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# Vaccine quality is a key factor to determine thermal stability of commercial Newcastle Disease (ND) vaccines.

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**Highlights:**

- preparation specific factors of ND- vaccines dominated influence of thermal stability
- Infectivity of solubilized ND- vaccine withstand at 37°C for days.
- Infectivity is destroyed within hours when exposed to elevated temperatures (51°C / 61°C).
- regardless of the ND strain used, the appropriate cold chain is mandatory for live ND-vaccines

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## Abstract

Vaccination against Newcastle disease (ND), a devastating viral disease of chicken, is often hampered by thermal inactivation of the live vaccines, in particular in tropical and hot climate conditions. In the past “thermostable” vaccine strains (I-2) have been proposed to overcome this problem. In the current study, we compared the thermal stability of 6 commercially available ND vaccines. Subjected to 37°C as lyophilized preparation, two vaccines containing I-2 strains were more sensitive to inactivation than a third I-2 vaccine or when compared to three other vaccines based on different strains. However, after reconstitution strains proved to have a comparable tenacity. Interestingly, all vaccines retained a sufficient virus dose for protection ($10^6$ $EID_{50}$) after 1 day at 37°C, still. However, experiments exposing ND-vaccines to elevated temperatures of 51°C and 61°C, clearly demonstrated inactivation of all dissolved vaccines within 2 to 4 hours. The data indicate preparation that specific factors may influence thermal stability rather than strain specific characteristics. Regardless of the ND strain used, the appropriate cold chain is mandatory for live ND-vaccines.

## Introduction

Newcastle disease (ND) is one of the most devastating endemic viral diseases of poultry in many countries worldwide (Bagust, 2014; Ganar et al., 2014). The economic impact is severe, for example with estimated losses of 288,49 million US dollars annually in Bangladesh (Khatun et al., 2018), 23 million US dollars in Nigeria (Shittu et al., 2016) and 162 million US dollar in USA (Cattoli et al., 2011). It is caused by *avian orthoavulavirus 1* (AOAV-1, NDV) within a subfamily *Avulavirinae* of the family *Paramyxoviridae* (ICTV, 2019). Depending on the virulence of the virus NDV infections can cause a wide spectrum of clinical signs ranging from asymptomatic (apathogenic, lentogenic pathotypes) or mild clinical respiratory signs or drop of egg production (mesogenic pathotypes) to up to 100% mortality (velogenic pathotype) (Samal, 2011). One of the hallmarks for ND-prevention was the discovery of lentogenic NDV viruses in America (Goldhaft, 1979; Hitchner, 1975). The subsequent development of the vaccine-strains La Sota and B1 with derivatives like e.g. clone30, are the basis for the majority of ND vaccines. Some strains of NDV

isolated in Australia between 1967 and 1978, were also found to be avirulent (Simmons, 1967) and the V4 strain with the I-2 strain as its variant claiming enhanced thermostability was used to develop live ND-vaccines .

Today, ND vaccination is applied worldwide and is effective in reducing the impact of the disease. In particular for smallholder chicken productivity in the developing world, backyard chicken ND vaccination is an important tool to poultry health (Alders et al., 2010; Bessell et al., 2020). However, easy thermal inactivation of the live vaccine virus in hot climates and distant regions can affect the efficacy (Aini et al., 1990; Allan et al., 1978, Young et al., 2012). Like most live vaccines, paramyxovirus based ND-vaccines are heat labile and require a cold chain to preserve the quality of vaccines during transport and storage. Reliability of the cold chain is a challenge and temperature excursions outside the optimal temperature range are frequently observed during transport and storage (Matthias et al., 2007; Nelson et al., 2006). Inappropriate equipment, human error or power shortages are important causes of cold chain breaking (Milhomme, 1993; Setia et al., 2002). It is estimated that roughly 50% of all lyophilized vaccines are discarded annually and poor thermostability is an important contributing factor in this (Schlehuber et al., 2011).

To tackle this problem several so-called thermostable strains have been described, including the V4, TS09-C; and I-2 (Wen et al., 2013). All these strains belong, like the Ulster strain, phylogenetically to class 1 viruses, genotype I. The I-2 strain was selected based on recovery of infectious virus after exposure to 56°C for 3 hours (Bensink and Spradbrow, 1999), while the strain TS09-C was developed by serial passages of strain V4 in BHK-21 cells (Wen et al., 2012). Vaccines prepared from some of these strains claim to be safe, thermostable, immunogenic, able to spread between chickens, and suitable for delivery on food (Mazija et al., 2010; Wambura and Kataga 2011).

