AVIAN DISEASES 60:779–783, 2016

Thermal Stability Study of Five Newcastle Disease Attenuated Vaccine Strains

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Received 4 May 2016; Accepted 6 August 2016; Published ahead of print 8 August 2016

SUMMARY. Newcastle disease (ND) is a big concern throughout the world because of the devastating losses that can occur with commercial and backyard poultry. The major problem in many countries is the loss of the vaccine's effectiveness due to inadequate use or storage conditions, particularly in hot climates. In the present study, stability of the five, most-used NDV vaccine strains (I-2, LaSota, B1, Clone 30 [C30], and VG-GA) was tested comparatively at different storage temperatures (4 and 37 C for the freeze-dried form and 4, 24, 37, and 45 C for the freeze-dried vaccine reconstituted in diluents). The vaccine stability was evaluated by the cumulative infectious titer drop and the theoretical shelf life at particular temperatures. Results showed that I-2 and LaSota are the most stable vaccine strains compared to B1, C30, and VG-GA; they registered the lowest titer drops and the longest shelf life whether at cool, high, or room temperatures and for both freeze-dried and reconstituted vaccines.

RESUMEN. Estudio de estabilidad térmica de cinco cepas vacunales atenuadas del virus de la enfermedad de Newcastle.

La enfermedad de Newcastle (ND) es una enfermedad que genera preocupación importante en todo el mundo debido a las pérdidas devastadoras que pueden ocurrir en la avicultura comercial y en las aves de traspatio. El principal problema en muchos países es la pérdida de efectividad de las vacunas debido al uso o almacenamiento en condiciones inadecuadas, especialmente en climas cálidos. En el presente estudio se evaluó de manera comparativa la estabilidad de las cinco vacunas más utilizadas contra la enfermedad de Newcastle (I-2, LaSota, B1, Clone 30 [C30] y VG-GA) bajo temperaturas de almacenamiento diferentes (4C y 37C para la forma liofilizada y 4C, 24C, 37C, y 45C para las vacunas liofilizadas reconstituidas en sus diluyentes). La estabilidad de la vacuna se evaluó mediante la disminución del título infeccioso acumulado y por la vida útil calculada teóricamente bajo temperaturas particulares. Los resultados mostraron que las cepas vacunales I-2 y LaSota fueron las cepas más estables en comparación con las cepas B1, C30 y VG-GA, ya que registraron las menores diminuciones en los títulos y la vida útil más larga ya sea a bajas o altas temperaturas ambientales y tanto en sus formas liofilizadas y reconstituidas.

Key words: poultry, vaccine, Newcastle, temperature, stability, infectious titer

Abbreviations: CRBC = chicken red blood cell; EID_{50} titer = egg infective dose 50%; HAT = hemagglutination test; ND = Newcastle disease

Newcastle disease (ND) is a worldwide poultry pathogen of significant economic importance that may cause mortality of 75% to 100% in unvaccinated flocks (13). Severity of the disease varies with host and virus strain. The disease is generally characterized by difficulties in breathing, with distress and gasping, marked decrease in egg production, conjunctivitis, and nasal discharge (1). However, in some circumstances high mortality without apparent signs of illness may be observed in chickens infected with extremely virulent strains (9).

Vaccination is considered the only economically feasible way to control ND endemicity. There are quite a number of conventional ND live vaccines suitable for use in commercial chickens and which are available on the international market. These vaccines have efficiently reduced the incidence of the disease in poultry farms (1,11). However, maintenance of a cold chain during storage and distribution is of big concern for all live vaccines. The disease is highly prevalent in tropical regions, where it continues to spread in poultry farms, resulting in huge economic impacts. This is partly due to the lack of biosecurity measures but also to poor storage conditions of the vaccine in hot climates and breaks in the cold chain during distribution and use. In addition, the vaccine may need to be transported for hours in countries with unreliable electric supplies and weak infrastructure (2,5).

To support the poorer communities relying on backyard poultry for their livelihood, thermostable ND vaccines are recommended. Different ND thermostable vaccines have been developed and tested successfully in many countries, even if their use in large industrial farms remains limited. Several ND thermostable strains have been described or used in different developing countries including the V4, I-2, and TS09-C (7,10,16). However, there are very few data on temperature stability under laboratory or field conditions for both conventional and thermostable vaccines.

