Evolution of Newcastle Disease Virus Quasispecies Diversity and Enhanced Virulence after Passage through Chicken Air Sacs

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ABSTRACT

It has been reported that lentogenic Newcastle disease virus (NDV) isolates have the potential to become velogenic after their transmission and circulation in chickens, but the underlying mechanism is unclear. In this study, a highly velogenic NDV variant, JS10-A10, was generated from the duck-origin lentogenic isolate JS10 through 10 consecutive passages in chicken air sacs. The velogenic properties of this selected variant were determined using mean death time (MDT) assays, intracerebral pathogenicity index (ICPI), the intravenous pathogenicity index (IVPI), histopathology, and the analysis of host tissue tropism. In contrast, JS10 remained lentogenic after 20 serial passages in chicken eggs (JS10-E20). The JS10, JS10-A10, and JS10-E20 genomes were sequenced and found to be nearly identical, suggesting that both JS10-A10 and JS10-E20 were directly generated from JS10. To investigate the mechanism for virulence enhancement, the partial genome covering the F0 cleavage site of JS10 and its variants were analyzed using ultradepth pyrosequencing (UDPS) and the proportions of virulence-related genomes in the quasispecies were calculated. Velogenic NDV genomes accumulated as a function of JS10 passaging through chicken air sacs. Our data suggest that lentogenic NDV strains circulating among poultry might be a risk factor to future potential velogenic NDV outbreaks in chickens.

IMPORTANCE

An avirulent isolate, JS10, was passaged through chicken air sacs and embryos, and the pathogenicity of the variants was assessed. A virulent variant, JS10-A10, was generated from consecutive passage in air sacs. We developed a deep-sequencing approach to detect low-frequency viral variants across the NDV genome. We observed that virulence enhancement of JS10 was due to the selective accumulation of velogenic quasispecies and the concomitant disappearance of lentogenic quasispecies. Our results suggest that because it is difficult to avoid contact between natural waterfowl reservoirs and sensitive poultry operations, circulating lentogenic NDV strains may represent a potential reservoir for emergent velogenic NDV strains that could cause outbreaks in chickens.

Newcastle disease (ND) is one of the most serious infectious diseases of poultry. The causative agent of the disease is Newcastle disease virus (NDV), an enveloped virus belonging to the genus Avulavirus within the family Paramyxoviridae (1). NDV contains a single-stranded, negative-sense, nonsegmented genome of approximately 15.2 kb containing six genes that encode nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase protein (L) (2). NDV strains are classified as highly virulent (velogenic), intermediate virulent (mesogenic), or nonvirulent (lentogenic) on the basis of their pathogenicity for chickens. The molecular basis for NDV pathogenicity is mainly the amino acid sequence of the cleavage site in the fusion (F0) protein. Lentogenic NDV strains contain dibasic residues at the cleavage sites $^{112}_{117}$E(G)RQE(G)RL, which are sensitive only to the blood clotting factor Xa-like and which are limited to the respiratory and enteric tracts. In velogenic strains, F0 usually contains polybasic amino acids at the cleavage sites $^{112}_{117}$R(K)RQR(K)RF, which are the preferred recognition sites for furin-like proteases present in most cells. F0 protein cleavage in a wide range of tissues is responsible for the systemic spread of velogenic NDV, as well as for its virulence (1, 3, 4).

Wild waterfowl are generally considered to be natural reservoirs of NDV, and the majority of the isolates are lentogenic or only potentially pathogenic (3–7). NDVs can be transmitted from wild waterfowl, via domestic waterfowl, to terrestrial poultry. A close phylogenetic relationship was found between numerous avirulent isolates obtained from live bird markets (LBMs) and wild birds, which suggests that NDVs were circulating in domestic poultry and wild birds (8–12). In eastern Asia, most domestic waterfowl are raised in semienclosed areas, which share ecological contact interfaces between wild aquatic birds and terrestrial poultry. Lentogenic NDVs can be isolated from apparently healthy domestic waterfowl that intermingled with terrestrial poultry. Thus, favorable natural conditions appear to exist for the generation of virulent NDV isolates (7, 13–16).