What determines the stability of a certain vaccine might depend on intrinsic properties of the strain, but other factors as manufacturing technology and lyophilisation process can have an equally important impact. To address this question for ND vaccines, we tested a number of commercially available freeze-dried preparations, comparing three conventional- and three so-called thermostable vaccines, containing the I-2 strain. The results revealed important differences between the vaccines and emphasize the importance of the quality of a certain vaccine preparation rather than the strain it contains.

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Studying commercially available ND vaccines will elucidate further possible reasons for vaccination failure, addressing in particular the impact of manufacturing aspects of freeze-dried vaccines. The results are important to develop successful vaccination strategies for hot climates based on appropriate supply chains, the selection of adequate ND-vaccine strains and application routes to ensure that animals will be vaccinated with sufficiently high doses of life ND vaccine virus.

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## 2 Material and Methods

For this investigation, six commercial vaccines against ND were tested, four of them claiming enhanced thermostability (A; D-F) and two not (B,C), (Table 1). Vaccine shipment was organized by GALVmed and reached the FLI in good conditions. All vaccines were used well within shelf life. Upon arrival, vaccines were stored at 4°C. Exposure to elevated temperatures (37°C, 41°C, 51°C and 61°C) was done in a water bath for the indicated periods, using either the original lyophilized material or vaccine vial diluted in 2 ml sterile distilled water (dd$H_2O$). Temperatures were chosen based on temperature in an incubator for cell cultures (37°C), analogue to body temperature of chickens (41°C) and with increase of 10°C (i.e. 51°C and 61°C). After indicated exposure times, all vials were stored at -70°C until tested for infectivity and hemagglutinating activity. As reference, lyophilized vaccines vials were kept at 4°C and tested on day 0, 7 and 21 after start of the experiment for an overview of investigated exposure time intervals at different temperature.

### Determination of infectivity

Infectivity was determined for vaccine vials diluted in 2 ml dd$H_2O$ and subsequent inoculated either on LMH-cell culture (ATCC® CRL-2117™; Kawaguchi et al., 1987) or using specific pathogen free (SPF) chicken eggs (VALO BioMedia, Germany) following standard procedures (OIE, 2008). In short, tenfold serial dilutions were prepared in cell culture medium (DMEM) and 0.2 ml of each dilution was inoculated in the allantoic sac of five 10 day old embryonated SPF-chickens eggs. After 5 days, eggs were chilled at 4°C for 2 hours and amnion-allantoic fluid (AAF) was harvested and subsequently tested for heamagglutinating (HA) activity. Eggs with HA-positive AAF but negative for bacteria were considered AOAV-1 infected.

For determination of the infectious dose in cell culture, LMH cells were freshly transferred to 96-well plates in medium supplemented with TCPK-Trypsin (2µg /ml) but without fetal calf serum. The addition of exogenous protease enabled multi-cycle replication of lentogenic vaccine strains, with the induction of multinucleated giant cells and subsequently cell necrosis. For each time point two independent tenfold virus dilutions in cell culture medium were prepared and 2x4 replicates for each

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dilution with 50µl each were transferred to the cells. Three days after infection wells were investigated by microscopy for induced cytopathic effect.

Egg infectious dose 50 ($EID_{50}$) and tissue culture infectious dose 50 (TCID50/ml) were calculated according to the method by Reed and Muench (1938). Values were normalized to vaccine dose within vaccine vials.

**Hemagglutination assay.**

All samples from different time / temperature intervals were investigated in parallel to infectivity titers for haemagglutination according standard protocols (OIE, 2008).

## 3 Results

Investigation of vaccines stored at 4°C over a period of 21 days resulted in virus titers well above $10^6$ $EID_{50}$ per dose at any time (Fig.1 B). Fluctuation of the virus-titer was within a limit of one dilution (log10), and can be considered as an indication for the variations of the biological titration system in embryonated chicken eggs. Viral titers measured by cell-culture were lower, but clearly support the notion of stability of lyophilized ND vaccines at 4°C (Fig 1A). Results of the HA units (HAU) were in line with titration as HAU remained stable for each of the vaccine over entire observation period (sTab. 1a)

At 37° C lyophilized vaccines again started out with virus-titers well above $10^6$ $EID_{50}$ per dose ($9.5x10^6$- $2.7x10^8$ $EID_{50}$/dose) (Fig. 2B). Titration on cells resulted in lower virus-titers ($3x10^5$- $9.7x10^6$ $TCID_{50}$/dose), in average observing a 36-fold difference (5-85 fold), compared to titers obtained from egg culture, (Fig. 2A). The ratio between $EID_{50}$ and $TCID_{50}$ remained within this range for the subsequent time points, with 14-, 28- and 11-fold higher $EID_{50}$ for samples from day 10, 14 and 21 after exposure (dpe) to 37° C respectively. Over this 3-week time period, $EID_{50}$ remained above $10^5$ for 4 out of the 6 vaccines tested ($1.3x10^5$-$1.3x10^6$). Infectivity of two out of three vaccines containing the I-2 strain (D and F), dropped dramatically, with vaccine F becoming negative by day 14 after exposure to 37° C, and the vaccine D with only minimal residual viral-titers in samples from day 10 to 21 ($1.4\text{-}2x10^1$ $EID_{50}$). The decay of infectivity became apparent on day 5 (vaccine F) and 10 (vaccine D) (Fig 2A). In

contrast, HA-activity remained at the same level for the entire observation period (sTab. 1b)

