Research paper

Onset and long-term duration of immunity provided by a single vaccination with a turkey herpesvirus vector ND vaccine in commercial layers

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A R T I C L E   I N F O

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Newcastle disease
Vaccination
Recombinant vaccine
rHVT-ND vaccine

A B S T R A C T

The onset and duration of immunity provided by a recombinant ND vaccine using HVT virus as vector (rHVT-ND) was followed up to 72 weeks of age in commercial layer chickens after single application or as part of two different vaccination regimes including conventional live and killed ND vaccines. Efficacy of the different vaccination programmes was checked, from 3 to 72 weeks of age, by serology as well as by challenges with a recent velogenic NDV isolate belonging to genotype VII. Assessment of protection was done based on the prevention of clinical signs and reduction of challenge virus shedding via the oro-nasal and cloacal routes.

Single vaccination with the rHVT-ND vaccine at one day of age provided complete or almost complete (95–100%) clinical protection against NDV challenges from 4 weeks of age up to 72 weeks of age when the latest challenge was done. Shedding of challenge virus both by the oro-nasal and cloacal route was significantly reduced compared to the controls. Booster vaccination of rHVT-ND vaccinated birds with conventional ND vaccines significantly increased the level of anti-NDV serum antibodies and further reduced the oro-nasal excretion of challenge virus.

1. Introduction

Newcastle disease (ND) is one of the most important diseases of poultry and other bird species and is a global threat to commercial poultry production. Velogenic strains of ND virus (NDV) cause a devastating disease of poultry throughout Asia, Africa, Middle East, Central and South America till today. NDV, also known as avian paramyxovirus serotype 1 (APMV-1) virus, is a member of the genus Avulavirus in the Paramyxoviridae family. NDV strains are classified into velogenic (highly virulent), mesogenic (medium virulent) and lentogenic/apathogenic (mild or non-virulent) pathotypes on the basis of their pathogenicity for chickens (Cattoli et al., 2011). The molecular basis for pathogenicity of NDV is mainly determined by the amino acid sequence of the protease cleavage site of the F protein, but other proteins (e.g., V and HN) also contribute to the determination of virulence (Dortmans et al., 2011). The principal antigens that elicit protective immune response are HN and F (Kumar et al., 2011).

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Considerable genetic diversity has been detected among NDV strains, but viruses sharing geographical and/or epidemiological relations tend to fall into specific lineages or clades. Phylogenetic analysis revealed that two major separations occurred during the history of ND. An ancient division in the original reservoir (wild waterfowl species) led to two basal sister classes, class I and II. Ancestors of only class II viruses colonized the chicken populations and subsequently converted to virulent forms. Division continued to occur in the secondary host (chicken) resulting in the branching-off class II viruses, compromising the recent velogenic genotypes (Czeglédy et al., 2006). Different genotypes of class II show geographical region specific occurrence and temporal distribution with apparent links to well defined epizootics (Miller et al., 2010). In the past decades, there has been a major shift in the genotypes of NDV strains that have been identified as prevalent in poultry.

Control of Newcastle disease, in addition to good biosecurity practices, primarily relies on preventive vaccination of flocks and culling of infected and at risk of being infected birds (protection zone). Since all ND viruses belong to a single serotype, thus by definition any NDV strain utilized to prepare a vaccine should induce protection against morbidity and mortality following challenge with any virulent NDV strains (Bwala et al., 2009; Perozo et al., 2012). Most countries, where poultry is raised commercially and where the disease is endemic, rely on vaccination to keep the disease under control. At the present time, most vaccination programmes for ND include the use of live (containing lentogenic or apathogenic NDV strains) or inactivated (killed) vaccines in order to induce a good protective immunity while producing minimal adverse effects in the birds. Both types of vaccine have their advantages and disadvantages, but the occurrence of continuous ND outbreaks in commercial poultry flocks in many part of the world indicate that routine vaccination in the field often fails to induce sufficiently high levels of immunity to control ND. Current ND vaccines widely used in commercial poultry can protect the vaccinated birds from disease and reduce virus shedding, but cannot prevent vaccinated birds from being infected and subsequently shedding the virus, and potentially transmitting it to susceptible birds (Dortmans et al., 2012). The presence of maternally derived antibodies (MDA) also interferes with the establishment of an early and persisting immunity after single or even repeated vaccination during the first 2–3 weeks of life.

A further consideration regarding conventional ND vaccines is that they might induce a better protection against viruses isolated in past epizootics than against the ones causing the recent outbreaks (Hu et al., 2009; Kapczynski and King, 2005; Miller et al., 2009, 2007; van Boven et al., 2008). The newly emerging virulent NDV strains (genogroup V and VII) have been suggested to have the ability to overcome vaccination barriers. While the causes of the apparent vaccine failures in the field have not clearly been identified in most cases, the efficacy of available conventional vaccines is being questioned based on the findings of the above referred papers. On the contrary, results of Dortmans et al. (2012) indicated that poor vaccination practices and or concurrent infection with immunosuppressive pathogens rather than antigenic variation may be responsible for poor immunity levels.

