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Disparate thermostability profiles and HN gene domains of field isolates of Newcastle disease virus from live bird markets and waterfowl in Uganda

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Abstract

Background: Uganda poultry production is still faced with frequent outbreaks of Newcastle disease (ND) in the backyard free-range systems despite the accessibility of cross protective vaccines. Live bird markets and waterfowl has long been reported as a major source of disease spread as well as potential sources of avirulent strains that may mutate to virulent strains. ND-virus has been reported enzootic in Ugandan poultry but limited studies have been conducted to ascertain thermostability phenotypes of the Ugandan ND-virus strains and to understand how these relate to vaccine strains.

Methods: This study evaluated thermostability of 168 ND-virus field isolates recovered from live bird markets and waterfowls in Uganda compared to two live commercial vaccine strains (I_2 and LaSota) by standard thermostability procedures and Hemagglutinin-Neuraminidase (HN) gene domains. The known pathotypes with thermostability profiles were compared at HN amino acid sequences.

Results: Field isolates displayed disparate heat stability and HN gene domains. Thermolabile isolates were inactivated within 15 min, while the most thermostable isolates were inactivated in 120 min. Four thermostable isolates had more than 2 $\log_2$ heamaglutinin (HA) titers during heat treatment and the infectivity of 9.8 geometric mean of $\log_{10} EID_{50\%}$ in embryonated eggs. One isolate from this study exhibited a comparable thermostability and stable infectivity titers after serial passages, to that of reference commercial vaccine was recommended for immunogenicity and protection studies.

Conclusion: The occurrence of ND-virus strains in waterfowl and live bird markets with disparate thermostability and varying HN gene domains indicate circulation of different thermostable and thermolabile ND-virus pathotypes in the country.

Keywords: Thermostability, Infectivity, Thermostable isolate, Hemagglutionation assay, HN gene

Background

Newcastle disease (ND) is a contagious poultry infection that usually invokes trade barriers and exerting great economic threats [1]. Newcastle disease virus (ND-virus) genome contains 6 genes encoding for structural proteins positioned from 3′ to 5′: Nucleoprotein (NP), Phosphoprotein (P), Matrix (M), Fusion (F) Hemagglutinin-Neuraminidase (HN) and polymerase (L) [23]. The physico-chemical properties of these proteins have been extensively used to categorize strains. According to World Organization for Animal Health [27], for a ND-virus to be notified as pathogenic, it has to meet the criteria of Intracerebral pathogenicity Index (ICPI) of beyond 0.7. Today, field strains are categorized by virulence into five pathotypes [3, 5, 6], confirmed by multiple basic amino acid sequences in the fusion (F) protein cleavage site (FPCS) [12, 26].

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Great importance is attached to the thermostability of live vaccinates because of their retained potency in areas with poor transport, storage facilities, human error and power shortages. Studies in search of new thermostable isolates assess virulence of pathotypes by determining the amino acid sequences at the F protein cleavage site (FPCS), mean death time (MDT) and Intracerebral pathogenicity indices (ICPI). In areas where there is frequent use of vaccines, selective pressure leading to new strains or failed vaccination has been recorded due to the use of phylogenetically divergent vaccine strains from the circulating local ND-viruses [24, 28]. Besides, ND-virus isolates with amino acids at FPCS typical of low-virulent type and ICPI values typical of highly virulent strains have been reported [37]. Such places are experiencing continual outbreaks of velogenic ND in backyard and vaccinated flocks [1, 4].

HN gene is known to play crucial multifunctions in infectivity [14, 42]. It has also been confirmed through reverse genetics using cDNA clones (ICS) as a crucial determinant of NDV thermostability with conferred complete protection of birds [43]. Thermostability characterization of heat-stable ND-viruses relating to HN gene will improve understanding of the molecular basis of ND-virus thermostability. This will augment rational design of NDV vaccines to solve managerial problems in scavenging rural chicken [18] or further evaluate performance of established vaccine strains.

Recovery of virulent ND strains in birds vaccinated with avirulent vaccines strains or in ND-virus endemic countries raises concerns whether these strains are derived from indigenous or vaccine strains [2]. In Uganda, available ND vaccines used are namely: LaSota strain and I_2 strain with well-defined genotypes. However, the recent ND-virus isolates from Ugandan live bird markets indicate a high pathogenicity and low evolution [8] with no thermostability phenotypes. Several studies have recovered low-virulent, thermostable ND-virus in several species of birds [5, 33]. Such strains have been evaluated as vaccine candidates to protect village birds in the tropics where temperatures are high and local farmers either lack or are unable to pay for the cold-chain needed to sustain live thermolabile ND-virus vaccine usage [35].

Thermostability of hemagglutinins of ND-virus is retained through many serial passages in embryonating eggs and can distinguish one culture from another [13]. The basis of thermostability testing of ND-virus is that all strains have hemagglutinin surface proteins, which agglutinates chicken RBCs in vitro, and Neuraminidase enzyme that promotes virus release from infected cells. The activity of these surface glyco-proteins is used to detect hemagglutinating viruses in the family [27], and to follow their stability when the virus is exposed at different temperatures [1]. Since the development of a thermostable

Australian V4 ND vaccine strain, several thermostability testing methods have been used to evaluate live ND-virus isolates and vaccine candidates for ability to survive under different temperatures [19, 21, 22, 36].

The aim of the current study was to determine the thermostability of ND-virus isolates recovered from live bird markets (LBM) and waterfowls and relate this phenotype to HN gene sequence.

Materials and methods

ND-virus isolates

A total of 168 ND-virus isolates were used in the current study. These field ND-viruses were isolated in Uganda during 2011 from LBM comprised of local chickens, turkeys and ducks. The isolates from waterfowl were recovered from freshly voided fecal droppings of migratory and resident waterfowls in selected landing sites. Sixty seven percent (112/168) of the viruses of LBM were isolated from cloacal (C) and/oro-pharyngeal (P) swabs, while thirty three percent (56/168) of isolates from waterfowls were recovered from fresh fecal (F) matter. Initially, viruses were passaged twice in 10–day-old embryonated chicken eggs (ECE) obtained from ND-free flocks and clean isolates obtained in chicken embryonated fibroblasts (CEF). The allantoic fluid was aseptically harvested, virus presence confirmed by HI test according to standard procedures and analyzed further or stored at −20 °C as virus stocks. Biological characterization for chicken ND isolates were described elsewhere [8]. Two live commercial vaccines (I_2 and LaSota) were used as controls throughout the study.

The presence of the ND-virus was confirmed by PCR and serologically with positive sera in HI test [8, 27]; using 0.5 % (v/v) washed ND-virus–free chicken RBCs according to standard procedures. Serum was generated in in-house rabbits immunized with ND-virus - LaSota vaccine strain (Laborotorios Hipra, S.A, Spain).

Assessment of thermostability

HA and infectivity titers for all the stock viruses were determined by standard microtiter plate hemagglutionation assay [27] and the viability of selected isolates by end point infectivity assay [30]. Nine paired sealed vials containing 0.5 mL aliquots of each ND-virus isolate were thawed once. One pair was left on ice while the other eight were incubated on a water bath (Polypro Bath° CA, USA) kept at a constant temperature (56 ± 0.5 °C). At regular interval of 15, 30, 45, 60, 75, 90, 105 and 120 min, a pair of vials was removed and chilled quickly on ice to stop heat inactivation. All the aliquots were assayed for HA activity by standard methods [27, 30]. The isolates that showed HA titers at 56 °C after 2 h were further analyzed for regression analysis of HA activity using rate constants (k) and among these, avirulent isolates were passaged five times without heating

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intervals. Additionally, to determine the effect of heat on the viability or multiplication of the selected viruses, the vials incubated at 56 °C for 0, 30, and 60 min were assayed for virus viability and compared to the thermostable I_2 strain. Briefly, 0.3 mL of the virus aliquot from each mentioned treatment was injected into three sets of eggs and incubated for 4 days before allantoic fluid was harvested, checked for the mean $\log_2$ HA titer and assayed for infectivity.