Testing stability for dissolved vaccines (Fig. 2 C and D), it was remarkable, that at the end of the 4-day observation period, the viral titers of 4 out of 6 viruses were above $10^5$ $EID_{50}$ ($1.1\ 10^5$ -$9.5x10^6$ $EID_{50}$) (Fig 2D). Vaccine B and F had still viral-titers of above $10^6$ $EID_{50}$, an amount that is indicated as vaccine dose ($2.7x10^6$ $EID_{50}$ and $9.5x10^5$ $EID_{50}$ respectively), while vaccine A, D and E contained $2.5x10^5$, $1.1x10^5$ and $5.8x10^4$ $EID_{50}$ per dose. Loss of infectivity higher than $10^4$ $EID_{50}$was observed only for vaccine C, resulting in a final virus-titer of $5.5x10^2$ $EID_{50}$ per dose. The same trend was evident also for the samples tested by cell culture (Fig. 2C). Again, after vaccine dissolution, HA-activity remained at the same level for the entire observation period (sTab. 1c).

Comparing the data from both experimental settings, it became apparent, that displayed loss of infectivity for vaccine D and F was particularly evident with lyophilized vaccine preparations, while losses in solution where less pronounced. To verify these results, fresh virus stocks from all vaccine strains were prepared by propagating viruses in the allantoic cavity of embryonated SPF-chicken eggs. The obtained AAF was frozen at -70° C as virus stocks. Viral-titers were determined from one aliquot and ranged between $1.3x10^8$ $TCID_{50}$ (Vaccine A) to $1.3x10^9$ $TCID_{50}$ (Vaccine D).

To test strain thermostability fresh aliquots of virus stocks were diluted with DMEM medium to give a final titer of $10^8$ $TCID_{50}$ and subsequently exposed to 37° C in a water bath. Comparable to the previous experiment with the original vaccine, loss of infectivity remained moderate for the first day from $2.0x10^7$ to $1.3x10^8$ $TCID_{50}$ representing 16-135 % residual infectivity (Fig 2F). Exception to this trend is virus-stock A, with dropping of the virus-titer down to $5.x10^6$ $TCID_{50}$ representing 0.97 % residual infectivity. However, on day 3, decay of infectivity slowed down for virus-stock A, resembling results of 4 other virus strains, with virus-titer of $1.3x10^6$ to $1.0x10^7$ $TCID_{50}$ representing 0.3-5.8 % residual infectivity (Fig 2F). At this point of time, virus-stock B showed the most prominent decline in virus-titer, dropping down to $6.x10^4$ $TCID_{50}$ representing 0,068 % residual infectivity on day 3 and becoming negative on day 5. While at day 7 after temperature exposure infectivity was present in all other vaccine virus stocks, at the end of the experiment on day 10, residual

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infectivity was detectable only in viral stocks from vaccine D, E and A with viral-titer of $3.7x10^0$, $1.4x10^2$, and $1.6x10^2$ $TCID_{50}$ respectively.

The results obtained at 37° C demonstrate that some NDV-vaccines, irrespective of the strains tested, are remarkable stable at a temperature close to physiological environment encountered during virus replication. However, in hot climates more elevated temperatures might also be relevant with live ND vaccination. Therefore the effect of exposure of vaccines and vaccine virus stocks to 51°C and 61° C was investigated. Beside the original vaccine B, viral vaccine-stocks were tested (Fig 3). It became evident that lyophilized vaccine could withstand 41°C and even 51°C for one day with still $1.5x10^5$ $TCID_{50}$ per dose (Fig 3A). At day three at 51°C, viral-titer dropped to $2.3x10^3$ $TCID_{50}$ and after that only residual infectivity was left. At 61°C infectivity declined more rapidly, but was still present on day 1 ($1.3x10^2TCID_{50}$) and 3 ($1.3x10^1$ $TCID_{50}$). Dissolved vaccine could not withstand elevated temperatures: Already at the first time point tested, i.e. 6 hours of exposure, infectivity was below detection limit (Fig 3B). In subsequent experiments, testing different fresh stocks of vaccine strains at 51°C (Fig 3C) and 61°C (Fig 3D) rapid decay was verified. Already 2 hours after exposure to 51° C residual viral-titers were between $3.7x10^1$ $TCID_{50}$ (vaccine virus B) to $1.5x10^4$ $TCID_{50}$ (vaccine virus F). After 4 hours only for vaccine virus D and E residual infectivity was detected ($3.7x10^1TCID_{50}$). At 61°C, inactivation was complete already after 2 hours, irrespective of the strain within a vaccine.