RNA viruses exist in the host as a group of variants, known as “quasispecies,” a concept considered to play a critical role in pathogenesis (17–20). Phylogenetic analysis of NDV evolution

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showed that velogenic viruses likely emerged from a lentogenic progenitor virus via a change of two amino acids at the F0 cleavage site (21, 22). Subsequently, a reverse transcription–real-time PCR analysis demonstrated that velogenic F0 sequences exist in lentogenic isolates (23). However, there is no detailed analysis of NDV quasispecies yet, and the relationship between emergent virulent strains and NDV quasispecies has not been clarified. Ultradeep pyrosequencing (UDPS) has emerged as an important tool with which to investigate viral diversity and to detect mutants in a group of quasispecies (24–29).

To investigate the role of NDV evolution in virulence mechanisms, we passaged a domestic duck-origin lentogenic class I isolate, JS10, through chicken airsacs and embryos and then assessed the pathogenicity of the variants. UDPS was used to monitor changes to the virulence-related F0 cleavage site sequences, with the aim of establishing possible correlations between viral pathogenesis and quasispecies composition.

MATERIALS AND METHODS

Embryos and chickens. Specific-pathogen-free (SPF) chicken eggs (White Leghorns) were obtained from Merial (Merial, Beijing, China), hatched in an incubator (Deguang, Shandong, China) in our laboratory, and used for experimentation following hatching. The chicks were housed in isolators under biosecurity conditions and a 12-h light/dark cycle with free access to food and water until the days of the experiments. Care and maintenance of all animals were in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines set by the Chinese Academy of Agricultural Science (permit numbers SHVRI-Po-0099 for virus air sac passage, SHVRI-Po-0116 for virus pathogenicity index tests, SHVRI-Po-0120 for virus shedding and cohabitation infection examination, and SHVRI-Po-0142 for tissue tropism and histopathology determination). Before infection with live NDVs, birds were bled and the serum hemagglutination inhibition (HI) titers were determined to confirm that the flock was negative for NDV antibodies. All inoculation experiments with live NDVs were conducted within the animal biosecurity level 3 (ABSL3) facility in the Veterinary College of Yangzhou University.

Viruses and passage series. A lentogenic NDV strain, Duck/JS/10 (JS10), was isolated from a nonvaccinated duck (30). After plaque purification three times on DF-1 cells, JS10 virus stock was prepared by inoculating 10-day-old SPF chicken embryos only and then sequentially passed 10 times in air sacs of 3-day-old chicks or 20 times in 10-day-old chicken embryos as described previously (31, 32). Briefly, each caudal thoracic air sac of three chicks was inoculated with 0.2 ml of allantoic fluid containing 10^5 50% egg infectious doses (EID50) of the virus. The chicks from early passages (1 to 4 times) and moribund chicks from late passages (5 to 10 times) (clinical signs included diarrhea, rough hair coat, no eating or drinking, etc.) were sacrificed humanely with an intravenous injection of sodium pentobarbital at a dose of 100 mg/kg of body weight at day 4 or on days on which the clinical signs emerged postinfection, and air sacs were collected, homogenized, and pooled. Serial air sac passages in chicks (three birds per passage) were performed with 0.2 ml pooled air sac homogenate every 5 days. For passage in chicken embryos, an aliquot of 0.2 ml allantoic fluid containing 10^5 EID50 was inoculated into the allantoic cavity of three 10-day-old embryos. After incubation for 5 days (or until embryo death) at 37°C, the embryos were chilled, and the allantoic fluids were collected for the next passage. The generated isolates were named by the virus passage numbers and the origin (23). However, there is no detailed analysis of NDV genicities yet, and the relationship between emergent virulent strains and NDV quasispecies has not been clarified. Ultradeep pyrosequencing (UDPS) has emerged as an important tool with which to investigate viral diversity and to detect mutants in a group of quasispecies (24–29).

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Virulence-related quasispecies detection by UDPS

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Virulence-related quasispecies detection by UDPS