Stability of infectivity

The infectivity stability of the selected avirulent viruses was evaluated and compared to vaccine strains (I_2 and LaSota) in 10-day-old embryonated eggs. The infectivity evaluation of the isolates involved five passages. Briefly, tenfold serial dilutions were made in normal saline solution (Phosphate Buffered Saline, PBS) and five dilutions were selected. One hundred microliters (0.1 mL) of each of these dilutions: 10^{-1}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} was inoculated in duplicate into five eggs, incubated for 4 days, and harvested for HA and median embryo infectivity dose at fifty percent ($\log_{10}EID_{50}$) assays. This was carried out for five passages where both HA and infectivity assays were carried out for each passage. Additionally, the heamagglutionation units were measured following passages, to evaluate the replication competence of the isolates.

Viral RNA extraction, RT-PCR and DNA sequencing

Viral RNA was extracted from all the HI positive allantoic fluid samples by using the QIAamp Viral RNA mini kit (Qiagen, USA) according to the manufacturer's instructions and sequenced to obtain full HN sequences as described in [8].

HN gene sequence analysis

To predict the HN relationship between the field ND-virus isolates and vaccine strains, multiple sequence alignment of HN gene of representative thermostable ND-virus isolates was done. Using the following accession numbers previously deposited in the Gene Bank for isolates; NDV/UG/MU/007- HG937536, NDV/UG/MU/010-HG937538, NDV/UG/MU/022- HG937542, NDV/UG/MU/039- HG937548, NDV/UG/MU/059- HG937553, NDV/UG/MU/098- HG937564 and NDV/UG/MU/111- HG937566. Together with other representative thermostable vaccines: TS09C- JX110635, I_2 – AY935499, I_2 progenitor – AY935500, NDV4-C - JX443519 and thermolabile: Lasota –JF950510, LaSota C5 – KC844235, and D58 strain- EU305607 and others were blast searched from NCBI GeneBank using the Bio-Edit software (North Carolina State University, USA) to generate sequence analysis data. The parameters used for the sequences analysis were multiple alignment (Clustal W), sequence identity plotter and sequence matrix at both amino acid and nucleotide levels. The B- cell epitope of HN gene was predicted in a computer algorithm using web server based on software www.bioinfo.tsinghua.edu.cn/epitope/EPMLR. The transmembrane domains were predicted using Dense Alignment Surface (DAS) transmembrane domain prediction server– http:www.sbc.su.se/-miklos/DAS/maindashtml [11].

Statistical analyzes

Thermostability was analyzed by calculating the slope of the regression curve of HA activity. When appropriate, the data of exponential decline in activity of HA were presented as rate constants (k, expressed as
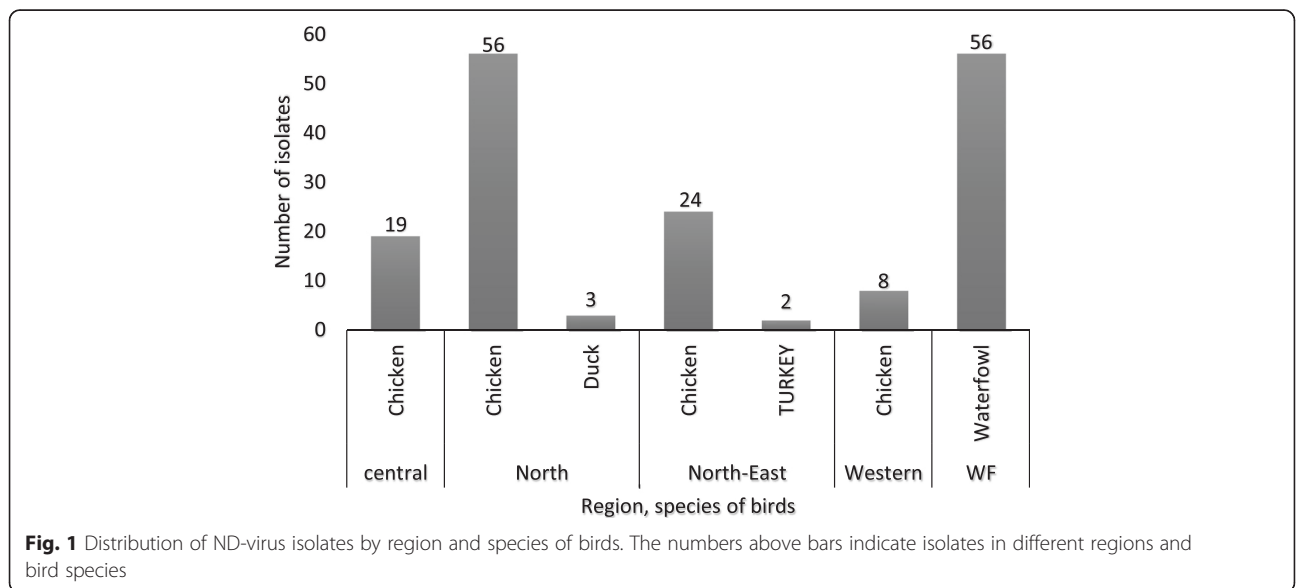

Fig. 1 Distribution of ND-virus isolates by region and species of birds. The numbers above bars indicate isolates in different regions and bird species

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Table 1 Thermostability profiles with numbers of NDV isolates by HA activity determined at 56 °C

REGIONS	District	Site	15	30	45	60	75	90	105	120	All
Central	KALUNGU	C	–	1	–	1	–	–	–	–	2
		P	–	1	–	2	1	–	–	–	4
	KIBOGA	C	–	1	–	–	–	–	–	–	1
		P	–	1	–	–	–	–	–	–	1
	LWENGO	C	2	–	–	–	–	–	–	–	2
		P	–	2	–	–	–	–	–	–	2
	MASAKA	C	–	1	–	–	–	–	–	1	2
		P	1	1	–	–	–	–	–	–	2
	MUKONO	P	1	–	–	–	–	–	–	–	1
	WAKISO	C	2	–	–	–	–	–	–	–	2
East	BUGIRI	C	–	1	1	–	–	–	–	–	2
		P	1	1	–	–	–	–	–	–	2
	IGANGA	C	–	1	1	–	–	–	–	–	2
		P	–	–	–	–	–	1	–	–	1
		T	–	2	–	–	1	–	–	–	3
	MAYUGE	P	–	1	–	–	–	–	–	–	1
	MBALE	C	1	–	–	–	–	–	–	1	2
		P	–	1	–	–	–	–	–	–	1
North	APAC	C	1	–	–	–	–	–	–	–	1
	ARUA	C	2	1	–	–	–	–	–	–	3
		P	3	–	1	–	–	–	–	–	4
	KOBOKO	C	–	2	–	–	–	–	–	–	2
		P	–	–	2	1	1	–	–	–	4
	KOLE	P	1	–	–	–	–	–	–	–	1
	LIRA	C	1	–	–	–	–	–	–	–	1
		P	1	–	–	–	–	–	–	–	1
	MARACH	C	1	1	–	–	–	–	1	–	3
		P	–	–	2	–	1	1	–	–	4
	OTUKE	C	–	–	1	–	–	–	–	–	1
	OYAM	C	5	1	–	–	–	1	–	2	9
		P	7	1	2	–	1	–	–	2	13
	YUMBE	C	1	–	–	–	–	–	–	–	1
		P	2	–	–	–	–	–	–	1	3
	ZOMBO	C	3	1	–	1	–	1	–	–	6
		P	–	–	1	–	–	–	–	–	1
North-East	ABIM	C	–	1	–	2	–	–	–	1	4
		P	–	1	–	–	–	–	–	1	2
	KOTIDO	C	–	1	–	–	–	–	–	–	1
		P	2	–	–	–	–	–	–	–	2
	KUMI	C	–	–	–	–	–	–	–	1	1
		P	–	–	1	1	2	–	–	–	4
	NAMUTUMBA	C	–	1	–	1	1	–	–	–	3

Table 1 Thermostability profiles with numbers of NDV isolates by HA activity determined at 56 °C (Continued)

