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Phylogenetic and Biological Characterization of Newcastle Disease Virus Isolates from Pakistan▽†

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Eight Newcastle disease virus isolates from Pakistan were sequenced and characterized. A PCR matrix gene assay, designed to detect all avian paramyxovirus 1, did not detect four of the isolates. A new matrix gene test that detected all isolates was developed. Phylogenetic analysis and pathotyping confirmed that virulent viruses of different genotypes are circulating in Pakistan.

Newcastle disease (ND) is a devastating infection of chickens caused by Newcastle disease virus (NDV), an avian paramyxovirus (1). Since ND is highly contagious and is clinically similar to the highly pathogenic avian influenza (2), accurate and rapid diagnosis of an outbreak is important. Prompt detection and differentiation from lentogenic NDV are necessary.

Eight previously uncharacterized NDV isolates were obtained from commercial and backyard poultry flocks surrounding Karachi, Pakistan, showing clinical signs of ND and experiencing relatively high rates of mortality. The samples were propagated in 9- to 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. RNA was extracted from infected allantoic fluid samples with TRIzol LS (Invitrogen, Carlsbad, CA) as described previously (3, 4). Clinical isolates were tested for NDV using the USDA-validated fusion (F) gene real-time reverse transcription-PCR (rRT-PCR) assay (F-gene assay) protocol (8). The F-gene assay detected all of these Pakistani isolates, except for the LaSota vaccine strain, as expected (data not shown). The F-gene test is designed to differentiate lentogenic (low-virulence) NDV from mesogenic (moderate-virulence) and velogenic (high-virulence) NDVs based on sequence changes at the fusion cleavage site (8), a well-characterized virulence determinant for NDV (6). The F-gene test is negative against avian influenza.

The 8 isolates and 49 additional ones were also tested for NDV using the USDA-validated matrix (M) gene rRT-PCR assay (M-gene assay) protocol (Table 1; see also Table S1 in the supplemental material) (8), designed to detect all NDV class II strains. In contrast to the results from the F-gene test, only five of eight Pakistani field isolates and the LaSota vaccine strain, but not the Mukteswar vaccine strain, were detected (Table 1). Because the validated M-gene assay is typically used to screen clinical samples, the failure of this test could result in an unacceptable false-negative diagnosis. While the test has failed to detect class I viruses before (5), this is the first case where the test has failed to detect class II NDVs and shows that constant monitoring of NDV genomic sequences is needed to detect future variability worldwide. This outcome is not surprising, given the difficulty of developing a single test for such a large, diverse group of viruses that continue to evolve (reviewed in reference 7). The test was modified by lowering the annealing temperature from 56°C to 52°C and 50°C, which allowed for the detection of NDV/27 and NDV/32, respectively. However, validated M-gene tests using annealing temperatures as low as 48°C were negative for the two remaining undetected viruses.

The sensitivity of the standard M probe (M+4169) was compared to that of a new M probe. The new probe, M+4169Pak (5′–[6-carboxyfluorescein] TTY TCT AGC AGY GGG ACA GCY TGC [black hole quencher_1]–3′) (Fig. 1), was designed using the consensus from an alignment of 50 samples with different origins and hosts (data not shown). For all class II viruses, the performance of the new M-gene assay was comparable (average threshold cycle [C_T] value for M+4169Pak = 16.7; average C_T value for M+4169 = 16.5). For the detection of class I viruses, a slightly improved performance for the new test was observed; however, the test does not provide dependable detection because of its very high C_T values (average C_T value = 36.5). Sequencing of the matrix genes of the Mukteswar vaccine strain (1974/PK/1) and 2007/PK/33 indicated that mismatches to the probe were likely responsible for the test failure (Fig. 1). These strains have four nucleotide differences relative to the probe (two of the mismatches are shared). The two other matrix genes sequenced were from strains that were detected by the validated M-gene assay, 2008/PK/43 and 2005/PK/26. Both of these strains had fewer mismatches with the matrix probe, three and two, respectively, than the two strains that failed the test. Moreover, the test did not likely fail because of differences in the primer sequences, since there was only one mismatch, and it was not near the 3′ end (data not shown).