Noninvasive detection of NDV partial genome covering F0 cleavage site. Viral RNA was isolated from allantoic fluid containing viruses with the QiAamp viral RNA minikit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The same amount of RNA (100 ng) for each sample was used for CDNA synthesis, which was performed separately by using random hexamers, together with Superscript III reverse transcriptase, according to the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). Following first-strand synthesis, cDNA samples were used as the templates to generate UDPS amplicons. To minimize the error rate of the PCR process (false nucleotide substitutions), a high-fidelity polymerase, Pfu Ultra-II (Stratagene, La Jolla, CA, USA), was used. The study was designed for analysis of the amplicon at the maximum length of 400 nucleotides (nt) by the FLX+ platform. PCR primers were selected for amplification of a specific 387-bp NDV fragment, which included the F0 cleavage site. To compensate for random sampling and substitution errors, reverse transcription-PCRs (RT-PCRs) were performed in triplicate and then the reaction mixtures were mixed. Samples from mixed RT-PCR mixtures were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) to remove unincorporated deoxynucleoside triphosphates (dNTPs) and primers, according to the manufacturer’s instructions. Purification was performed using the QIAquick PCR purification kit (Qiagen). Quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The emulsion PCR (emPCR) was performed with Roche GS FLX Titanium emPCR kits and the GS FLX Titanium sequencing kit (Roche, Mannheim, Germany), with 5 ×
10\(^{10}\) copies of DNA as the initial templates in each sample. To determine the error rate of the UDPS procedure, deep sequencing was conducted under similar conditions with a plasmid containing the JS10 sequence (JS10-P) that was prepared in our laboratory.

<table>
<thead>
<tr>
<th>Strain(^a)</th>
<th>MDT(^b) (h)</th>
<th>ICPI(^c)</th>
<th>IVPI(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duck/JS/10</td>
<td>(\geq120)</td>
<td>0.13</td>
<td>0</td>
</tr>
<tr>
<td>Duck/JS/10-A3</td>
<td>109</td>
<td>0.76</td>
<td>0.69</td>
</tr>
<tr>
<td>Duck/JS/10-A6</td>
<td>72.8</td>
<td>1.75</td>
<td>1.26</td>
</tr>
<tr>
<td>Duck/JS/10-A10</td>
<td>48.4</td>
<td>1.91</td>
<td>2.08</td>
</tr>
<tr>
<td>Duck/JS/10-E10</td>
<td>(\geq120)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Duck/JS/10-E20</td>
<td>(\geq120)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Newcastle disease virus original isolate and its variants.

\(^b\) Mean death time for chicken embryos infected with a single minimal lethal dose of virus.

\(^c\) Intracerebral pathogenicity index in 1-day-old chicks.

\(^d\) Intravenous pathogenicity index in 6-week-old chickens.

**Sequence analysis.** The raw data were treated as described previously with minor refinements (36). Reads obtained from UDPS were demultiplexed corresponding to each sample combination of a multiplex identifier and primer to generate a FASTA file with each sample. These reads were subsequently processed by the Quantitative Insights into Microbial Ecology pipeline. Sequences that were less than 300 or greater than 400 bp in length, contained incorrect primer sequences, or contained more than 10 ambiguous bases were discarded. In addition, an extra chimera-checking step against the default reference database was performed. The resulting sequences were aligned using the Burrows-Wheeler Aligner (BWA-MEM, version 0.7.5a) (http://bio-bwa.sourceforge.net) to analyze the variation of all the sequenced bases that contained the NDV F0 cleavage site. In this step, any sequences containing insertion/deletion errors in this core domain were discarded.

**Statistical analysis.** Virus titers were analyzed by using analysis of variance (ANOVA) in GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA); a \(P\) value of <0.05 was considered statistically significant. Those response variables were subjected to comparisons for all pairs by using the Tukey-Kramer test.

In UDPS, nucleotides 169 to 556 of the F gene, including cleavage sites...
of all valid sequence in each sample, were extracted from the alignment file and translated into amino acids by a script to compare the variation between JS10 and the variants. To obtain the percentage of amino acid variability in each sample, the total number of amino acid substitutions was divided by the total number of amino acids analyzed. According to the F0 cleavage site and phenotype, the variants were divided into three broad categories to statistically analyze the variability of the quasispecies. The Mann-Whitney U test was used to determine statistical differences in numerical variables.

**Nucleotide sequence accession numbers.** The complete genomes of JS10-A10 and JS10-E20 were sequenced and submitted to the GenBank database (accession numbers KT124544 and KT124545).