	SERERE	P	–	1	–	–	–	–	–	–	1
Western	BUNDIBUJO	C	–	–	1	–	–	–	–	1	2
	KABALE	P	–	1	–	–	–	–	–	–	1
	KABAROLE	C	1	–	–	–	–	–	–	–	1
	KASESE	F	1	–	–	–	1	–	1	2	5
		P	–	–	1	–	–	–	–	–	1
	KYENJOJO	P	–	1	–	–	–	–	–	–	1
	MBARARA	P	–	1	–	–	–	–	–	–	1
Waterfowl	JINJA	F	2	1	–	–	–	–	1	2	6
	RAKAI	F	10	1	1	–	–	–	–	3	15
	WAKISO	F	8	5	3	1	–	2	1	5	25
Number of Isolates			60	38	18	10	9	6	4	23	168

mins^{-1}), defined by first-order inactivation equation below as described [41].

$$2.303 \, log10 \, (V/Vo) = -kt$$

Isolates whose late time points, i.e., 105 or 120 min did not contain HA were excluded from analysis. The survival analysis for the isolates were titrated by standard median $EID_{50\%}$ according to standard protocols [27]. All statistical analyzes were performed in GraphPad Prism, version 5.

Results

Thermostability of field ND-virus isolates at 56 °C

The 168 field isolates showed HA titers greater than 16 log_2 in embryonated eggs during the initial culture and were confirmed as ND-viruses by HI test and PCR. Regarding the source of poultry isolates, Northern region had the highest number of isolates compared to other regions. ND-viruses occurring in different species of poultry existing in the four regions of Uganda (Fig. 1). All the isolates used showed disparate thermostability at 56 °C ranging from 15 to 120 min (Table 1). Of these study isolates, 13.7 % (23/168) retained more than 2 log_2 of their initial HA titers after 120 min and were regarded as thermostable while 86.3 % (145/168) without residual HA titer after the above time were considered thermolabile isolates. Of the 23 thermostable isolates, 13 were from LBM while 10 were from waterfowls (Table 1). Based on the heat inactivation rate constant (k) of HA activity, the thermostable strains were included in Table 2. When compared by rate constant of HA activity, 26.1 % (6/23) of the 23 thermostable ND-virus isolates were inactivated at a lower rate (higher heamagglutinin activity) than the reference I_2 vaccine strain. Also all these isolates were from waterfowls.

Table 2 Hemagglutination titers and heat inactivation rate constant (*k*) of thermostable ND-viruses incubated at 56 °C

Virus Isolates	Arithmetic mean HA titer (log2) at various time intervals of heat inactivation (minutes)									Rate constant (k)
	0	15	30	45	60	75	90	105	120	(10³ K/min⁻¹)
NDV007/UG/MU/2011	5	5	5	5	5	5	4	4	4	2.23
NDV015/UG/MU/2011	6	6	6	6	5	5	4	4	4	4.26
NDV022/UG/MU/2011	8	8	8	8	8	8	8	8	3	4.36
NDV023/UG/MU/2011	7	7	6	5	5	4	4	3	1	12.62
NDV039/UG/MU/2011	8	8	8	8	7	7	6	5	4	5.44
NDV059/UG/MU/2011	8	8	7	7	6	6	5	4	3	7.59
NDV073/UG/MU/2011	5	5	5	5	4	4	3	2	1	11.59
NDV089/UG/MU/2011	3	3	3	3	3	3	2	1	1	9.45
NDV092/UG/MU/2011	5	5	4	3	2	2	2	1	1	14.51
NDV097/UG/MU/2011	8	4	4	4	4	4	4	4	4	3.08
NDV098/UG/MU/2011	5	4	4	3	2	2	2	2	2	8.38
NDV099/UG/MU/2011	5	5	4	4	4	3	3	3	1	9.82
NDV111/UG/MU/2011	6	5	4	4	3	2	2	2	2	10.25
NDV115/UG/MU/2011	6	6	6	6	6	6	6	6	4	1.80
NDV118/UG/MU/2011	7	7	7	7	7	7	7	7	7	0.00[b]
NDV119/UG/MU/2011	5	5	5	5	4	4	4	3	2	6.52
NDV133/UG/MU/2011	6	6	6	6	6	6	6	6	6	0.00[b]
NDV152/UG/MU/2011	6	6	6	6	6	6	6	6	6	0.00[b]
NDV158/UG/MU/2011	4	3	3	3	3	3	3	2	1	7.51
NDV173/UG/MU/2011	6	6	6	6	6	6	6	6	5	0.81[b]
NDV177/UG/MU/2011	6	6	6	6	6	6	6	5	5	1.22[b]
NDV178/UG/MU/2011	6	6	6	6	6	6	6	6	4	0.81[b]
NDV180/UG/MU/2011	6	6	6	5	5	4	4	3	3	6.54
I₂ vaccine	11	10	10	10	10	10	10	9	9	1.24[a]
LaSota vaccine	8	≥1	–	–	–	–	–	–	–	245.97[a]

[a]Reference vaccines
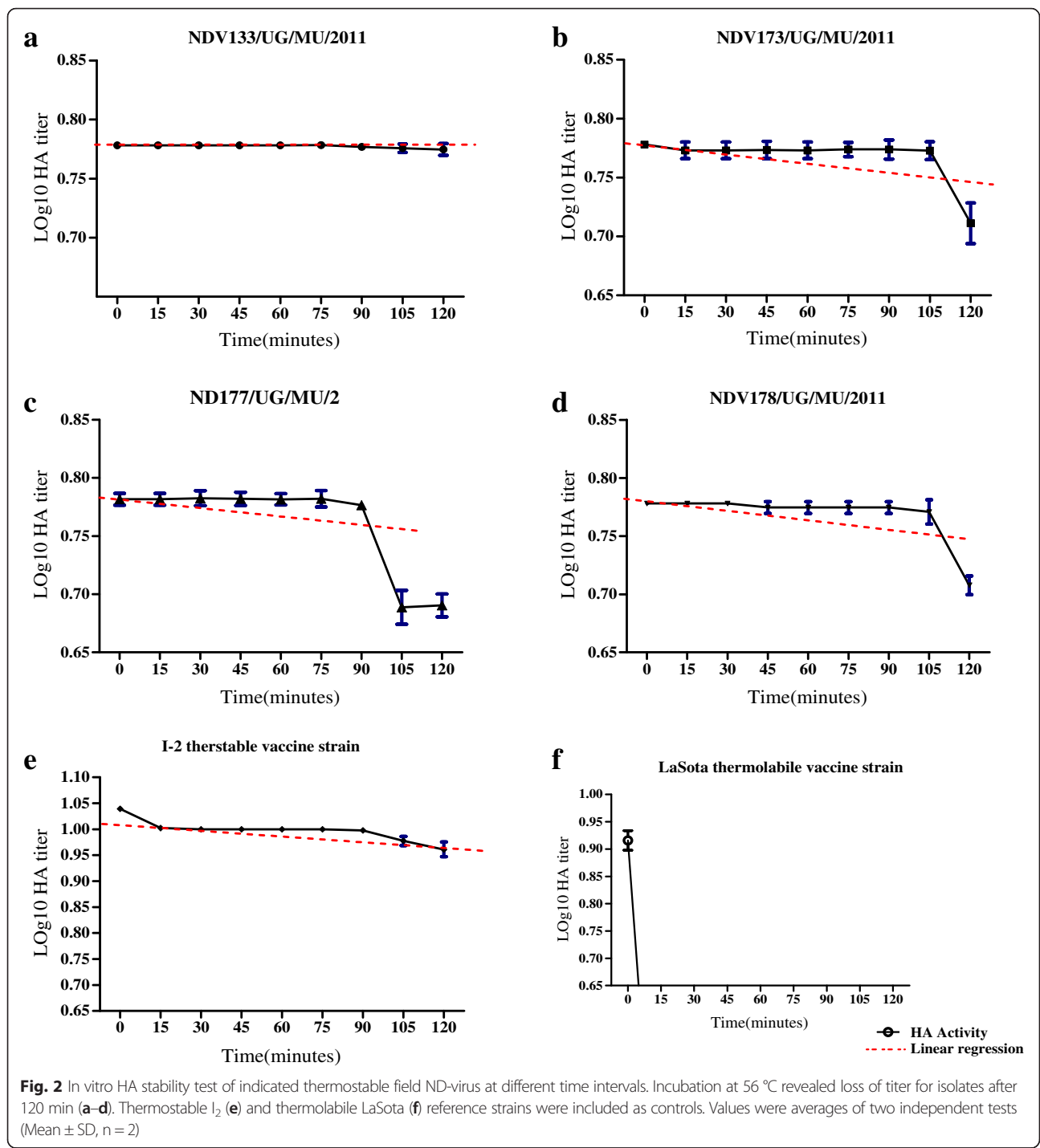[b]Most thermostable ND-virus, below I_2 reference thermostable strain

