also allowed the inference of the amino acid motif of the fusion protein cleavage site and the virulence of the sequenced viruses.

We used both MinION and MiSeq sequencing platforms when studying these viruses isolated from Mexico. The consistency of the topologies of the phylogenetic trees and the identity of the consensus sequences obtained through both methods demonstrate the reliability of this MinION protocol. This is in agreement with a previous report utilizing the Oxford Nanopore technology to study NDV [38] that also demonstrates high accuracy of the sequences obtained by MinION. As seen from this study, the larger consensus sequences obtained from MinION were almost identical to those from MiSeq. The low initial investment and the demonstrated high accuracy of the MinION device make this a sequencing tool that can be utilized by most labs and across

different platforms. The MinION approach has some flaws; for example, there are insertions and deletions in the raw reads after homopolymeric sequences that may also be present in the final consensus and the error rate at read level is still high. However, as we demonstrate here and it has also been shown in previous reports [38, 50], this can be overcome as deep sequencing data produce consensus sequences comparable to those obtained by Illumina instruments.

In summary, our study reports the first detection and complete genome of genotype VI NDV in Mexico, and an epidemiological connection between US and Mexican pigeon-adapted NDV. It also reported that viruses of sub-genotype Vc continue to circulate and evolve locally in Mexico, and their detection in both chickens and wild birds suggest a spillover from poultry. Our data suggest that the described MinION protocol with the newly designed primers could be used for robust and rapid genotype identification of the NDV as the sequences obtained here provide phylogenetic inferences consistent with those obtained using complete genome MiSeq data.

Compliance with Ethical Standards

References

1. Amarasinghe GK, Bao Y, Basler CF, Bavari S, Beer M, Bejerman N, Blasdell KR, Bochnowski A, Briese T, Bukreyev A, Calisher CH, Chandran K, Collins PL, Dietzgen RG, Dolnik O, Durrwald R, Dye JM, Easton AJ, Ebihara H, Fang Q,

Formenty P, Fouchier RA, Ghedin E, Harding RM, Hewson R, Higgins CM, Hong J, Horie M, James AP, Jiang D, Kobinger GP, Kondo H, Kurath G, Kurath G, Lamb RA, Lee B, Leroy EM, Li M, Maisner A, Muhlberger E, Netesov SV, Nowotny N, Patterson JL, Payne SL, Paweska JT, Pearson MN, Randall RE, Revill PA, Rima BK, Rota P, Rubbenstroth D, Schwemmle M, Smither SJ, Song Q, Stone DM, Takada A, Terregino C, Tesh RB, Tomonaga K, Tordo N, Towner JS, Vasilakis N, Volchkov VE, Wahl-Jensen V, Walker PJ, Wang B, Wang D, Wang F, Wang LF, Werren JH, Whitfield AE, Yan Z, Ye G, Kuhn JH (2017) Taxonomy of the order Mononegavirales: update 2017. Arch Virol. https://doi.org/10.1007/s00705-017-3311-7

2. Lamb RA, Parks G (2013) Paramyxoviridae. In: Knipe DM, Howley PM (eds) Fields virology. Lippincott Williams & Wilkins, Philadelphia, pp 957–995

3. Miller PJ, Koch G (2013) Newcastle disease. In: Swayne D (ed) Diseases of poultry. Wiley, Ames, IA, pp. 89–107; 120–130

4. 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

5. Diel DG, da Silva LH, Liu H, Wang Z, Miller PJ, Afonso CL (2012) 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

6. de Almeida RS, Hammoumi S, Gil P, Briand FX, Molia S, Gaidet N, Cappelle J, Chevalier V, Balanca G, Traore A, Grillet C, Maminiaina OF, Guendouz S, Dakouo M, Samake K, Bezeid Oel M, Diarra A, Chaka H, Goutard F, Thompson P, Martinez D, Jestin V, Albina E (2013) New avian paramyxoviruses type I strains identified in Africa provide new outcomes for phylogeny reconstruction and genotype classification. PLoS ONE 8:e76413. https://doi.org/10.1371/journal.pone.0076413

7. Snoeck CJ, Owoade AA, Couacy-Hymann E, Alkali BR, Okwen MP, Adeyanju AT, Komoyo GF, Nakoune E, Le Faou A, Muller CP (2013) High genetic diversity of newcastle disease virus in poultry in West and Central Africa: cocirculation of genotype XIV and newly defined genotypes XVII and XVIII. J Clin Microbiol 51:2250–2260. https://doi.org/10.1128/Jcm.00684-13

8. Dimitrov KM, Ramey AM, Qiu X, Bahl J, Afonso CL (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

9. Villegas P (1998) Viral diseases of the respiratory system. Poult Sci 77:1143–1145. https://doi.org/10.1093/ps/77.8.1143

10. OIE (2018) Detailed country (ies) disease incidence. http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail

11. Cardenas Garcia S, Navarro Lopez R, Morales R, Olvera MA, Marquez MA, Merino R, Miller PJ, Afonso CL (2013) Molecular epidemiology of Newcastle disease in Mexico and the potential spillover of viruses from poultry into wild bird species. Appl Environ Microbiol 79:4985–4992. https://doi.org/10.1128/AEM.00993-13