## Discussion

Vaccines are an effective tool in disease prevention, but efficacy of live vaccines is hampered by thermal inactivation in particular in hot climates (Alders et al., 2010). To minimize temperature-induced degradation, virus strains like I-2 strains that are considered thermostable, were proposed as most suitable for ND vaccination in rural areas in hot climates (Boumart et al., 2016). Studies on that subject have mainly focused on the use of different vaccine strains, but the intrinsic quality of vaccine preparations and the importance of freeze-dry technology have not been equally considered. Here we report on six commercial products that represent four vaccines claiming increased thermostability, three based on the use of the I-2 strain and one is containing an improved freezed-dry process with the LaSota strain. Two vaccines,

not claiming enhanced thermostability are based on La Sota derivatives. Results revealed that all vaccines contained well above $10^6$ $EID_{50}$ per dose in the original material and were stable as lyophilized preparation at 4°C. However, after incubation at 37°C infectivity dropped dramatically for two lyophilized vaccines containing I -2 (D and F), whereas the other four vaccines retained a viral titer above $10^5$ $EID_{50}$ until the end of the investigation period of 21 dpe. The obtained data suggested, that inactivation was not a continuous process, rather than an abrupt decay. For example for vaccine F, a sudden 1000 fold decrease became evident between four and five dpe and for vaccine D between 7 and 10 dpe. Interestingly some residual infectivity remained for vaccine D until 21 dpe, while for vaccine F no infectivity was detectable at 14 dpe, a result obtained both with the cell culture system as well as by SPF-chicken egg culture.

Our results revealed that the La Sota vaccine and vaccines based on its derivatives are relatively stable at this ambient temperature of 37°C. For La Sota this is in agreement with previous work (Boumart et al., 2016) but for Clone 30 our data contradict their findings. While in the Boumart study complete loss of infectivity of the freeze-dried clone30 was observed when tested at 14 dpe, our study resulted in a loss of only less than 1 log10 over this time period.

A possible explanation might be that in contrast to our study, Boumart and colleagues (2016) did not test commercial ready to use vaccines but freeze-dry preparations AAF using the same stabilizer for all strains specific laboratory virus preparation, thus disregarding the impact of quality and manufacturing aspects of individual manufacturers. Individual manufacturers potentially could have selected the most optimal stabilizer combinations and freeze-dry conditions depending on the strain in the vaccine and as such have considerably improved upon robustness of the vaccine preparation. This striking difference between both studies would point to the influence of manufacturing factors, which is in agreement with earlier investigations, comparing seven vaccines containing either La Sota or Hitchner B1 strains (Wood et al., 1988). Individual manufacturer potentially could have selected the most optimal stabilizer combinations and freeze-dry conditions depending on the vaccine strain and as such have considerably improved robustness of the vaccine preparation. Such differences of products may include vaccine formulation, stabilizers, methods of vaccine preparation and residual moisture of the freeze-dried pellet, factors that can

considerably influence quality on the level of vaccine batches (Pisal et al., 2006; Schlehuber et al., 2011; Kumru et al., 2014).

To elaborate further whether strain differences or manufacturing aspects were the basis of the observed differences between individual vaccines, fresh AAF stocks were prepared in the laboratory and subjected to elevated temperatures. A decrease of infectivity after 3 days was comparable to the dissolved commercial vaccines with a drop of two log10 $TCID_{50}$. But in this experiment clone30 preparation was most sensitive to heat treatment. This became even more pronounced after five dpe. At this point of time no residual infectivity was left in the clone30 stock, while all other viruses still yielded considerable infectivity. In conjunction with the first set of experiments, this indicates that the quality of the harvested AAF has an impact on stability of the virus. Altogether, it is remarkable that in five out of the six supernatants tested, NDV stayed infectious in liquid for a week when exposed to 37°C and for three preparations residual infectivity was detectable even after 10 dpe. Beside vaccine application, this high tenacity of NDV should be kept in mind concerning the spread of ND. In this respect, thermostability might still be a relevant factor. Originally used to distinguish field isolates (Lomniczi, 1975), thermostabilty was suggested early on for epidemiology studies (Hanson and Spalatin, 1978). Later it was demonstrated, that NDV variants with increased thermosstability can be recovered after heat treatment (King, 2001). Further studies on thermostability of emerging NDV genotypes could address the relevance of tenacity for the dissemination of viral populations.