Using residual infectivity, in the present study we compared the thermostabilty of five of the most-used NDV vaccine strains, on

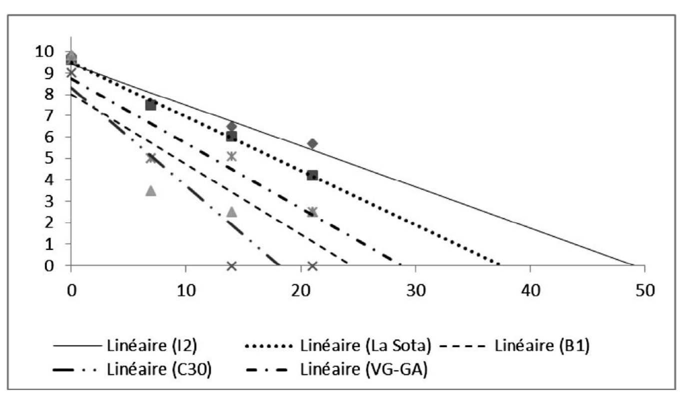

Fig. 1. Theoretical shelf life of the five NDV freeze-dried vaccines maintained at 37 C.

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All the authors have seen and approved the content and contributed significantly to the work.

Table 1. Comparative stability for five NDV freeze-dried vaccine strains maintained at 4 C for 2, 4, and 6 mo. The cumulative titer drop represents the aggregate of titer drops obtained at each incubation period.

ND strains	Infectivity titers (EID_{50}/ml)				Cumulative titer drop
	Day 0	2 Mo	4 Mo	6 Mo	
I-2	9.5	9.3	9.3	9.1	0.8
LaSota	9.6	9.3	9.1	9.1	1.3
B1	9.5	9	9.3	9	1.2
C30	9.3	9.1	9.0	9.0	0.8
VG-GA	8.6	8.6	8.8	8.5	0.3

freeze-dried and liquid forms (reconstituted), after storage or exposure to different temperatures that simulated field conditions in the tropics. The objective was to determine resistance to high ambient temperatures and propose the most appropriate vaccine strain for backyard poultries in hot climate conditions or seasons.

MATERIALS AND METHODS

Vaccine strains. The following vaccine strains were used in this study: I-2, B1, LaSota, VG-GA, and C30. As all live virus ND vaccines are produced in specific-pathogen-free eggs (9), these strains were propagated in 9-day-old embryonated chicken eggs for one passage. The allantoic fluid of each virus was harvested from infective eggs, clarified by centrifugation (1000 g for 30 min), and freeze-dried at equal volume with a stabilizer (peptone sucrose in phosphate buffer).

Storage temperature. For the freeze-dried form, the stability of the five vaccines was tested at 4 C (normal storage temperature) and at 37 C (accelerated process). The following temperatures were chosen to test stability of the five vaccines after dissolution in mineral water: 4, 24, 37, and 45 C.

Titration of infectivity. Two vials of each freeze-dried vaccine were maintained at 37 C for 7, 14, and 21 days and at 4 C for 2, 4, and 6 mo. Vial contents were reconstituted in peptone water (peptone 1% and sodium chloride 0.5%, pH 7.2) and 10-fold diluted until 10^{-8}. The different dilutions were inoculated into the allantoic cavity of 9-day-old embryonated eggs using five eggs in each dilution. Inoculated eggs were placed in an incubator at 37 C and 60% humidity. The embryos found dead before 24 hr were discarded and the allantoic liquid of the others were sampled and submitted to hemagglutination test (HAT) after 5 days of incubation. Only HAT-positive samples were considered positive for infectivity. The titer expressed by egg infective dose 50% (EID_{50}/ml) was calculated in accordance with the Reed and Muench method (12).