Pathotyping showed that all of the Pakistani isolates from chickens collected between 1995 and 2008 are velogens (Table 1). The pathotypes of NDV isolates are determined by either

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TABLE 1. Comparison of new and validated (old) matrix rRT-PCR assays

Isolate name[k]	Fusion tree name[a]	Species/strain	Year	Matrix rRT-PCR		Pathotype results	
				Old	New	MDT (h)	ICPI
SPVC/Karachi/NDV/1[e]	G/1974/PK/290918495	Mukteswar	1974[d]	0	18.42	50	1.4
SPVC/Karachi/NDV/2	G/1995/PK/290918503	LaSota	1995[d]	12.26	12.28	110	0.4
SPVC/Karachi/NDV/16	G/1995/PK/290918487	Chicken[b]	1995	13.2	13.13	48.2	2
SPVC/Karachi/NDV/22	G/2004/PK/290918499	Chicken[b]	2004	22.5	18.07	48	1.7
SPVC/Karachi/NDV/23	G/2004/PK/290918491	Chicken[b]	2004	18.56	13.3	48.3	1.9
SPVC/Karachi/NDV/26[f]	G/2005/PK/290918497	Chicken[b]	2005	29.68	23	51.6	1.75
SPVC/Karachi/NDV/27	G/2006/PK/290918505	Chicken[b]	2006	19.42[i]	17.14	64.8	1.7
SPVC/Karachi/NDV/32	G/2007/PK/290918493	Chicken[b]	2007	14.88[j]	12.24	50	1.8
SPVC/Karachi/NDV/33[g]	G/2007/PK/290918501	Chicken[b]	2007	0	12.56	54.5	1.85
SPVC/Karachi/NDV/43[h]	G/2008/PK/290918489	Chicken[c]	2008	14.16	12.57	49	1.7

[a] Tree name shown for isolates included in phylogenetic analysis trees (see Fig. S2 in the supplemental material).
[b] Collected from commercial poultry.
[c] Collected from a backyard flock.
[d] Years when Mukteswar and LaSota vaccines were first used by Sindh Poultry Vaccine Center (SPVC), Karachi, Pakistan.
[e] The matrix tree name (see Fig. S2B in the supplemental material) for this isolate is G/1974/PK/290918481.
[f] The matrix tree name (see Fig. S2B) for this isolate is G/2005/PK/290918485.
[g] The matrix tree name (see Fig. S2B) for this isolate is G/2007/PK/290918483.
[h] The matrix tree name (see Fig. S2B) for this isolate is G/2008/PK/290918479.
[i] At 52°C, negative at 56°C.
[j] At 50°C, negative at 56°C.
[k] All isolates are class II NDV isolates from the vicinity of Karachi, Pakistan.

the intracerebral pathogenicity index (ICPI) found in 1-day-old chicks, mean death time (MDT) for embryonated eggs, or sequence of the fusion protein cleavage site. The ICPI ranged from 1.7 to 2.0, and the MDT ranged from 48 to 64 h; therefore, no clear change in pathogenicity over time was measured.

The complete M gene and the 374-bp region (cleavage site) of the F gene were sequenced. Gene sequences were obtained by RT-PCR using the Qiagen OneStep RT-PCR kit with gene-specific primers. The USDA-validated matrix probe (M+4169) sequence was compared to all available ($n = 167$) NDV matrix gene sequences in GenBank (Fig. 1). These sequences were aligned and analyzed for mismatches at the probe site. All of the sequences analyzed had less than four mismatches, with the exception of samples from class I waterfowl viruses with lentogenic cleavage sites, which had four or more mismatches. These viruses have already been shown to fail the test. Several isolates had three mismatches, and these should be followed more carefully, as they, too, will likely not be detected with the M-gene test.

Phylogenetic analysis of the eight NDV isolates in comparison to two vaccine strains and other reference isolates was done to determine the relationship between the Pakistani isolates and other class II sequences. The maximum likelihood PHYML version 2.4.4 software was used with a general time-reversible (GTR) model of nucleotide substitution, as shown previously (4), for 50 matrix and 71 partial fusion coding sequences.