The results of our study highlight that thermostability of a given vaccine does not depend solely on the used vaccine strain, but the overall excellence of the manufacturing process can be as or even more important for the quality of the vaccine. This was also clearly demonstrated by the important differences found between the 3 vaccines, all using the I-2 strain but clearly different with respect to their thermostability aspects. It is striking that two out of three vaccines based on the I-2 strain and claiming enhanced thermostability were considerably less stable at elevated temperatures compared to the other vaccines and that none of the vaccines claiming enhanced thermostability performed better compared to the vaccines containing the Clone 30 and Cl/79 strain not claiming any thermostability properties.

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A specific definition and criteria for thermostability (time, temperature, acceptable losses) in the literature and in regulatory requirements are currently lacking. It is clear from our data that for the benefit of the user, strict criteria would be urgently needed and properly controlled in case certain manufacturers want to make such claims for commercial reasons. However, our results also clearly demonstrate that enhanced thermostability or any other characteristic of a certain vaccine should be evaluated as a property of the product and should not solely rely on a part of the content of such product. In evaluating quality of a vaccine one cannot only rely on a single ingredient e.g. the strain, but every individual aspect will finally have its impact on the overall outcome. The LMH cell culture appears to be a suitable test system for such characterization process. Compared to embryonated egg culture, results in both systems gave very comparable results and were well correlated (sFig 1). The demonstrated higher sensitivity of the egg culture has still to be considered the “gold standard” for detection of residual infectivity. However, for time response studies, the cell culture system seems very suitable as it generated synchronous inactivation courses to the egg-culture results. Using the LMH test system has additional considerable benefits. Not in the least because no SPF eggs or eggs free of ND antibodies are used which not only makes the test considerably less expensive, but in some areas where SPF eggs are difficult to obtain, logistically less challenging.

From the practical point of view, the studies on temperatures above physiological range clearly show that infectivity is diminished within hours after exposure to 51°C and 61°C for all vaccines tested. This is in line with earlier observations (Rani et al., 2014) and should be considered for application of live ND vaccines. Not only in hot climates but also for hot days in moderate climates, water pipes and reservoirs might become warmed up and accelerate degradation of the ND vaccine. Thus, beside errors due to inappropriate storage temperature, human miss-handling or defective transport equipment (Matthias et al., 2007; Nelson et al., 2006), inappropriate application may account for vaccination failure. However, studies on proper vaccination in hot climates are missing. For monitoring purposes, testing whether vaccine virus is reaching the drinker nipples and subsequently the animals would be of uttermost importance to evaluate vaccination. At the end, successful vaccination depends on delivery of adequate vaccine dose to the animal. Our study clearly show,

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that regardless of the ND strain used, the appropriate cold chain is mandatory for live ND-vaccines.

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests

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### Authors' contributions

| | |
|---|---|
| Nabila Osman | Investigation; Data curation; Formal analysis; Writing - original draft |
| Danny Goovaerts | Conceptualization; Writing - editing |

| | |
|---|---|
| Serageldeen Sultan | data curation; Writing - editing |
| Christain Grund | Conceptualization; Supervision; Validation; Visualization; Writing |

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**Tab. 1: Tested live Newcastle disease vaccines**

| Code | vaccine | | strain | Dose/vial | Vol.[1] | EID[2] | TCID[2] | HAU[3] | EID/TCID | EID50/HAU |
|---|---|---|---|---|---|---|---|---|---|---|
| A | La Sota thermostable ND | Hester | La Sota | 100 | 2 ml | $4.7x10^6$ | $9.3x10^5$ | 128 | 5.1 | $3.7x10^6$ |
| B | ND Clone 30 | MSD AH | clone 30 | 1000 | 2 ml | $6.3x10^6$ | $6.3x10^6$ | 128 | 10.0 | $4.9x10^7$ |
| C | Hipraviar CLON | Hipra | CI /79 | 5000 | 2 ml | $1.3x10^7$ | $1.3x10^5$ | 512 | 84.8 | $1.2x10^8$ |
| D | ND-Kukustar | Brentec | I-2 | 500 | 1 ml | $8.3x10^7$ | $6.3x10^8$ | 256 | 65.8 | $1.6x10^8$ |
| E | Avivax ND I-2 lyophilisé | MCI Santé Animale | I-2 | 100 | 2 ml | $9.6x10^6$ | $1.3x10^6$ | 512 | 7.6 | $1.9x10^6$ |
| F | Avivax I-2 | Kevevapi | I-2 | 100 | 2 ml | $1.4x10^8$ | $4.9x10^6$ | 64 | 28.0 | $2.1x10^6$ |