By plotting the logarithmically transformed (log_{10}) HA of the selected avirulent isolates against time, the inactivation rates were further compared (Fig. 2). Except for NDV133/UG/MU/2011 and LaSota that yielded a monophasic curve (Fig. 2a and d), other selected thermostable isolates produced biphasic curves (Fig. 2b-d). For biphasic curves, from zero to 90 time points, a plateau that was observed was followed by a linear decline. Overall, isolate NDV133/UG/MU/2011 had better survival rate compared to NDV173/UG/MU/2011, NDV177/UG/MU/2011 and NDV178/UG/MU/2011 as avirulent thermostable isolates.

Stability of infectivity upon passage

Four clean thermostable isolates were passaged five times and their infectivity shown in Fig. 3. Infectivity of the isolates decreased along the five serial passages. At the end of 3rd passage, the infectivity of isolate NDV177/UG/MU/2011 rapidly declined to a geometric median log_{10} $EID_{50\%}$/mL titer of 1.25, while that of NDV178/UG/MU/2011 was at 4.55 at the same passage. The infectivity titers of NDV133/UG/MU/2011 and NDV173/UG/MU/2011 isolates remained fairly close to that of I_2 vaccine strain at geometric median log_{10} $EID_{50\%}$ of 7.4. A greater change in infectivity of the isolates was at the fourth and fifth passages. Comparatively, one isolate NDV133/UG/MU/2011 retained more than 6.5 $log_{10}EID_{50\%}$/mL of its infectivity titer compared to I_2 and LaSota of 8.05 $log_{10}EID_{50\%}$/mL and 7.5 $log_{10}EID_{50\%}$/mL respectively at the 5th passage. The infectivity titers of isolates following a less than one hour time heat exposure were compared in Table 3. The ND-virus isolates NDV133/UG/MU/2011, NDV173/UG/MU/2011 and ND178/UG/MU/2011 retained more than 50 % of their $log_{10}EID_{50\%}$ infectivity within one hour of exposure at 56 °C. LaSota (thermolabile) vaccine strain had no detectable infectivity after 30 min of heat exposure while I_2 (thermostable) strain retained its infectivity.

HN gene sequences

The HN gene of Ugandan isolates contains coding sequence (cds) comprising of 1713 nucleotides coding for 571 amino acids with the stalk and globular head major areas comprising of 1-143 amino acids and 125-571 amino acids respectively. The transmembrane domains of the selected thermostable were variable with three to five predictable domains at positions 24–47, 25–45, 96–97, 101–107 and 557–563. 13 cysteine (C) residues were conserved for all our isolates at position 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531 and 542. Six glycosylation sites were also conserved across all isolates at positions G_1-119, G_2-341, G_3-433, G_4-481, G_5-508 and G_6-538. The details is provided in Table 4. The sequence identity matrix for HN gene of the selected thermostable isolates and other strains of ND-virus are provided in Table 5 and the predicted B-cell epitopes for HN genes are given in Table 6

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Fig. 2 In vitro HA stability test of indicated thermostable field ND-virus at different time intervals. Incubation at 56 °C revealed loss of titer for isolates after 120 min (**a**–**d**). Thermostable I$_2$ (**e**) and thermolabile LaSota (**f**) reference strains were included as controls. Values were averages of two independent tests (Mean ± SD, n = 2)

Discussions

Thermostability and infectivity of isolates

Several thermostable strains like I$_2$, HR-V4 and V4 have been isolated and characterized [7, 16, 34]. This specific study profiled the thermostability of the Ugandan ND-virus isolates and compared them at HN protein to differentiate local strains from existing vaccines strains. While thermostability is considered to be a natural

property of ND-virus isolates used to rapidly characterize field ND-virus isolates in epizootiological studies in the absence of any other marker [13], Ugandan field ND-virus isolates showed disparate thermostability profiles which is not related to the virulence of isolates, site of isolation in the bird species or geographical locality of isolation. This confirms the findings from other studies [20, 33]. Although poultry samples were
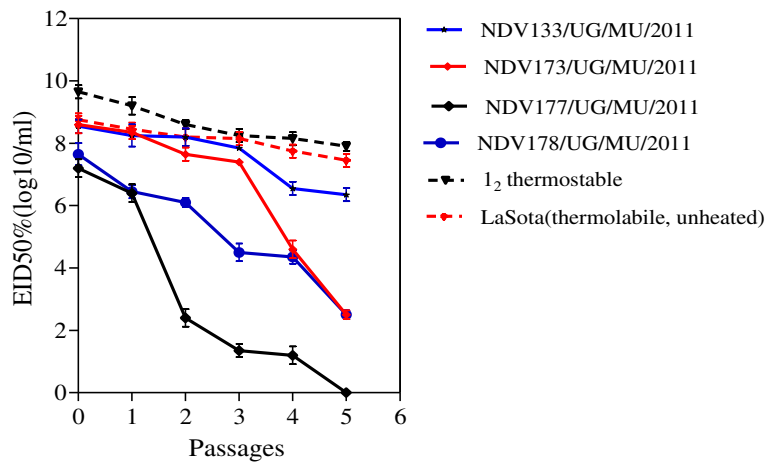
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Fig. 3 Stability of infectivity of indicated ND-virus strains by geometric mean $\log_{10}EID_{50\%}$ following serial passages. A greater change in the titers of field strains occurred at 4th and 5th passages compared to I_2 reference vaccine. LaSota, a thermolabile vaccine was unheated initially but included to compare its viability with that of thermostable isolates. Values indicated are means of three independent tests (Mean ± SD, n = 3)

collected from places of different climatic conditions of their natural sources, thermostability profiles of isolates did not reflect these conditions. The average thermostability of isolates from climatically hotter North and North-East regions of Uganda was 45 min, while that from cooler central and western regions was 60 min. This observation is in agreement with other studies supporting the fact that heat resistance in ND-virus is not a climatically determined trait [15].

The natural spread of clonal derivatives of vaccines strains in a locality following massive vaccination has

Table 3 HA and infectivity titers of avirulent, thermostable ND-viruses at 56 °C for 0, 30, 60 min

Virus	Parameter	Heat treatment time (minutes.) at 56 °C		
		0	30	60
NDV133/UG/MU/2011	[a]HA titer (log2)	2^6	2^6	2^6
	[b]Infectivity titer	$10^{8.53}$	$10^{8.35}$	$10^{8.05}$
NDV173/UG/MU/2011	[a]HA titer (log2)	2^6	2^6	2^6
	[b]Infectivity titer	$10^{8.4}$	$10^{8.25}$	$10^{7.7}$
NDV177/UG/MU/2011	[a]HA titer (log2)	2^6	2^6	2^6
	[b]Infectivity titer	$10^{7.3}$	$10^{6.3}$	$10^{2.25}$
NDV178/UG/MU/2011	[a]HA titer (log2)	2^6	2^6	2^6
	[b]Infectivity titer	$10^{7.8}$	$10^{6.45}$	$10^{6.2}$
I_2 thermostable strain	[a]HA titer (log2)	2^{11}	2^{10}	2^{10}
	[b]Infectivity titer	$10^{9.8}$	$10^{9.5}$	$10^{9.3}$
LaSota thermolabile	[a]HA titer (log2)	2^9	2^9	2^9
	[b]Infectivity titer	$10^{9.6}$	–	–

[a]Hemagglutination assay (HA) titer presented as arithmetic mean HA $\log_2$ titer; and infectivity as $\log_{10}$
[b]50 % embryonated egg infectious dose (EID_{50}/ml)

been observed [3]. In the present study, thermostable isolates were recovered from unvaccinated flocks. This suggests some degree of natural area spread which explains disparate thermostability profiles of both waterfowl and live bird market bird isolates. This is further confirmed by presence of new genotypes our previous study of genotypic characterization of LBM isolates [8]. Taken together, there is a spread of field viruses with varied pathogenicity, thermostability and antigenicity in tropical developing countries, which require a carefully designed control measures.