The shortcomings faced when current ND vaccines and vaccination schemes are used necessitated the search for more potent vaccines, which can be applied more efficiently in the control of ND. A promising approach to achieve the above goals is the development of vector vaccines. The first and foremost advantage for using a vector vaccine is its safety. Some live vaccines used in the poultry industry have some undesirable side-effects, such as horizontal transmission, reversion to virulence and vaccine reactions, any of which may result in disease or production loss (Alexander, 2008). With a vector vaccine, the gene(s) of the donor pathogen is inserted into a ‘safe’ vector, thus separating the key protective antigen from the live donor organism and its undesirable side-effects.

Herpesvirus of turkeys (HVT) has already been used worldwide both as live vaccine and as vector for recombinant polyvalent vaccine in poultry. Recombinant vaccines against ND using the herpesvirus of turkeys (rHVT) as vector contain and express the protective antigens, typically the F and/or HN glycoprotein (Morgan et al., 1992), HVT-based recombinant vaccine containing the F protein (rHVT/F) elicited immune response and provided protection against lethal challenge with a velogenic strain of NDV (Morgan et al., 1992). As in case of HVT itself (Purchase et al., 1971), long term virus persistence was shown for rHVT also in inoculated chickens (Reddy et al., 1996), and furthermore, the expression of the F gene was measurable even after 30 weeks of a single s.c. inoculation of day-old chickens (Saitoh et al., 2003). Additionally, the immune response evoked by the rHVT/F construct appeared to be less sensitive to interference with MDA, which adds further useful characteristic to this vector vaccine (Morgan et al., 1993). Beyond that, the application of this kind of vaccine proved to be safe since it did not have adverse effects on hatchability or the survival of in-ovo and post-hatch vaccinated specified-pathogen-free (SPF) chickens (Morgan et al., 1992; Reddy et al., 1996).

Efficacy of a commercialized rHVT-NDV vaccine (Saitoh et al., 2003) expressing the F protein of the avirulent D26/76 genotype I NDV strain (Sato et al., 1987, GenBank accession number: M24692) has already been shown in SPF layers and in commercial broilers with MDA in previous publications (Rauw et al., 2010; Palya et al., 2012). The aim of the study presented here was to evaluate the onset and duration of immunity of the same vaccine in commercial layers up to 72 weeks of age after single ND vaccination at day old or as a component in ND vaccination programmes including conventional live and killed vaccines.

2. Materials and methods

2.1. Chickens

Layers (Lohmann, Brown Lite) with MDA to NDV and MDV were purchased from a commercial hatchery. Chickens were randomly assigned to four groups according to their vaccination programme against ND. Number of day-old birds was 296, 280, 180 and 223 in groups 1, 2, 3 and 4, respectively. All groups were kept in isolated
lab-scale experimental poultry houses on deep litter. Groups 1 and 4 were kept in the same house sharing the same air space with physical separation to avoid direct contact with the drinkers, feeders and the litter of the other group. The other two groups (groups 2 and 3) were reared in isolated experimental poultry houses at two different locations (more than 10,000 m from each other) attended by different animal keepers. Biosecurity measures were in place to prevent transmission of live ND vaccine-viruses to the environment. Water and feed was provided ad libitum. Lighting parameters were set according to the management manual for Brown Lite breed provided by Lohmann. Required number of nests was introduced from the start of lay.

Preventive vaccinations of all birds against coccidiosis, infectious bronchitis (IB), infectious bursal disease (IBD), fowl pox (FP) and egg drop syndrome (EDS) were performed using commercial vaccines. Immunization against Marek’s disease with Rispens strain was applied for all groups while the rHVT-ND vaccine was used for all groups except the control group.

The animal study was conducted according to the national and European regulations.

2.2. Vaccines tested

One dose of the cell-associated rHVT-ND vaccine (Vectormune® ND, Ceva Sante Animale) was applied with its special diluent subcutaneously at the day of hatch for all ND vaccinated groups. The vaccine was back-titrated at the end of immunization procedure, when 2500 pfu/dose was measured. Live vaccine containing an apathogenic strain (Cevac® Vitapest L, Ceva Sante Animale) was applied at day old via the intra-ocular route for groups 2 and 3. Booster vaccination of group 3 at six weeks of age was performed with another live conventional vaccine based on the lentogenic LaSota strain (Cevac® New L, Ceva Sante Animale) using coarse spray application. Booster vaccination before lay (at 15 weeks of age) with an oil-emulsion trivalent killed vaccine containing ND, IB and EDS antigens (Cevac® ND IB EDS K, Ceva Sante Animale) was done subcutaneously. All conventional vaccines (live and killed) were applied in one dose according to the manufacturer’s instruction for use.