1: Volume used to dissolve the vial; 2: Infection dose 50 per vaccine dose;
3: haemagglutination units in dissolved preparations

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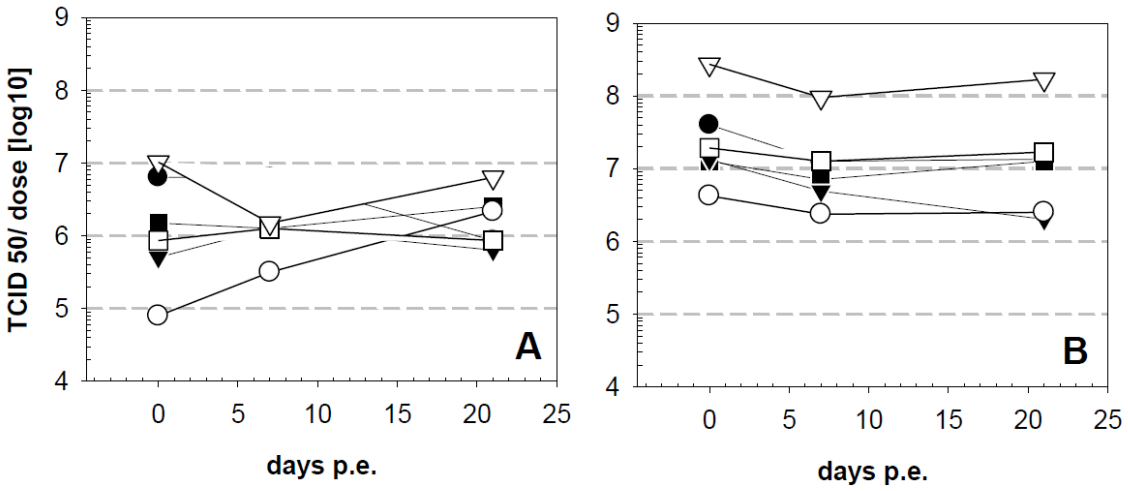


**Figure 1: Stability of commercial ND-vaccine stored at 4° C**

Lyophilized original vaccine vials were stored at 4° C and at indicated time points after the start of the experiments vials were investigated for infectivity on LMH-cells (A) and on SPF-chicken eggs (B). Symbols represent vaccine A (●), vaccine B (■), vaccine C (▲), vaccine D (○) vaccine E (□), vaccine F (Δ)

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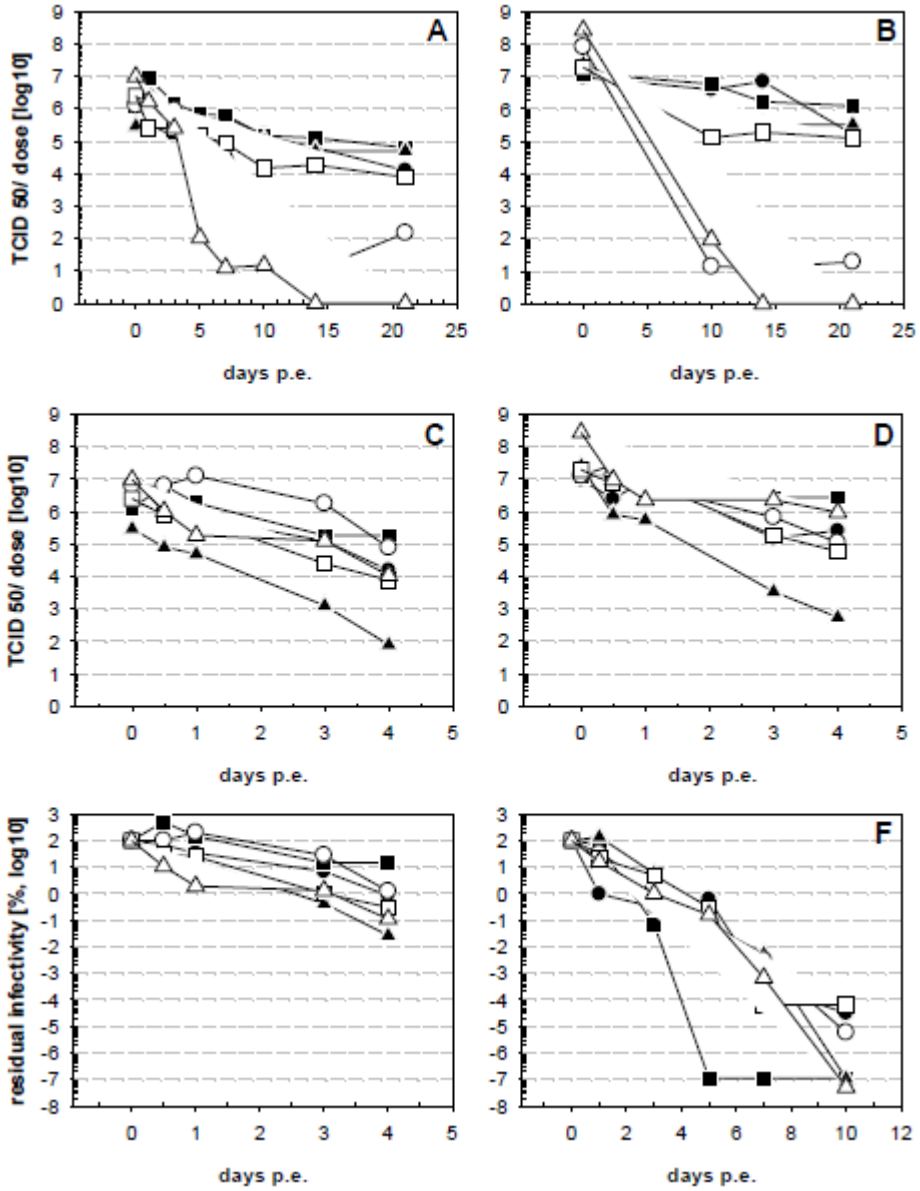