A prolonged exposure of ND-virus isolates to a high temperature of 56 °C has been preferred to short time exposure at lower temperature during heat selection [17, 41]. This is to ensure selection of a more thermostable ND-virus from a heterogenous population containing a mixture of heat-stable and heat labile strains [19, 22]. We recovered 13.7 % (23/168) of field isolates (LBM and waterfowl) with residual HA titers after 2 h exposure at 56 °C. Of the 23 isolates, 26.2 % (6 out of 23 isolates) had greater heamagglutinin titers greater than reference I_2 strain. These results are in conformity with other studies especially [19], who reported 38 % of the field ND-virus isolates with a greater heamagglutinin than reference thermostable strains. By regression analysis, we noted thermostable and thermolabile phenotypes of strains that was not influenced by site of isolation. Isolates from oro-pharyngeal (P) and cloaca (C) of the same bird had varied thermostability profiles. This confirms the idea that thermostability is not truly a genotypic character but just a trait that can readily be increased by selection involving heat shock [35].

Like in the Australian experience of enhancing thermostability of V4 vaccine strain from a subpopulation of known heat-stable virus also adopted by [16], we

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Table 4 Predicted transmembrane amino acid sequences analysis for HN gene of thermostable ND-viruses and vaccine strains

Potential Transmembrane Domains of thermostable field strains and vaccine strains					
NDV strain	Start	End	Cutoff	Length	Predicted amino acid sequence
NDV/UG/MU/007	24	47	1.7	24	VFRIAVLSLIIMILVISVSILVYS
NDV/UG/MU/010	25	46	2.2[a]	22	FRIAVLSLIIMILVISVSILVY
NDV/UG/MU/022	96	97	1.7	2	LL
NDV/UG/MU/039	101	105	1.7	5	STIMN
NDV/UG/MU/059	557	563	1.7	7	RIVPLLV
NDV/UG/MU/111					
NDV/UG/MU/098	24	47	1.7	24	VFRIAVLSLIIMILVISVSILVYS
	25	46	2.2[a]	22	FRIAVLSLIIMILVISVSILV
	96	97	1.7	2	LL
	101	104	1.7	4	SVIM
	557	563	1.7	7	RIVPLLV
LaSota Thermolabile strain	23	47	1.7	25	IFRIAILFLTWTLAISVASLLYS
	25	45	2.2[a]	21	FRIAILFLTWTLAISVASLL
	209	210	1.7	2	GV
	557	563	1.7	7	TTIMN
I-2 Thermostable strain	23	44	1.7	22	VFRIAILFLTWTLAVSAAALAYS
	25	42	2.2[a]	18	FRIAILFLTWTLAVSAAALA
	424	429	1.7	6	ALLYPM
	557	563	1.7	7	RIVPLLV
NDV4-C Thermostable	24	44	1.7	21	VFRIAILLSTWTLAISAAAL
	25	42	2.2[a]	18	FRIAILLSTWTLAISAA
	210	210	1.7	1	L
	424	430	1.7	7	ALLYMI
	557	563	1.7	7	RIVPLLV
NDV TS09-C thermostable	24	44	1.7	21	VFRIAILLSTWTLAISAAAL
	25	42	2.2[a]	18	FRIAILLSTWTLAISAA
	210	211	1.7	2	VL
	424	430	1.7	7	ALLYPMI
	557	563	1.7	7	RIVPLLV
NDV B1 strain	24	47	1.7	24	VFRIAILLSTWTLAISAAALAYS
	25	45	2.2[a]	21	FRIAILLSTWTLAISAAALA
	557	563	1.7	7	RIVPLLV

Sequences in boxes show variation in consensus with avirulent strains. The transmembrane domains marked with ([a]) were significant and considered to different isolates and vaccinates

subjected clean ND-virus isolates to the same temperature (56 °C), followed HA titers until 2 h and selected four most thermostable isolates. Previous studies have attempted to isolate avirulent, thermostable ND-viruses from the feces of wild migratory birds for vaccine candidates [32, 33, 40]. Here, the present study identified thermostable ND-viruses from waterfowls and this could provide a promising line of research in thermostable vaccine development in Uganda and providing a base to develop an affordable thermostable ND vaccine.

DNA sequence analysis of the HN gene

HN is a type II homotetrameric glycoprotein with a monomer length of 577 amino acids for most NDV strains [25]. The heamagglutinin, Neuraminidase and thermostability are one of the multifunctional functions of HN protein that plays key roles in the steps of the NDV life cycle [14]. Mutations within the HN gene has been reported to contribute to thermostability phenotype and immunogenicity of ND-virus [39, 43]. Deletions of amino acid, R 403 were thought to influence thermostability but attempts by several studies to find alterations at such a position was futile in thermostable strains. However, evaluation of chimeric ND-viruses confirmed that thermostability phenotype of ND-virus was dependent upon the origin of HN segment and not any other ND-virus genome [43]. Still no study has offered a clear mechanism or sequence based analysis of HN gene for thermostability. By analyzing the sequences of field thermostable and thermolabile viruses, we noted no deletions at the suggested site but observed increased proportion of charged amino acid residues at the expense of uncharged polar amino acid among the thermostable isolates. This could confer rigidity and stability by minimizing deamination and backbone cleavage.

The change to a higher proportion of isoleucine (I), leucine (L), valine (V) and arginine (R) at the stalk and globular regions of NDV HN ectodomain of thermostable isolates offers an explanation to the thermostability since these aliphatic amino acid residues contribute to hydrophobic interaction, a force that maintains the conformational internal protein integrity. Besides, it has been observed that substitution of amino acids at the conserved stalk spike especially the leucine zipper motif affect the Neuraminidase activity of the globular domain where HN molecule interacts with sialic acid binding and F protein to initiate fusion. Protein stabilities have been predicted by sequence feature-based predictions upon amino acid substitutions using models [38]. Our observed amino acids substitutions at V24I, I43S, V45L, T48A, R62A, T73L, V266A, R269S, G293K, S310D, S315P, I404V, I477V and N440S in our thermostable isolates all decreased protein stability with prediction confidence of >80 % which in our case partly explains thermostability phenotype shown in Fig. 4.