Phylogenetic analysis using the 374-nucleotide partial fusion gene sequence shows that the recent Pakistani isolates 2007/PK/32, 2007/PK/33, 2006/PK/27, and 2008/PK/43 form a distinct cluster within genotype VII viruses and are most related to a 1989/Japan isolate (see Fig. S2A and Table S2 in the supplemental material). The Mukteswar (1974/PK/1) vaccine virus is distant from these and grouped with many recent Asian

			Matrix probe M+4169	T T C T C T A G C A G T G G G A C A G C C T G C
			Pakistan-specific probe M+4169Pak	T T **Y** T C T A G C A G **Y** G G G A C A G C **Y** T G C
			NDV B1	C C T T T C T T C T C T A G C A G T G G G A C A G C C T G C T A T C
			Sequence position	4163 4173 4183 4193
class	genotype	Virus name		▼ ▼ ▼ ▼
II	VII	SPVC/Karachi/NDV/43		. . . . . T . . C . . . . T . . . . .
II	III	SPVC/Karachi/NDV/1		. . . . . A . . A C . . . . . T . . . . . C .
II	VI	SPVC/Karachi/NDV/26		. . . . . T . . . . . C . . . . .
II	VII	SPVC/Karachi/NDV/33		. . . . . T . . C . . . . . C . . . . . T . . . . .
I	6	duck/US/119535-1/2001		. . . . . A . . T . . C . . A . . C . . A . . T . . . .
I	5	duck/US/154979-1/2001		. . . . . A . . . . . C . . A . . C . . A . . T . . . .
I	6	chicken/US/101250-2/2001		. . . . . A . . . . . C . . A . . C . . A . . T . . . .
II	VII	chicken/IT/3286/2000		. . . . . T . . C . . . . . A . . . . . T . . . .
II	IIa	32C/T.98		. . . . . C . . T . . . . A . . . . .
II	IIa	126C.00		. . . . . C . . T . . . . A . . . . .
II	VIII	AF2240		. . . . . A G C . . . . .
II	VIIb	tern/RU/2755/2001		. . . . . C . . . . . C . . . . . T . . . .

FIG. 1. Comparison of sequences at the validated M-gene assay probe site. Four Pakistan isolate sequences and eight previously characterized sequences with significant numbers of mismatches (≥3) with the validated M-gene assay probe are shown. Conserved nucleotides are shown as dots; nucleotide changes introduced into the Pakistan-specific M-gene assay probe are in boldface.

genotype III to IV viruses. The remaining four isolates obtained from 1995 to 2005, 2004/PK/23, 1995/PK/16, 2005/PK/26, and 2004/PK/22, are closely grouped among genotype VI viruses.

Phylogenetic analysis of the full matrix sequence shows a grouping similar to that found with analysis of fusion gene sequences, with some differences due to the availability (or lack) of sequences in GenBank. Of the four Pakistan NDV viruses for which matrix sequences were available (see Fig. S2B and Table S2 in the supplemental material), 2007/PK/33 and 2008/PK/43 are tightly grouped in genotype VII viruses near a 2001/Russia tern isolate. Mukteswar (1974/PK/1) is distant from recent isolates and groups with other vaccine strains, as was seen with the 374-nucleotide partial fusion tree. 2005/PK/26 is grouped with genotype VI pigeon viruses and not with the other Pakistani viruses.

Overall, these results indicate that there are multiple velogenic genotypes circulating in Pakistan and causing outbreaks in poultry. Newcastle disease viruses have historically showed great mobility with viruses of Asian, American, or European origin, eventually achieving worldwide distribution (7). This report represents the first failure of the validated M-gene assay to detect virulent viruses. Therefore, providing sequences of isolates and the development of an rRT-PCR assay should aid diagnostic labs worldwide if these viruses move out of Pakistan. The need for continued modification of rRT-PCR test primer/probe sets should be expected.

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Mention of trade names or commercial products in this work is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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