**Figure 2: Stability of ND vaccine at 37° C**

ND vaccines were exposed to 37° C in a water bath for indicated days (days p.e). In the first experiments the vaccines were incubated as lyophilized material in the original package and tested for residual infectivity using LMH-cells (A) or SPF-eggs (B). The second experiment tested vaccines dissolved in 2 ml dd$H_2O$ and determined residual infectivity with LMH-cells (C) or SPF-eggs (D). The infectivity is normalized per dose of the original package. In a third set of experiments used AAF from virus stocks generated in the laboratory in SPF-eggs were used and tested for residual infectivity on LMH-cells (F). To normalize results, residual infectivity is given as % of the original virus stock. For comparison, data from dissolved vaccines (C) are

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included as % residual infectivity (E). Symbols represent vaccine A (●), vaccine B (■), vaccine C (▲), vaccine D (○) vaccine E (□) and vaccine F (Δ)

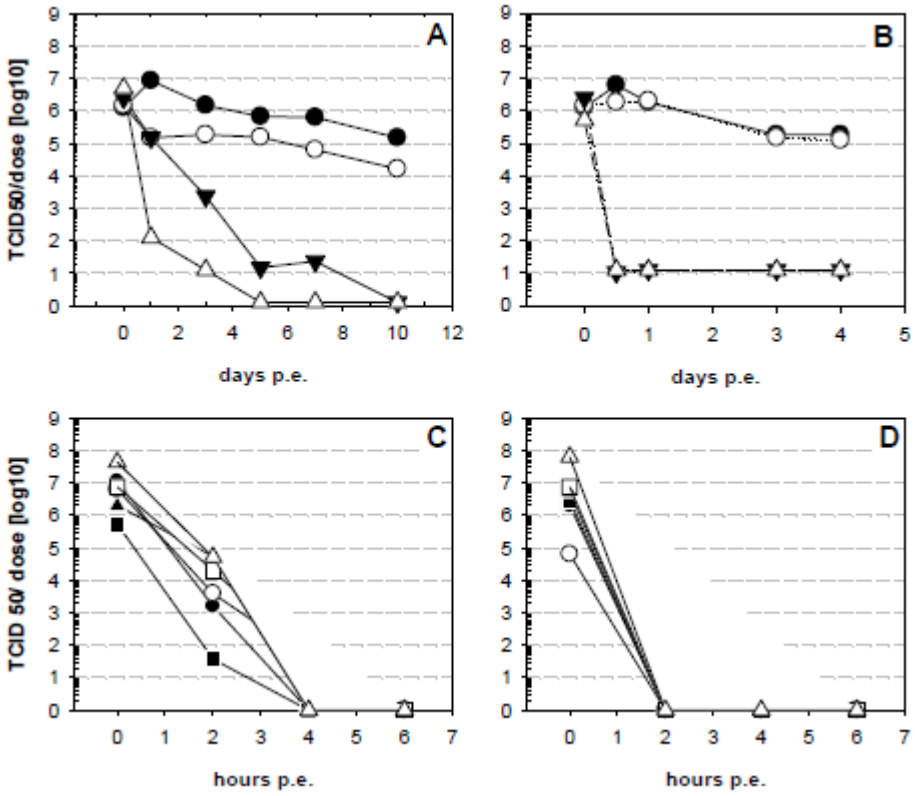


**Figure 3: Stability of ND-vaccines at elevated temperatures.**

Vaccine B was tested at elevated temperatures as lyophilized preparation in the original tubes (A) or dissolved (B) at 37°C (●), 41°C (○), 51°C (▼), 61°C (Δ). Viral titer was normalized to virus dose. In addition, prepared AAF stocks of all six vaccines were tested after exposure to 51° C (C) or at 61° C (D). Symbols represent vaccine virus A (●), vaccine virus B (■), vaccine virus C (▲), vaccine virus D (○) vaccine virus E (□) and vaccine virus F (Δ)