To test stability after dissolution of the vaccine, two vials of each freeze-dried vaccine were reconstituted in mineral water (anions: SO_4^{2-}, Cl^-, HCO_3^-, NO_3^-; and cations: $Ca2^+$, Na^+, Mg^{2+}, K^+) and stored in incubators at 37 C for 12, 24, and 36 hr and at 24 C for 4,7, and 10 days; and in a water bath at 45 C for 3, 6, and 9 hr and at 4 C for 4s, 7, and 10 days. The vials were removed at the end of exposure and titrated on eggs as described above.

Table 2. Comparative accelerated stability for five NDV freeze-dried vaccine strains maintained at 37 C for 7, 14, and 21 days.

ND strains	Infectivity titers (EID_{50}/ml)				Cumulative titer drop
	Day 0	Day 7	Day 14	Day 21	
I2	9.8	7.6	6.5	5.7	9.6
LaSota	9.6	7.5	6	4.2	11.1
B1	9.8	3.5	2.5	2.5	20.9
C30	9	5	0	0	22
VG-GA	9.6	5	5.1	2.5	16.2

Table 3. Comparative stability for five NDV freeze-dried vaccines maintained at 4 C for 4, 7, and 10 days after reconstitution.

ND strains	Infectivity titers (EID_{50}/ml)				Cumulative titer drop
	Day 0	Day 4	Day 7	Day 10	
I2	8.5	8.5	8.5	7.8	0.7
LaSota	8	7.8	7.8	7.6	0.8
B1	8.7	8.4	7.7	7.5	2.5
C 30	8.6	8.5	8.4	7.8	1.1
VG-GA	8.7	7.7	7.7	7.8	2.9

Hemagglutination test. Chicken red blood cells (CRBC) were collected with anticoagulant. CRBCs were washed and used to test for the presence of the virus in allantoic fluid by HAT: 100 µl of the fluid was added to 100 µl of 1% washed CRBCs on a white plate. After 5 min incubation at room temperature, agglutination could be observed.

Statistical analyses. Data were analyzed using the SPSS SmartViewer 20.0 software (IBM Corporation, Armonk, NY). Differences between titer drops were determined using a one-way ANOVA followed by a student *t*-test. Values of $P \leq 0.05$ were considered significant.

RESULTS

Stability of the freeze-dried vaccine at 4 C. The infectious titer of the five ND freeze-dried vaccines was evaluated at 4 C after 2, 4, and 6 mo. No significant differences were observed between the five strains, which remained stable during the 6 mo of storage ($P > 0.05$; Table 1).

Stability of the freeze-dried vaccine at 37 C. Accelerated stability of the five ND freeze-dried vaccines was evaluated by titration after incubation at 37 C for 7, 14, and 21 days. The five vaccines registered a variable decrease in their infectivity titers ($P < 0.05$). For C30 we noted an important drop at day 7 with complete inactivation after 14 days. The VG-GA and B1 strains showed an intermediate stability with cumulative titer drops of 16.2 and 20.9, respectively, after 21 days of incubation. Although the B1 vaccine showed the most-important drop of titer at day 7 (6.3 log), it remained stable within the 21 days at 37 C. I-2 and LaSota seem to be the most stable vaccines at 37 C; they lost less than 2.3 log at day 7 and registered the lowest cumulative titer drops; 9.6 for I-2 and 11.1 for LaSota after 21 days of incubation (Table 2). Figure 1

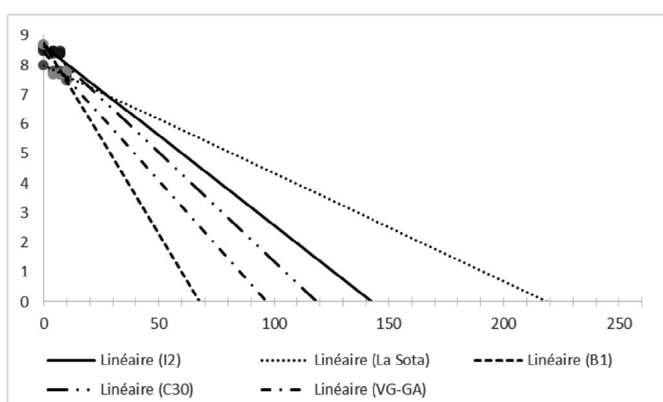

Fig. 2. Theoretical shelf life of the five NDV vaccines maintained at 4 C after reconstitution of the freeze-dried form.