**RESULTS**

**JS10 virulence was enhanced during passaging in chick air sacs.** JS10 and its variant JS10-A3 induced no clinical signs or mortality after inoculation into chickens. However, variant JS10-A6 induced clinical signs of illness, such as mouth breathing and lethargy, and variant JS10-A10 produced 100% mortality within 48 h p.i. In contrast, no clinical signs or death was recorded in any case when JS10 was passaged in embryos 20 times (JS10E1 to JS10E20). These findings demonstrate that lentogenic NDVs in domestic waterfowl became highly pathogenic after consecutive passages in chicken air sacs. When the viruses were passaged in embryos, however, no changes in pathogenesis were observed. In addition, no cytopathic effect (CPE) was observed in JS10- or JS10-E20-infected cells; however, effacement and syncytia were observed in JS10-A10-infected cells on day 3 p.i. During the passage, the hemagglutinin (HA) and EID50 titers of all the generated variants showed no significant difference, indicating that virus replication was not altered.

**Determination of the virus pathogenicity.** MDT, ICPI, and IVPI values were determined to quantify the virulence of selected viral variants. No significant changes were observed in MDT and IVPI between JS10 and JS10-A3, but ICPI values for JS10 and JS10-A3 were determined as 0.13 and 0.76, respectively, indicating that the virulence of JS10-A3 increased. The pathogenicity index

**FIG 2** Histopathology examination of JS10 and its variants in chickens (tissues stained with hematoxylin and eosin). (A to C) PBS-injected chickens. (D to F) JS10-infected chickens. (G to I) JS10-A10-infected chickens. Pneumonorrhagia, inflammation, and obvious hemorrhage in lung (G); slight esophagitis and mild hemorrhagic proventriculitis with infiltration of lymphocytes and macrophages in intestine (H); and severe necrosis, marked lymphocyte depletion, and infiltration of macrophages in spleen (I) were observed. (J to L) JS10-E20-infected chickens. No histopathological changes were observed.
increased significantly as a function of additional air sac passages (Table 1).

No enhancement of virulence was observed when JS10 was passaged in chicken embryos. Indeed, JS10-E20 was even slightly attenuated compared with JS10 (Table 1). Thus, consecutive passage of JS10 through air sacs enhanced viral virulence, while passage through chicken embryos attenuated virulence.

**Tissue tropism and histopathology detection.** Tissue tropism was investigated by determining the viral titers in the organs of infected chickens through oral, nasal, intramuscular, and intravenous inoculation on day 3 p.i. JS10 was recovered from the trachea, lung, air sac, and intestine of chickens infected through oral and nasal routes, while viral replication was limited to the intestine following intramuscular inoculation (Fig. 1). When the virus was inoculated intravenously, the virus was found in the lung, air sac, spleen, and intestine. JS10-A10, whether inoculated orally, nasally, or intramuscularly, was isolated from all organs except for the brain, though it could also be recovered from the brain following intravenous inoculation. In contrast, JS10-E20 was recovered only from the trachea in the orally and nasally inoculated groups. These results indicated that the tissue tropism of JS10-A10 expanded dramatically and ultimately became pantropic, another characteristic of velogenic viruses. However, the replication ability of JS10-E20 in SPF chickens was significantly reduced.

Histopathological examination showed that severe lesions, including hemorrhages, proliferation of periparabronchial lymphoid tissues, and infiltration of macrophages and lymphocytes, were observed in the lungs, intestines, and spleens of chickens infected with JS10-A10 (Fig. 2). In contrast, no histopathological change was observed in the lungs, intestines, or spleens of chickens infected with JS10 or JS10-E20 (Fig. 2A to F and J to L).

**Virus shedding and cohabitation infection examination.** Virus shedding of JS10, JS10-A10, or JS10-E20 was examined through virus isolation from oropharyngeal and cloacal swabs of infected SPF chickens. Results showed that JS10 and JS10-A10 isolation was positive from 80% and 100% of oropharyngeal swabs on day 2 p.i. and 60% and 80% on day 4 p.i., respectively. For cloacal swabs, JS10 and JS10-A10 isolation was positive from 60% and 70% on day 4 p.i. and 80% and 100% on days 7 p.i., respectively. In contrast, JS10-E20 was isolated from only 40% and 30% of oropharyngeal swabs on days 2 and 4 p.i., respectively. No JS10-E20 was isolated from cloacal swabs on time points examined in this study (Fig. 3A and B).