Several amino acid residues have been reported as functional amino acid residues, F220, S222, L224, E401, R416, and Y526 in HN protein. 13 cysteine amino acid residues at position 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531 and 542. Six potential

Table 5 Sequence identity matrix for CDS of HN gene of thermostable ND-viruses and vaccine strains

SEQUENCE	LASOTAC5	TZ	D58	MU001	MU007	MU010	MU022	MU039	MU059	MU098	MU111	LASOTA	I-2	BEAUDC	C/P/01	I-2PRO	TS09-C	NDV4C	CLONE30
LA SOTA C5	**ID**	**83.2**	**93.4**	**81.1**	**81.2**	**81.2**	**81.2**	**80.9**	**81.2**	**80.9**	**81.1**	**99.5**	**91.3**	**9.6**	**0.8**	**0.8**	**0.8**	**0.9**	**60.7**
TZ060107	76.2	**ID**	**88.0**	**90.8**	**90.7**	**90.5**	**90.7**	**90.5**	**91.0**	**90.8**	**90.8**	**82.8**	**83.2**	**9.4**	**1.5**	**0.7**	**0.7**	**0.8**	**53.0**
D58	93.5	81.3	**ID**	**85.9**	**86.1**	**86.1**	**86.1**	**85.7**	**86.1**	**85.7**	**85.9**	**93.8**	**87.1**	**10.0**	**0.6**	**0.8**	**0.8**	**0.8**	**60.2**
NDV/MU001	76.4	85.8	81.4	**ID**	**98.5**	**98.4**	**99.1**	**99.2**	**98.9**	**98.7**	**99.2**	**80.9**	**80.7**	**8.2**	**1.5**	**0.7**	**0.7**	**0.8**	**51.5**
NDV/MU007	76.7	85.8	81.7	98.4	**ID**	**99.4**	**99.1**	**98.9**	**98.9**	**98.7**	**98.9**	**81.1**	**80.7**	**8.2**	**1.4**	**0.7**	**0.7**	**0.8**	**51.5**
NDV/MU010	76.7	85.8	81.7	98.3	99.8	**ID**	**98.9**	**98.4**	**98.4**	**98.5**	**98.7**	**81.1**	**80.7**	**8.2**	**1.4**	**0.7**	**0.7**	**0.8**	**51.5**
NDV/MU022	76.4	85.8	81.4	99.2	98.8	98.7	**ID**	**99.1**	**99.1**	**98.9**	**99.4**	**81.1**	**80.7**	**8.4**	**1.4**	**0.7**	**0.7**	**0.8**	**51.5**
NDV/MU039	76.4	85.7	81.4	99.3	98.4	98.3	99.1	**ID**	**99.2**	**98.7**	**99.2**	**80.7**	**80.4**	**8.2**	**1.5**	**0.7**	**0.7**	**0.8**	**51.3**
NDV/MU059	76.6	85.9	81.6	99.0	98.5	98.4	99.3	99.0	**ID**	**98.7**	**99.2**	**81.1**	**80.4**	**8.2**	**1.4**	**0.7**	**0.7**	**0.8**	**51.3**
NDV/MU098	76.4	85.8	81.4	99.0	98.6	98.4	99.3	98.9	99.0	**ID**	**99.1**	**80.7**	**80.7**	**8.2**	**1.4**	**0.7**	**0.7**	**0.8**	**51.5**
NDV/MU111	76.6	86.0	81.6	99.1	98.6	98.5	99.4	99.1	99.2	99.2	**ID**	**80.9**	**80.6**	**8.2**	**1.4**	**0.7**	**0.7**	**0.8**	**51.5**
LASOTA	99.7	76.2	93.6	76.3	76.6	76.6	76.3	76.3	76.5	76.3	76.5	**ID**	**90.9**	**9.4**	**0.6**	**0.8**	**0.8**	**0.9**	**60.5**
I-2	87.9	78.6	82.7	77.2	77.3	77.3	77.3	77.1	77.2	77.4	77.3	87.7	**ID**	**10.4**	**1.3**	**0.8**	**0.8**	**0.8**	**56.9**
BEAUDETTE C	9.3	9.3	9.9	9.2	9.3	9.3	9.3	9.2	9.2	9.3	9.2	9.3	10.2	**ID**	**1.5**	**<.001**	**<.001**	**<.001**	**9.5**
C/P/0103/01	5.2	5.7	5.5	5.5	5.5	5.5	5.4	5.5	5.5	5.4	5.5	5.1	5.2	12.9	**ID**	**0.2**	**0.12**	**0.2**	**0.8**
I-2PROGENITOR	3.0	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	3.0	3.0	0.2	0.6	**ID**	**89.0**	**89.2**	**0.8**
TS09-C	3.1	2.8	2.9	2.9	2.9	2.9	2.8	2.8	2.9	2.8	2.9	3.1	3.0	0.3	0.5	95.4	**ID**	**99.5**	**0.7**
NDV4-C	3.1	2.8	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	3.1	3.0	0.3	0.5	95.5	99.8	**ID**	**0.8**
CLONE 30	68.5	55.0	66.8	55.1	55.2	55.2	55.0	55.1	55.1	55.2	55.2	68.5	61.4	9.0	5.0	3.2	3.1	3.1	ID

Amino acid differences are indicated in bold and nucleotide differences are in normal font. The difference with commonly used vaccine strains are high lightened

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Table 6 B-cell epitopes of HN gene of representative thermostable field ND-viruses

HN GENE B-CELL EPITOPES

NO	Start	End	Thermostable field isolates Peptide	NDVUG/MU/059 Peptide
1	13	18	EEREAK	EEREAK
2	48	58	TGASTPSDLAS	GASTPSDLAS
3	63	69	ISKAEDR	ISKTEDR
4	79	79	E	D
5	115	135	NGAANTSGCGAPVHDPDYIGG	NGAANTSGCGAPVHDPDYIGG
6	146	147	SD	SD
7	150	156	SFYPSAY	SFYPSAY
8	164	174	PAPTTGSGCTR	PAPTTGSGCTR
9	179	180	DM	DM
10	198	201	DHSH	DHSH
11	229	240	LDDTQNRKSCSV	LDDTQNRKSCSV
12	242	244	ATP	ATP
13	255	267	TETEEEDYRSVAP	TETEEEDYRSVAP
14	279	284	GQYHEK	GQYHEK
15	297	307	ANYPGVGGGSL	ANYPGVGGGSL
16	319	331	GLKPNSPSDAAQE	GLKPNSPSDAAQE
17	341	351	NNTCPDEQDYQ	NNTCPDEQDYQARMAKSSYKPGRFGGK
18	357	367	SSYKPGRFGGK	
19	381	395	SLGEDPELTVPPNTV	SLGEDPELTVPPNTV
20	418	420	SSY	SSY
21	436	439	ATLH	ATLH
22	447	461	FTRPGSVPCQASARC	FTRPGSVPCQASARC
23	469	473	VYTDP	VYTDP
24	494	494	N	N
25	496	499	QARL	QARL
26	517	527	VSSSSTKAAYT	VSSSSTKAAYT

A Alanine, *R* Arginine, *N* Asparagine, *T* Threonine, *V* Valine, *C* Cysteine, *Q* Glutamine, *E* Glutamate, *D* Aspartate, *G* Glycine, *H* Histidine, *I* Isoleucine, *L* Leucine, *K* Lysine, *M* Methionine, *F* Phenylalaine, *P* Proline, *S* Serine, *W* Tryptophan, *Y* Tyrosine

glycosylation sites at G_1-119 G_2-341, G3-433, G_4-481, $G_5$508 and G_6-538, together with the different length of HN cds [29, 31]. The salient features of HN protein of our strains include: 571 amino acid size; conserved amino acids: R 174, I 175, R 197, D 198, K 236, R 416, R 498, Y 526 and E 547; potential glycosylation sites being G_1-119, G_2-341, G_3-433, G_4-481, G_5-508 and G_6-538, which is similar to genotype II vaccine strains Beaudette C, B1 and Ulster. Replacement of glycosylation site G_5-508 in other ND-virus by tryptophan and not serine (G_5-N508Y) has been used to pathotype ND-virus as avirulent [29]. All our selected thermostable isolates had this glycosylation site confirming their high pathogenicity reported earlier [8]. Further, a conserved amino acid E347 present in all thermostable and thermolabile isolates, a feature common to all vaccine strains of genotype II ND- viruses was observed. The percent similarity

in relation to amino acids between isolates and thermostable I_2 and TS09C is 80.6 % and that of thermolabile LaSota is 80.5 % suggesting our isolates share some attributes of these vaccine strains shown in Table 5. It could not be establish why isolates obtained from unvaccinated birds share this similarity more so with I_2 vaccine that has not been widely used in the country at the time of isolation.