12. Courtney SC, Susta L, Gomez D, Hines NL, Pedersen JC, Brown CC, Miller PJ, Afonso CL (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

13. Absalon AE, Mariano-Matias A, Garcia LJ, Morales-Garzon A, Toscano-Contreras A, Lucio-Decanini E, Cortes-Espinosa DV (2014) Complete genome analysis of velogenic Newcastle disease virus reference strain "Chimalhuacan": evolution of viral lineages in Mexico. Virus Genes 49:233–236. https://doi.org/10.1007/s11262-014-1082-8

14. Fernandes CC, Varani AM, Lemos EG, de Miranda VF, Silva KR, Fernando FS, Montassier MF, Montassier HJ (2014) Molecular and phylogenetic characterization based on the complete genome of a virulent pathotype of Newcastle disease virus isolated in the 1970s in Brazil. Infect Genet Evol. https://doi.org/10.1016/j.meegid.2014.05.014

15. Walker JW, Heron BR, Mixon MA (1973) Exotic Newcastle disease eradication program in the United States. Avian Diseases 17:486–503

16. Pedersen JC, Senne DA, Woolcock PR, Kinde H, King DJ, Wise MG, Panigrahy B, Seal BS (2004) Phylogenetic relationships among virulent Newcastle disease virus isolates from the 2002-2003 outbreak in California and other recent outbreaks in North America. J Clin Microbiol 42:2329–2334. https://doi.org/10.1128/Jcm.42.5.2329-2334.2004

17. Perozo F, Merino R, Afonso CL, Villegas P, Calderon N (2008) Biological and phylogenetic characterization of virulent Newcastle disease virus circulating in Mexico. Avian Dis 52:472–479. https://doi.org/10.1637/8276-022908-Reg.1

18. Xiao S, Paldurai A, Nayak B, Mirande A, Collins PL, Samal SK (2013) Complete genome sequence of a highly virulent newcastle disease virus currently circulating in Mexico. Genome Announc 1:e00177-00112. https://doi.org/10.1128/genomeA.00177-12

19. Absalon AE, Mariano-Matias A, Vasquez-Marquez A, Morales-Garzon A, Cortes-Espinosa DV, Ortega-Garcia R, Lucio-Decanini E (2012) Complete genome sequence of a velogenic Newcastle disease virus isolated in Mexico. Virus Genes 45:304–310. https://doi.org/10.1007/s11262-012-0782-1

20. SAGARPA SdA, Ganadería, Desarrollo Rural, Pesca y Alimentación (2016) Acuerdo mediante el cual se enlistan las enfermedades y plagas de los animales, exóticas y endémicas de notificación obligatoria en los Estados Unidos Mexicanos. Diario Oficial 4 de mayo de 2016.

21. Ayala AJ, Dimitrov KM, Becker CR, Goraichuk IV, Arns CW, Bolotin VI, Ferreira HL, Gerilovych AP, Goujgoulova GV, Martini MC, Muzyka DV, Orsi MA, Scagion GP, Silva RK, Solodiankin OS, Stegniy BT, Miller PJ, Afonso CL (2016) Presence of Live Newcastle Disease Vaccines in Wild Birds. PLoS One. https://doi.org/10.1371/journal.pone.0162484

22. Devlin JM, Vaz PK, Coppo MJ, Browning GF (2016) Impacts of poultry vaccination on viruses of wild bird. Curr Opin Virol 19:23–29. https://doi.org/10.1016/j.coviro.2016.06.007

23. Kaleta EF, Alexander DJ, Russell PH (1985) The first isolation of the avian PMV-1 virus responsible for the current panzootic in pigeons ? Avian Pathol 14:553–557. https://doi.org/10.1080/03079458508436258

24. He Y, Taylor TL, Dimitrov KM, Butt SL, Stanton JB, Goraichuk IV, Fenton H, Poulson R, Zhang J, Brown CC, Ip HS, Isidoro-Ayza M, Afonso CL (2018) Whole-genome sequencing of genotype VI Newcastle disease viruses from formalin-fixed paraffin-embedded tissues from wild pigeons reveals continuous evolution and previously unrecognized genetic diversity in the U.S. Virol J. https://doi.org/10.1186/s12985-017-0914-2

25. Alexander DJ (2011) Newcastle disease in the European Union 2000 to 2009. Avian Pathol 40:547–558. https://doi.org/10.1080/03079457.2011.618823

26. Chong YL, Lam TT, Kim O, Lu H, Dunn P, Poss M (2013) Successful establishment and global dispersal of genotype VI avian paramyxovirus serotype 1 after cross species transmission. Infect Genet Evol 17:260–268. https://doi.org/10.1016/j.meegid.2013.04.025

27. Souza S, Fredo G, Dupont P, Leite-Filho R, Pavarini SC, Canal C, Driemeier D (2018) Pathological and molecular findings of avian avulavirus Type 1 outbreak in pigeons