Table 4. Comparative stability for five NDV freeze-dried vaccine strains maintained at 24 C for 4, 7, and 10 days after reconstitution.

ND strains	Infectivity titers (EID_{50}/ml)				Cumulative titer drop
	Day 0	Day 4	Day 7	Day 10	
I2	8.5	7.7	7.5	7.3	3
LaSota	8	7.3	7.3	6.8	2.6
B1	8.7	7	7	7	5.1
C 30	8.6	7.7	7.7	7.1	3.3
VG-GA	8.7	7.5	6.2	6.2	6.2

Table 5. Comparative stability for five NDV freeze-dried vaccine strains maintained at 37 C for 12, 24, and 36 hr after reconstitution.

ND strains	Infectivity titers (EID_{50}/ml)				Cumulative titer drop
	Hr 0	Hr 12	Hr 24	Hr 36	
I2	8.5	7.4	7.3	6.5	4.3
LaSota	8.5	7	7.1	6.5	4.9
B1	9.1	6.5	6.6	5.3	8.9
C30	9.1	7.5	7	6.5	6.3
VG-GA	8.3	7.5	6.8	5.8	4.8

shows that the I2 vaccine had the longest theoretical shelf life followed by LaSota and the other three vaccines.

Vaccine stability at 4 C after reconstitution. After dissolution of the vaccine, stability was evaluated by titration after 4, 7, and 10 days of storage. LaSota and I-2 strains were significantly different compared to the other ND vaccine strains ($P < 0.05$). They remained stable during 7 days at 4 C, registering a cumulative titer drop of 0.8 and 0.7, respectively, with the longest shelf life (220 days for LaSota and 150 days for I-2; Table 3; Fig. 2). Although the C30 strain remained stable during 7 days of storage, it showed a cumulative titer drop of 1.1 and a theoretical shelf life of only 120 days. The B1 vaccine lost 1.2 log after 10 days of storage. The VG-GA vaccine strain registered the most important titer drop (1 log) at day 4 and the highest cumulative titer drops after 10 days (2.9; Table3).

Vaccine stability at 24 C after reconstitution. The evaluation of NDV strains' stability at 24 C showed significant differences between the five strains ($P < 0.05$). After 10 days of incubation, LaSota, I-2, and C30 showed to be the most stable strains with a cumulative titer drop of 2.6, 3, and 3.3, respectively (Table 4). However, the theoretical shelf life of LaSota and I-2 vaccines seemed to be longer than C30 (Fig. 3). The B1 strain registered the most important titer drop after 4 days of incubation followed by the VG-GA strain, which registered the highest cumulative titer drop (6.2; Table 4).

Vaccine stability at 37 C after reconstitution. Accelerated stability of the five ND freeze-dried vaccines after reconstitution was evaluated at 37 C by titration after 12, 24, and 36 hr of incubation. The B1 strain was the most thermosensitive strain, showing significantly higher titer drops compared to the other vaccine strains ($P < 0.05$) and with the shortest theoretical shelf life (90 days; Table 5; Fig. 4). In contrast, the I-2 strain registered the lowest cumulative titer drops (4.3) and the longest shelf life (165 days) followed by LaSota (4.9, 165 days) and VG-GA vaccine strains (4.8, 120 days). C30 showed an intermediate level of stability with a cumulative titer drop of 6.3 (Table 5).

Vaccine stability at 45 C after reconstitution. Accelerated stability of the five ND freeze-dried vaccines after reconstitution was evaluated at 45 C by titration after 3, 6, and 9 hr of incubation. After 3 hr, the five vaccines registered a variable decrease in their infectivity titers ($P < 0.05$). As shown in Figure 5, the theoretical shelf life of C30, LaSota, VG-GA, and B1 vaccines seemed to be shorter than for I-2. However, I-2 and LaSota were the most stable strains, showing the lowest cumulative titer drop after 9, 4.4, and 5 hr, respectively. The VG-GA showed an intermediate stability level and the highest titer drops were noted for B1 and C30 strains (Table 6).