The most variable portion of NDV HN protein is present in the N-terminal 78 amino acids that included the transmembrane domain at position 24–47, 25–45, 557–563 using online bioinformatics webserver DAS-transmembrane prediction server [11]. The significant cut off value for the transmembrane domain considered was 2.2 at 25–45 length common to all isolates and vaccinates. However, the variable predicted number of transmembrane domains for thermostable isolates were similar to
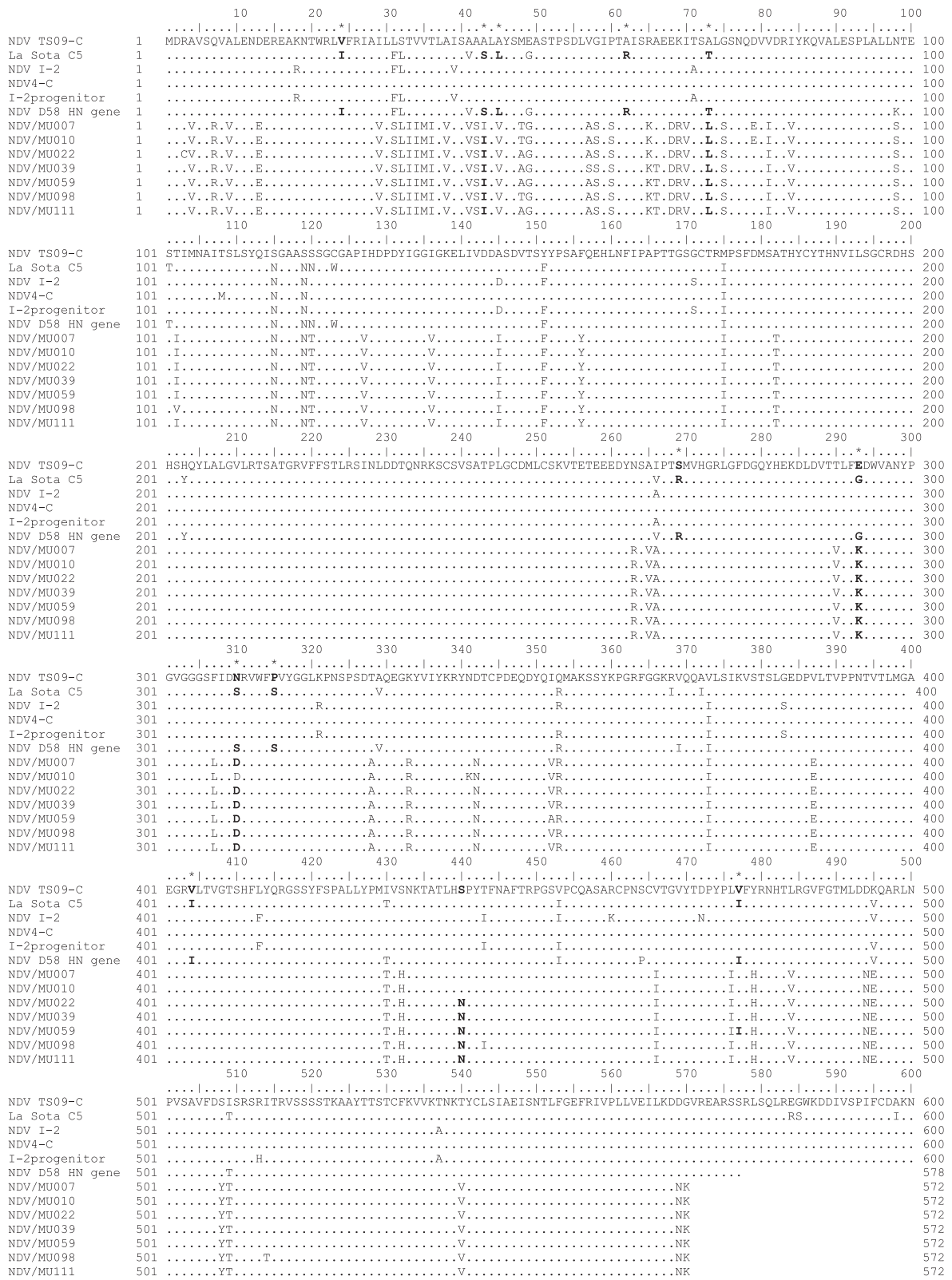
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                              10        20        30        40        50        60        70        80        90        100
                         ....|....|....|....|...*|....|....|....|..*.*...|....|....|..*..|....|..*.*|....|....|....|....|....|
        NDV TS09-C     1 MDRAVSQVALENDEREAKNTWRLVFRIAILLSTVVTLAISAAALAYSMEASTPSDLVGIPTAISRAEEKITSALGSNQDVVDRIYKQVALESPLALLNTE 100
        La Sota C5     1 .......................I......FL.......V.SL...G...........R.........T.......................... 100
        NDV I-2       1 ......................R..........FL.......V...........................A......................... 100
        NDV4-C        1 ......................................................................................................... 100
        I-2progenitor 1 ......................R..........FL.......V...........................A......................... 100
        NDV D58 HN gene 1 .......................I......FL.......V.SL...G...........R.........T................K.. 100
        NDV/MU007     1 ...V..R.V...E.............V.SLIIMI.V..VSI.V..TG........AS.S....K..DRV..L.S....E.I..V.............S.. 100
        NDV/MU010     1 ...V..R.V...E.............V.SLIIMI.V..VSI.V..TG........AS.S....K..DRV..L.S....E.I..V.............S.. 100
        NDV/MU022     1 ..CV..R.V...E.............V.SLIIMI.V..VSI.V..AG........AS.S....K..DRV..L.S.....I..V.............S.. 100
        NDV/MU039     1 ...V..R.V...E.............V.SLIIMI.V..VSI.V..AG........SS.S....KT.DRV..L.S.....I..V.............S.. 100
        NDV/MU059     1 ...V..R.V...E.............V.SLIIMI.V..VSI.V..AG........AS.S....KT.DRV..L.S.....I..V.............S.. 100
        NDV/MU098     1 ...V..R.V...E.............V.SLIIMI.V..VSI.V..TG........AS.S....KT.DRV..L.S.....I..V.............S.. 100
        NDV/MU111     1 ...V..R.V...E.............V.SLIIMI.V..VSI.V..AG........AS.S....KT.DRV..L.S.....I..V.............S.. 100
                             110       120       130       140       150       160       170       180       190       200
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
        NDV TS09-C   101 STIMNAITSLSYQISGAASSSGCGAPIHDPDYIGGIGKELIVDDASDVTSYYPSAFQEHLNFIPAPTTGSGCTRMPSFDMSATHYCYTHNVILSGCRDHS 200
        La Sota C5   101 T.............N...NN..W...............................F................I.................... 200
        NDV I-2     101 ...............N...N.................D....F..........................S...I.................... 200
        NDV4-C      101 .......M....N...N...........................................................I.................... 200
        I-2progenitor 101 ...............N...N.................D....F..........................S...I.................... 200
        NDV D58 HN gene 101 T.............N...NN..W...............................F.............................. 200
        NDV/MU007   101 .I.............N...NT......V.......V.......I...F...Y................I.....T.............. 200
        NDV/MU010   101 .I.............N...NT......V.......V.......I...F...Y................I.....T.............. 200
        NDV/MU022   101 .I.............N...NT......V.......V.......I...F...Y................I.....T.............. 200
        NDV/MU039   101 .I.............N...NT......V.......V.......I...F...Y................I.....T.............. 200
        NDV/MU059   101 .I.............N...NT......V.......V.......I...F...Y................I.....T.............. 200
        NDV/MU098   101 .V.............N...NT......V.......V.......I...F...Y................I.....T.............. 200
        NDV/MU111   101 .I.............N...NT......V.......V.......I...F...Y................I.....T.............. 200
                             210       220       230       240       250       260       270       280       290       300
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|...*|....|....|....|..*.|....|
        NDV TS09-C   201 HSHQYLALGVLRTSATGRVFFSTLRSINLDDTQNRKSCSVSATPLGCDMLCSKVTETEEEDYNSAIPTSMVHGRLGFDGQYHEKDLDVTTLFEDWVANYP 300
        La Sota C5   201 ..Y........................................................V..R........................G....... 300
        NDV I-2     201 ...............................................................A......................... 300
        NDV4-C      201 ......................................................................................................... 300
        I-2progenitor 201 ...............................................................A......................... 300
        NDV D58 HN gene 201 ..Y........................................................V..R........................G....... 300
        NDV/MU007   201 .............................................................R.VA....................V..K....... 300
        NDV/MU010   201 .............................................................R.VA....................V..K....... 300
        NDV/MU022   201 .............................................................R.VA....................V..K....... 300
        NDV/MU039   201 .............................................................R.VA....................V..K....... 300
        NDV/MU059   201 .............................................................R.VA....................V..K....... 300
        NDV/MU098   201 .............................................................R.VA....................V..K....... 300
        NDV/MU111   201 .............................................................R.VA....................V..K....... 300
                             310       320       330       340       350       360       370       380       390       400
                         ....|...*....*...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
        NDV TS09-C   301 GVGGGGSFIDNRVWFPVYGGLKPNSPSDTAQEGKYVIYKRYNDTCPDEQDYQIQMAKSSYKPGRFGGKRVQQAVLSIKVSTSLGEDPVLTVPPNTVTLMGA 400
        La Sota C5   301 ......S....S..........V.................R.........I..I.............. 400
        NDV I-2     301 ................R....................R.........I........S........... 400
        NDV4-C      301 ........................................................I.......................... 400
        I-2progenitor 301 ................R....................R.........I........S........... 400
        NDV D58 HN gene 301 ......S....S..........V.................R.........I..I.............. 400
        NDV/MU007   301 .....L..D..........A....R.....N.........VR........I.............E......... 400
        NDV/MU010   301 .....L..D..........A....R.....KN........VR........I.............E......... 400
        NDV/MU022   301 .....L..D..........A....R.....N.........VR........I.............E......... 400
        NDV/MU039   301 .....L..D..........A....R.....N.........VR........I.............E......... 400
        NDV/MU059   301 .....L..D..........A....R.....N.........AR........I.............E......... 400
        NDV/MU098   301 .....L..D..........A....R.....N.........VR........I.............E......... 400
        NDV/MU111   301 .....L..D..........A....R.....N.........VR........I.............E......... 400
                             410       420       430       440       450       460       470       480       490       500
                         ...*|....|....|....|....|....|....|....|....|....|....|....|....|..*..|....|....|....|....|
        NDV TS09-C   401 EGRVLTVGTSHFLYQRGSSYFSPALLYPMIVSNKTATLHSPYTFNAFTRPGSVPCQASARCPNSCVTGVYTDPYPLVFYRNHTLRGVFGTMLDDKQARLN 500
        La Sota C5   401 ...I..................................T..................I................I...............V.... 500
        NDV I-2     401 .......F.............................I...........I....K...........N.............V.... 500
        NDV4-C      401 ......................................................................................................... 500
        I-2progenitor 401 .......F.............................I...........I..................................V.... 500
        NDV D58 HN gene 401 ...I..................................T..................I........P.......I...............V.... 500
        NDV/MU007   401 ..................T.H.......................I........I..H....V.......NE.... 500
        NDV/MU010   401 ..................T.H.......................I........I..H....V.......NE.... 500
        NDV/MU022   401 ..................T.H......N................I........I..H....V.......NE.... 500
        NDV/MU039   401 ..................T.H......N................I........I..H....V.......NE.... 500
        NDV/MU059   401 ..................T.H......N................I........II.H....V.......NE.... 500
        NDV/MU098   401 ..................T.H......N................I........I..H....V.......NE.... 500
        NDV/MU111   401 ..................T.H......N................I........I..H....V.......NE.... 500
                             510       520       530       540       550       560       570       580       590       600
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
        NDV TS09-C   501 PVSAVFDSISRSRITRVSSSSTKAAYTTSTCFKVVKTNKTYCLSIAEISNTLFGEFRIVPLLVEILKDDGVREARSSRLSQLREGWKDDIVSPIFCDAKN 600
        La Sota C5   501 .......T.........................................................RS...........I.. 600
        NDV I-2     501 ...................A................................................................... 600
        NDV4-C      501 ......................................................................................................... 600
        I-2progenitor 501 .......H.........A................................................................... 600
        NDV D58 HN gene 501 .......T.................................................... 578
        NDV/MU007   501 .......YT.................V................NK 572
        NDV/MU010   501 .......YT.................V................NK 572
        NDV/MU022   501 .......YT.................V................NK 572
        NDV/MU039   501 .......YT.................V................NK 572
        NDV/MU059   501 .......YT.................V................NK 572
        NDV/MU098   501 .......YT....T............V................NK 572
        NDV/MU111   501 .......YT.................V................NK 572
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Fig. 4 Multiple amino acid sequence alignment of HN proteins. Alignment was performed by dividing amino acids into stabilizing and destabilizing groups. Black bold fonts marked by asterik (*) denote point variation in identity between thermostable and thermolabile viruses