Cohabitation infection was examined by determining the viral shedding from chickens in contact with infected chickens. After cohabitation with JS10-infected chickens, only 25% of oropharyngeal swabs and cloacal swabs were positive on day 7 p.i. However, virus was isolated from both oropharyngeal and cloacal swabs of all chickens that cohabited with the JS10-A10-infected group on day 7 p.i., indicating that JS10-A10 is transmissible.
TABLE 2 Amino acid substitutions among Duck/JS/10 and its passaged variants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide (amino acid) position</th>
<th>Codon (deduced amino acid)</th>
<th>Duck/JS/10</th>
<th>Duck/JS/10-A10</th>
<th>Duck/JS/10-E20</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>1088–1090 (323)</td>
<td>GTA (Ala)</td>
<td>GCA (Val)</td>
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<td></td>
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<tr>
<td>M</td>
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<td>GAC (Asp)</td>
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<tr>
<td></td>
<td>2067–2069 (61)</td>
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<tr>
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<td>2175–2177 (97)</td>
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<td>GCT (Ala)</td>
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<td>ATC (Ile)</td>
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<td>10790–10792 (800)</td>
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<td>GAG (Gl)</td>
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<td>AAT (Asn)</td>
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<td>13067–13069 (1541)</td>
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<td>GGG (Gly)</td>
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<td>ATT (Ile)</td>
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<tr>
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<td>14576–14578 (2062)</td>
<td>AGC (Ser)</td>
<td>AGA (Arg)</td>
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</tr>
</tbody>
</table>

However, no viruses were isolated from oropharyngeal and cloacal swabs of chickens in contact with JS10-E20-infected birds at 2, 4, and 7 dpi, demonstrating that JS10-E20 lost transmissibility (Fig. 3C and D).

Genetic comparisons between parent virus and passaged variants. The sequence homology among JS10, JS10-A10, and JS10-E20 was 99.7 to 99.9%, suggesting that all variants originated from the same parent virus, JS10. However, a total of 41 mutations from JS10 were identified in JS10-A10, of which 26 led to amino acid substitutions (Table 2). Three mutations in the cleavage site coding region of the F gene caused changes from the lentogenic cleavage site112ERQERL117 to the typical velogenic sequence112KQRQKRF117 and are likely to contribute to the increased viral pathogenicity of JS10-A10 in chickens. Another 22 mutations were dispersed throughout the rest of the viral genome. We also identified 25 mutations between JS10 and JS10-E20, 14 of which led to amino acid substitutions (Table 2). Three of these mutations were unique to JS10-E20, while 11 were also found in JS10-A10 and located mainly at the polymerase proteins (6 amino acids) or protein associated with virus polymerase complex (5 amino acids).

UDPS and calculation of background errors. To perform UDPS analysis of different virulence-related F0 cleavage site sequences of a series of NDV variants, simultaneous analyses were conducted using barcoded primers. A total of 366,732 sequence reads were obtained from JS10, JS10-P, and its variants using UDPS, and the average number of sequence reads was 26,195 (range, 19,767 to 51,341) per sample. An average of 20,726 UDPS sequence reads could be aligned with the standard sequence, which accounted for 79% of the total reads (Fig. 4A). The average read length was 378 bp per sample (Fig. 4B). The number of reads at each position in the tested region ranged from 6,000 to 10,000 (Fig. 4C). The background error rate of UDPS from nucleotides 169 to 556 of the F gene was calculated with JS10-P (Fig. 4D). Among 26,657 valid reads, each of which was 386 nucleotides (nt) long, the maximum error rate was 2.02% at the 462nd base, and the next highest error rate was 0.67% at the 457th base. These three-A homopolymers are a weak point of UDPS. Excluding the 462nd and 457th nucleotide positions, the error rate was 0.02% or lower. From repeated deep sequencing of the plasmid, the overall nucleotide error rate was calculated as 0.00031 ± 0.0004.
FIG 4 Summary of ultradepth pyrosequencing (UDPS) results. (A) Number of reads and aligned reads for each sample. (B) Average read length for each sample. (C) NDV F gene coverage. The dots represent the minimum, 25% quartile, 75% quartile, and maximum coverage of reads of samples at each amino acid position of the F protein. (D) JS10-P (F gene plasmid) was subjected to UDPS, and the background error rate of pyrosequencing was calculated.
Based on this analysis, a mixture of bases detectable above the background error of 0.034% (mean background error rate ± 2 SDs) was defined as a biologically significant mixture.

Variability of F gene cleavage site sequence in JS10 and its variants. Based on the 263,511 valid sequence reads, the F gene fragment, which was limited to codons 57 to 185, in JS10 and its variants was analyzed. Variability was analyzed as the percentage of changes in all codons of the limited region (Fig. 5A).