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**Supplemental Table 1: Stability of HAU in lyophilized ND-vaccines (A-F)**

**sTab 1c: Lyophilized ND-vaccines (A-F) at 4° C**

| | HA-Titer (log2) | | | | | |
|---|---|---|---|---|---|---|
| time (days) | A | B | C | D | E | F |
| 0 | 7 | 7 | 9 | 8 | 9 | 6 |
| 7 | 7 | 8 | 9 | 7 | 9 | 6 |
| 21 | 7 | 8 | 9 | 7 | 9 | 6 |

**sTab 1b: Lyophilized ND-vaccine (A-F) at 37° C**

| | HA-titer (log2) | | | | | |
|---|---|---|---|---|---|---|
| time (days) | A | B | C | D | E | F |
| 0 | 7 | 7 | 9 | 8 | 9 | 6 |
| 1 | 7 | 7 | 9 | 8 | 8 | 6 |
| 3 | 7 | 7 | 9 | 8 | 8 | 6 |
| 5 | 7 | 7 | 9 | 8 | 8 | 6 |
| 7 | 7 | 7 | 9 | 8 | 8 | 6 |
| 10 | 7 | 7 | 9 | 8 | 9 | 6 |
| 14 | 7 | 7 | 9 | 8 | 9 | 6 |
| 21 | 7 | 8 | 9 | 8 | 9 | 6 |

**sTab 1c: Dissolved ND-vaccines (A-F) at 37° C**

| | HA-Titer (log2) | | | | | |
|---|---|---|---|---|---|---|
| time (days) | A | B | C | D | E | F |
| 0 | 7 | 9 | 9 | 6 | 9 | 6 |
| 0,5 | 8 | 9 | 9 | 6 | 8 | 6 |
| 1 | 7 | 10 | 9 | 6 | 8 | 6 |
| 3 | 7 | 7 | 9 | 7 | 9 | 6 |
| 4 | 7 | 9 | 9 | 7 | 8 | 6 |

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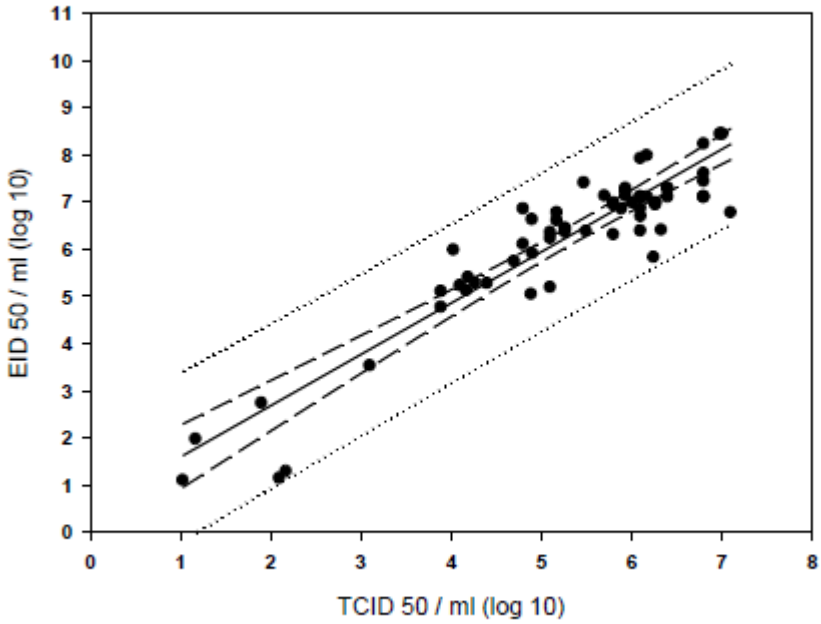


**Supplemental Figure 1: Correlation of titer obtained by egg or cell culture.** Supernatants of all lentogenic virus stocks (n= 67) were titrated in parallel in SPF embryonated eggs as well as on LMH cells. Depicted is the relation between titer as egg infectious doses (EID50) and tissue culture doses (TCID50) with the correlation as well as the 95% Confidence interval (dashed lines) and the prediction intervals (dotted lines).

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