DISCUSSION

Various private pharmaceutical agencies are producing ND vaccines consisting of live, lentogenic vaccine strains such as LaSota, B1, VG-GA, and Ulster. However, these vaccines are used mainly in the commercial poultry sectors and have limited applications in rural areas due to some problems of heat lability of the vaccine strain and difficulties in maintaining the cold chains during delivery and storage (5,11). In Australia, the avirulent thermostable ND vaccine strains I-2 (4) and NDV4-HR (1,8) were developed by researchers to provide rural poultry farmers with an effective, affordable means of controlling ND in their flocks. These vaccines have been used successfully in backyard poultry in many countries in Asia and Africa (3).

Although thermostability of NDV may vary between strains, there are very few data on comparative temperature stability of

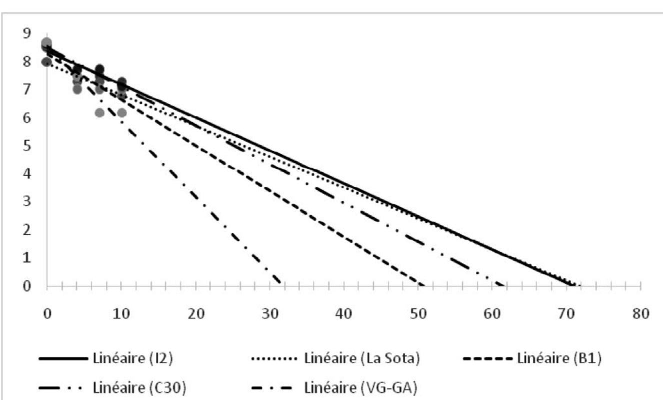

Fig. 3. Theoretical shelf life of the five NDV vaccines maintained at 24°C after reconstitution of the freeze-dried form.

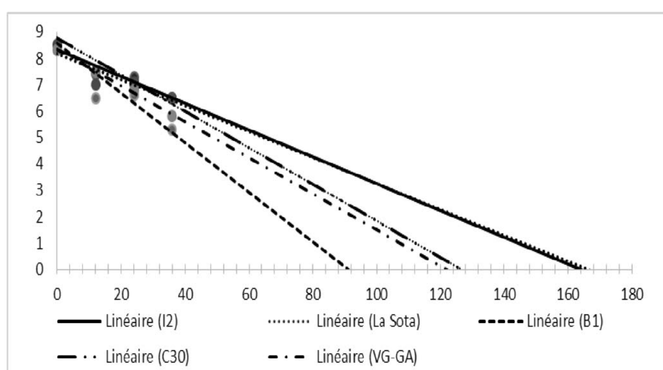

Fig. 4. Theoretical shelf life of the five NDV vaccines maintained at 37°C after reconstitution of the freeze-dried form.

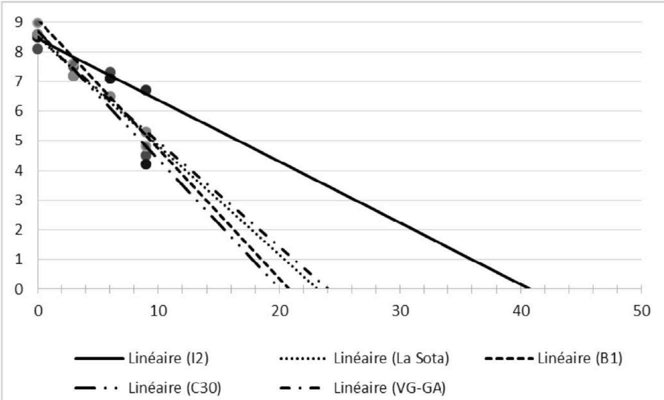

Fig. 5. Theoretical shelf life of the five NDV vaccines maintained at 45°C after reconstitution of the freeze-dried form.

conventional or thermostable vaccines. In our study, five most-used NDV vaccine strains were tested comparatively for stability at different storage temperatures (4 and 37 C for the freeze-dried form and 4, 24, 37, and 45 C for the freeze-dried vaccine after reconstitution). The vaccine stability was evaluated based on the cumulative infectious titer drops and the theoretical shelf life of the vaccine at particular temperatures. Our results showed a variable thermostability of freeze-dried and reconstituted vaccines. Indeed, I-2 and LaSota strains showed to be the most stable strains, with a great ability to resist to high temperature exposure when compared to B1, C30, and VG-GA. The tested strains are classified in Table 7 and show that the I-2 strain is the most stable, with a slight advantage over LaSota.