Ten air sac-passaged variants (JS10-A1 to JS10-A10) had a much higher variability at three codons (112, 115, and 117) of the F0 cleavage site, from 97.42 to 99.03%. The variability for other F0 codons ranged from 0.01 to 0.02%. In contrast, two embryo-pasaged variants, JS10-E10 and JS10-E20, showed differences at only amino acid 82, with 18 to 27% variability, respectively (Fig. 5A).

Linkage analysis was performed to determine the codon accounts for the most frequent amino acid substitution in the same viral sequence at the cleavage site. Sequence variability gradually increased with increasing passage times in air sacs (Fig. 5B). The JS10 isolate was shown to be a mixture of different sequences at codons 112, 115, and 117. Characteristic virulence codon ratios at positions 112, 115, and 117 in JS10 were 0.73%, 0.68%, and 0.49%, respectively. In JS10-A1, the ratios at codons were increased to 4.25%, 4.08%, and 0.64%, respectively. In JS10-A10, the ratios were dramatically increased to 99.03%, 98.89%, and 97.42%, respectively. In each passaged variant, codon 112 had the largest variation ratio, followed by codon 115 and then codon 117. In addition, the variation at codons 112, 115, and 117 emerged sequentially with increased passaging. The average variation ratios at codons 112, 115, and 117 of air sac-passaged variants were 76.35%, 73.28%, and 51.86%, respectively. In comparison, the mean variation frequency of the same three positions at the cleavage site of two selected embryo-passaged viruses was only 0.72%, 0.53%, and 0.49%, respectively (Fig. 5B).

Analysis of quasispecies constituting JS10 and its variants. Quasispecies analysis showed that the sequences at the F0 cleavage site of positions 112 to 117 could be divided among 32 different types (Table 3). The lentogenic cleavage sites consisted of 112ERQERL117, 112GRQGRL117, 112GRQERL117, and 112ERQGRL117, and the velogenic cleavage sites consisted of 112RRQRRF117, 112KRQKR117, 112KRQRRF117, and 112RRQKR117, respectively. The other 24 cleavage site sequences were classified as transitional types.

The dynamic changes of the quasispecies proportions in JS10 and its air sac-passaged variants were calculated (Fig. 6A). Based on the pyrosequencing data, the major portion of 99.30% at the F0 sequence in JS10 was lentogenic, while the velogenic and transitional F0 sequence proportions were 0.34% and 0.36%, respectively. After air sac passaging, the proportion of transitional F0 sequence in JS10 gradually increased from JS10-A1, and then the proportion of velogenic F0 sequence gradually increased from JS10-A3, while the proportion of lentogenic F0 sequence decreased. After 10 passages, the proportion of the velogenic F0 sequence accumulated to 94.87%. In contrast, the rate of the lentogenic F0 sequence detected in JS10-A10 declined to 0.28%.

From JS10-A1 to JS10-A5, the proportions of the total transitional F0 sequences continued to rise, and in JS10-A5, the proportion reached its peak at 82.58%. However, the proportion of the total transitional F0 sequence started to decrease at JS10-A6, and the trend became more obvious with additional passages, as the proportion in JS10-A10 declined to 4.83%. The proportions of transitional F0 sequence genotypes of 112ERQERL117, 112RRQRRF117, 112KRQKRF117, and 112GRQGRL117 were all under 0.5% during 10 air sac passages (not shown in Fig. 6A).

After 10 passages in embryos, the proportions of velogenic, lentogenic, and transitional F0 sequences changed from 0.34%, 99.30%, and 0.36% in JS10 to 0.10%, 99.45%, and 0.45% in JS10-E10, respectively (Fig. 6B). After another 10 passages, the proportions of velogenic and transitional F0 sequences decreased to 0 and 0.12%, respectively, but the proportion of lentogenic F0 sequence increased to 99.88%. Also, the proportions of transitional F0 sequence genotypes of 112ERQERL117, 112RRQRRF117, 112KRQKRF117, and 112GRQGRL117 were all under 0.5% (not shown in Fig. 6A).

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Composition of NDV quasispecies according to F0 cleavage site of JS10 and its air sac-passaged variants (A) and chicken embryo-passaged variants (B).