(Columba livia) of southern Brazil. Pesq Vet Bras. https://doi.org/10.1590/1678-5150-pvb-5528

28. Zanetti F, Mattiello R, Garbino C, Kaloghlian A, Terrera MV, Boviez J, Palma E, Carrillo E, Berinstein A (2001) Biological and molecular characterization of a pigeon paramyxovirus type-1 isolate found in Argentina. Avian Dis 45:567–571. https://doi.org/10.2307/1592896

29. Castro ER, Zanetti F, Arbiza J (2012) Genetic characterization of a pigeon paramyxovirus type 1 isolated from Columba livia in Uruguay. Avian Dis 56:243–248. https://doi.org/10.1637/9835-061611-Case.1

30. Dortmans JC, Rottier PJ, Koch G, Peeters BP (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

31. Dortmans JCFM, Koch G, Rottier PJM, Peeters BPH (2009) Virulence of pigeon paramyxovirus type 1 does not always correlate with the cleavability of its fusion protein. J Gen Virol 90:2746–2750. https://doi.org/10.1099/vir.0.014118-0

32. Dortmans JCFM, Rottier PJM, Koch G, Peeters BPH (2010) The viral replication complex is associated with the virulence of Newcastle disease virus. J Virol 84:10113–10120. https://doi.org/10.1128/Jvi.00097-10

33. Munir M, Linde AM, Zohari S, Stahl K, Baule C, Engstrom B, M Renström LH, Berg M (2011) Whole genome sequencing and characterization of a virulent Newcastle disease virus isolated from an outbreak in Sweden. Virus Genes 43:261–271. https://doi.org/10.1007/s11262-011-0636-2

34. Hanson RP, Spalatin J, Jacobson GS (1973) The viscerotropic pathotype of Newcastle disease virus. Avian Dis 17:354–361. https://doi.org/10.2307/1589219

35. Alexander DJ, Wilson GW, Russell PH, Lister SA, Parsons G (1985) Newcastle disease outbreaks in fowl in Great Britain during 1984. Vet Rec 117:429–434

36. Damena D, Fusaro A, Sombo M, Belaineh R, Heidari A, Kebede A, Kidane M, Chaka H (2016) Characterization of Newcastle disease virus isolates obtained from outbreak cases in commercial chickens and wild pigeons in Ethiopia. Springerplus 5:476. https://doi.org/10.1186/s40064-016-2114-8

37. Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, Kapczynski DR, 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

38. Butt SL, Taylor TL, Volkening JD, Dimitrov KM, Williams-Coplin D, Lahmers KK, Rana AM, Miller PJ, Suarez DL, Afonso CL, Stanton JB (2018) Rapid virulence prediction and identification of Newcastle disease virus genotypes using third-generation sequencing. Virol J. https://doi.org/10.1101/349159

39. Dimitrov KM, Sharma P, Volkening JD, Goraichuk IV, Wajid A, Rehmani SF, Basharat A, Shittu I, Joannis TM, Miller PJ, Afonso CL (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

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

41. 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

42. Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press, New York

43. 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, pp 555–574

44. Merino R, Villegas H, Quintana JA, Calderon N (2009) Characterization of Newcastle disease viruses isolated from chicken, gamefowl, pigeon and quail in Mexico. Vet Res Commun 33:1023–1030. https://doi.org/10.1007/s11259-009-9321-5

45. Sabra M, Dimitrov KM, Goraichuk IV, Wajid A, Sharma P, Williams-Coplin D, Basharat A, Rehmani SF, Muzyka DV, Miller PJ, Afonso CL (2017) Phylogenetic assessment reveals continuous evolution and circulation of pigeon-derived virulent avian avulaviruses 1 in Eastern Europe, Asia, and Africa. BMC Vet Res 13:291. https://doi.org/10.1186/s12917-017-1211-4

46. Aldous EW, Fuller CM, Ridgeon JH, Irvine RM, Alexander DJ, Brown IH (2014) The evolution of pigeon paramyxovirus type 1 (PPMV-1) in Great Britain: a molecular epidemiological study. Transbound Emerg Dis 61:134–139. https://doi.org/10.1111/tbed.12006

47. Alexander DJ, Parsons G, Marshall R (1984) Infection of fowls with Newcastle disease virus by food contaminated with pigeon faeces. Vet Rec 115:601–602

48. Abolnik C, Gerdes GH, Kitching J, Swanepoel S, Romito M, Bisschop SP (2008) Characterization of pigeon paramyxoviruses (Newcastle disease virus) isolated in South Africa from 2001 to 2006. Onderstepoort J Vet Res 75:147–152. https://doi.org/10.4102/ojvr.v75i2.13

49. Gardy J, Loman NJ, Rambaut A (2015) Real-time digital pathogen surveillance - the time is now. Genome Biol 16:155. https://doi.org/10.1186/s13059-015-0726-x

50. Boza V, Brejova B, Vinar T (2017) DeepNano: Deep recurrent neural networks for base calling in MinION nanopore reads. PLoS ONE 12:e0178751. https://doi.org/10.1371/journal.pone.0178751

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