Our study confirms previous reports on the thermostability of the I-2 vaccine strain. In fact in Vietnam, after extensive laboratory and village trials, it has been officially recognized as the ND vaccine for village chickens (14). In previous studies it has been reported that I-2 vaccine produced in Mozambique is able to retain its activity for 8 wk at 28 C when freeze-dried and stored in the dark (1). In addition, I-2 freeze-dried vaccine has been shown to lose about 1 log of infectivity when stored for 6 days at 26–32 C (15). Also, when reconstituted after storage for 24 days at 30–35 C, this vaccine strain still produced substantial protection in vaccinated chickens (11). Our results show that the I-2 vaccine, as does the LaSota strain, has a great ability to withstand exposure to high temperature; it maintained its infectious titer for more than 3 wk when incubated at 37 C and lost about 2 log of infectivity after 7 days of incubation. After reconstitution, the I-2 vaccine lost about 2 log after 36 hr of incubation at 37 C and after 9 hr of maintenance at 45 C. The two other vaccine strains C30 and VG-GA showed an intermediate stability, and B1 was reported to be the most-sensitive vaccine strain.

Table 6. Comparative stability for five NDV freeze-dried vaccine strains incubated at 45 C for 3, 6, and 9 hr after reconstitution.

	Infectivity titers (EID_{50}/ml)				Cumulative
ND strains	Hr 0	Hr 3	Hr 6	Hr 9	titer drop
I2	8.6	7.6	7.1	6.7	4.4
LaSota	8.1	7.5	7.3	4.5	5
B1	9	7.6	7.1	4.8	7.5
C 30	8.5	7.2	7.1	4.2	7
VG-GA	8.6	7.2	6.5	5.3	6.8

Table 7. Total of cumulative infectious titer drops of the five NDV freeze-dried vaccines (FD) maintained at 37 and 4C and reconstituted (R) vaccines maintained at 4, 24, 37, and 45 C.

	Cumulative titer drops						
ND strains	FD 37 C	FD 4 C	R 4 C	R 24 C	R 37 C	R 45 C	Total
I2	9.6	0.8	0.7	3	4.3	4.4	22.8
LaSota	11.1	1.3	0.8	2.6	4.9	5	25.7
B1	20.9	1.2	2.5	5.1	8.9	7.5	46.1
C30	22	0.8	1.1	3.3	6.3	7	30.5
VG-GA	16.2	0.3	2.9	6.2	4.8	6.8	37.2

Also, it is important to note that vaccine thermostability does not necessarily depend on the vaccine immunogenicity, ease of application, transportability, and safety. In our study, we showed that LaSota and I-2 vaccines have a greater stability at higher temperature and that B1 is the least-stable strain. Nevertheless, it has been reported that LaSota strain, although it induces a strong immune response, will cause considerably greater problems in young susceptible birds (postvaccination respiratory signs) than does the B1 strain—which can be used in chickens of all ages, the same as I-2 and the heat-resistant V4 strain (6). LaSota is thus used as a booster vaccine in flocks vaccinated with I-2 or B1. In fact, there is detectable variation in the antigenicity of different circulating strains, which may indicate a need to tailor vaccines more carefully (9).

CONCLUSION

In this study, we give stability data of the five, most-used NDV vaccine strains at different storage temperatures under freeze-dried or liquid forms. The results showed that I-2 and LaSota strains are the most robust vaccines—the ones that can resist high temperatures and thus be advantageously used in the rural areas and tropical or subtropical countries.

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ACKNOWLEDGMENT

We gratefully acknowledge the support for this study by MCI (Multi-Chemical Industry) Santé Animale, Mohammedia, Morroco.