FIG 6 Composition of NDV quasispecies according to F0 cleavage site of JS10 and its air sac-passaged variants (A) and chicken embryo-passaged variants (B).
DISCUSSION

We compared the potential impacts on virulence enhancement of passaging the avirulent NDV isolate JS10 in chicken air sacs versus chicken embryos. JS10-A10, the variant of JS10 after 10 consecutive passages through chicken air sacs, produced 100% mortality within 48 h p.i. and the classic virulent syncytia in infected cells on day 3 p.i. The pathogenicity indices of MDT, ICPI, and IVPI were all increased to the value of virulent NDV, suggesting that the velogenic variant JS10-A10 was evolved from the lentogenic JS10 after consecutive passages in chicken air sacs. Virus tissue tropism and transmission studies indicated that JS10-A10 produced an expanded tissue tropism and robust transmissible infection in chickens. Interestingly, we also identified changes in the JS10-A10 F0 cleavage sites associated with virulence by UDPS. With the passaging in air sacs, the lentogenic F0 genotypes of $^{112}{\text{E(G)QR}}^{117}$ were reduced from 99.30% to 0.28% after 10 consecutive passages. Meanwhile, the velogenic F0 genotypes of $^{112}{\text{R(K)QR}}^{117}$ were increased from 0.34% to 94.87%, which suggested that the emergence of velogenic F0 genotypes contributes to the virulence enhancement of JS10-A10. These findings were similar to the previous report (32), in which a wild-waterfowl-origin, avirulent class I NDV isolate, Goose/Alaska/415/91, was evolved to a velogenic virus, with the ICPI and IVPI increasing from 0 to 1.20 and 1.60, respectively, through nine consecutive passages in air sacs. This revealed that some avirulent NDVs maintained in the natural environment have the potential to evolve to the velogenic type, as happened in Australia (21, 22, 37–39).

To investigate whether virulence enhancement can appear in other lentogenic NDV isolates in the air sac model, we plaque purified two other avirulent isolates three times and conducted the same passage experiments. No virulence enhancement was observed after 10 consecutive air sac inoculations of these two isolates in our study (data not shown). Further sequence analysis of these two plaque-purified isolates by using UDPS showed that the proportion of the transitional genotypes was much lower than that of JS10, and no velogenic genotypes were detected, suggesting that the virulence enhancement of NDV during air sac passages is virus strain dependent. These findings, together with the previous results (32), suggest that among avirulent NDVs, a virulence evolution mechanism for some avirulent NDV strains may exist during air sac passages, which needs further investigation.

Conditions under which highly virulent viruses are generated from an avirulent strain through air sac passage are generally not found in nature. However, numerous lentogenic NDVs are carried by apparently healthy domestic waterfowl intermingling with terrestrial poultry (7, 13–16, 40). Air sacs are spaces where there is the constant presence of air; they form a connection between the lungs and bone cavities and aid in regulation of breathing in chickens (41–44). In our study, JS10 can be recovered from the air sac of chickens infected through oral and nasal routes. Therefore, avirulent NDV strains that grow efficiently in poultry respiratory and intestinal tracts may at times access chicken air sacs and evolve a virulent phenotype upon subsequent replication, eventually causing outbreaks in susceptible chickens.

NDV quasispecies are composed of “lentogenic” genomes and “velogenic” genomes in different proportions (23). To investigate the viral population dynamics during the virulence alteration, we used UDPS to analyze the quasispecies composition for JS10 and its variants. We found that amino acid variations mainly located at the F0 cleavage site and velogenic genomes accumulated as a function of serial passage. Furthermore, our results also confirmed that the virulence of NDVs evolved from the lentogenic type via the mesogenic type and ultimately changed to the velogenic type.

Single-point mutation can lead to large changes in viral phenotypes (45–48). Recently, it has been shown that quasispecies diversity can also determine the pathogenic potential of a viral population, especially for RNA viruses (49–53). Our results strongly suggest that the observed NDV pathogenesis was modulated by the proportion of avirulent and virulent genomes and their interactions. This is the first study to demonstrate experimentally that quasispecies stability is closely related to NDV pathogenesis. As the velogenic NDV perhaps directly evolves from lentogenic NDV in appropriate environments, employing next-generation sequencing methods to obtain the numerous gene sequences which can reflect the quasispecies status of the clinical isolates may contribute to surveillance of lentogenic NDVs.

As the molecular basis for how the interplay among viral genomes may affect host populations is still unknown, it seems desirable in the future to elucidate the unique ecological niche that exists in chicken air sacs.

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