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other thermostable vaccine isolates I$_2$, V4 more than those of thermolabile ones. The sialic acid binding sites were all conserved across isolates irrespective of their thermostability phenotypes. This finding is predictable since this site plays a key attachment role of the virus to cells. The presence of cysteine amino acid residue at position 123 has been reported to be essential for intramolecular disulphide bonds that stabilize the oligomeric HN structure gene [10]. Our indicated isolates had conserved cysteine amino acid at position 123 implying absence of chymotrypsin cleavage site and these could form disulphide-linked dimers increasing the hydrophobic properties of the entire HN molecule responsible for thermostability after exposure to 56 °C. The R416 essential for receptor binding, and Neuraminidase (NA) and haemagglutination (HA) activity was likewise conserved even after exposure to 56° C, as were the receptor binding site involving E401, R416 and Y526 [9]. The predicted amino acids present in the transmembrane domain of our isolates [25]FRIAVLSLIIMIL-VISVSILVY[46] which is different from less virulent thermostable I$_2$ or TS09C (Table 6). This variation could be due the virulence difference of these two categories.

The B-cell epitopes of thermostable isolates were 26 except for one isolate NDV/UG/MU/059 having 25 epitopes with four and 24 epitopes in the stalk and globular regions respectively (Table 6). This finding is consistent with earlier finding of the immunodorminant epitope concentrated in the globular region [10]. Earlier studies have confirmed that immune system responds to HN protein rather than the F gene and confirmed by use of neutralizing monoclonal antibodies with overlapping site at the HN gene. This variations of our isolates by HN gene suggests the existence of thermostable strains different from vaccine strains as already confirmed by our earlier findings using the HN phylogenetic tree analysis constructed by neighbor joining (NJ) algorithm with bootsrap along with distance.

Conclusion

From the current study, it can be concluded that field ND-virus (virulent or avirulent) circulating in LBM and waterfowl in Uganda display disparate thermostability profiles. Waterfowl provides a better source of avirulent, thermostable isolates with more than 4 logarithmic orders of HA within one hour of incubation at 56 °C and without loss of two logarithmic orders of infectivity titer within 30 min at serial passages. Chimeric NDV molecules has yielded new knowledge towards the understanding of NDV thermostability these require obtaining metastable HN molecules and performing mechanistic studies on how thermostability phenotype can be predicted by analysis of HN gene and such changes in amino acids of HN gene might

contribute to the stability of the ND-virus strain at high temperature.

Abbreviations
EID$_{50 \%}$: Median Embryo Infectivity Dose; HA: Hemagglutination test.; ND-virus: Newcastle disease virus; PBS: Phosphate buffered saline; RBCs: Red blood cells.

Availability of data and materials
This is not applicable since this is part of a large project pending settling the intellectual property issues.

Authors' contributions
JBO performed thermostability studies, analyzed data and sequences and drafted the manuscript; JBO, AW and KKM performed the virus isolation in eggs. DKB, JBO, MOO, JLN and HK designed this study; DKB, MOO planned and coordinated the study. All authors' have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
This study was part of a bigger study approved by Uganda National Council for Science and Technology under reference number HS776. This study was reviewed and approved by the College of Veterinary Medicine research and Higher Degrees Committee with regard to scientific content and ethics.

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