2.3. Trial design

Treatment (ND vaccinations) of the different groups and date of ND challenges is summarized in Table 1. Chicks in group 1 received only a single dose of rHVT-ND vaccine at day-old, while the chicks in group 2 were vaccinated with rHVT-ND and live vaccine at day-old, followed by booster vaccination with a killed vaccine at 15 weeks of age. The chicks in group 3 received rHVT-ND and live vaccine at hatch, then a booster vaccination with the LaSota vaccine at 6 weeks of age, followed by a second booster vaccination with a killed vaccine at 15 weeks of age.

2.4. NDV challenge and post-challenge samplings

Consecutive NDV challenges were performed from 3 to 72 weeks of age to check the onset and duration of immunity induced by the different vaccination programmes. Groups 1, 2 and 4 were included in all challenges (at 3, 4, 6, 10, 15, 25, 33, 40, 55 and 72 weeks of age), while group 3 was checked only at selected dates (at 10 weeks of age, i.e., 4 weeks after booster vaccination with the live vaccine; and at 55 weeks of age). At the earliest two challenge dates (i.e. 3 and 4 weeks of age) SPF controls of the same age were also included to validate the challenge.

The challenge strain used (D1524/1/1,2/MY/10) was isolated from Malaysia in 2010. The classification of this isolate as genotype VII strain was based on the analysis of the partial F-gene nucleotide sequence (Lomniczi et al., 1998). This strain belongs to the viscerotropic–velogenic pathotypes based on the mortality, clinical signs and gross-lesions induced in chickens. The strain caused 100% mortality in the SPF layers by 4–5 days post-infection (dpi), while in the non-immune commercial layers used in the study (group 4) the mortality reached 100% between 4 to 7 dpi (usually 5–6 dpi) after intra-nasal infection with a dose of 5.0 log10 median embryo lethal dose (ELD50)/bird.

All challenges were performed via the intra-nasal route with a dose of 5.0 log10 ELD50. Twenty chickens from each vaccinated group and twenty or ten control birds (in case of the challenges up to 15 weeks of age 20 chickens, later 10 hens) were submitted to challenge. The same dose of the virus-suspension was applied for challenge of 10 SPF-chickens at 3 and 4 weeks of age. Post-challenge observation period lasted for 14 days, during which all animals were observed for clinical signs and mortality daily. Clinical signs including listlessness, weakness, increased respiration, oedema around the eyes and head, green diarrhoea, muscular tremors, paralysis of legs and wings, and torticolis were considered as specific signs of ND. Birds dying due to aspecific causes were excluded from the evaluation. Clinical protection was calculated as the ratio of birds without ND specific clinical signs or mortality.

Swab samples were taken for the measurement of challenge virus replication and shedding from the choanal slit (oro-nasal swabs) and from the cloaca. Samplings were performed at 3 and 7 days post-challenge (dpi) after challenges at 3, 4, 6, 10, 40, 55 and 72 weeks of age. Head of the cotton swabs (EUROTUBO® collection swab, reference: 300203, Deltalab, Spain) were cut into an Eppendorf tube containing phosphate buffered saline (PBS) supplemented with a mixture of antibiotics (0.1 mg/ml gentamicin sulphate, 0.1 mg/ml colistin sulphate and 0.05 mg/ml norfloxacin; Sigma–Aldrich) right after sampling. The swabs were stored at −75 °C until further analysis.

Protection against egg drop after NDV challenge was tested only at 33 weeks of age (at the peak of egg production). Hens (30–30 birds from groups 1, 2 and 4) were transported to the challenge site at 32 weeks of age. Rearing conditions and feed was the same as in case of pre-challenge rearing sites. After an acclimatization period of 8 days, the follow up of egg production showed no persisting effect of transportation. Challenge infection was performed in groups 1 and 2, while 30 control birds of group 4 (to serve as control for the egg production) were mock-challenged and kept isolated from the vaccinated challenged groups. Further 10 hens from the control group (group 4) were transferred to another isolated site and were challenged
Table 1

Trial design.

<table>
<thead>
<tr>
<th>Group</th>
<th>ND vaccination</th>
<th>Booster (age in weeks)</th>
<th>NDV challenge dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (rHVT-ND)</td>
<td>rHVT-ND s.c.</td>
<td>–</td>
<td>3\textsuperscript{rd}, 4\textsuperscript{th}, 6\textsuperscript{th}, 10\textsuperscript{th}, 15, 25\textsuperscript{th}, 33\textsuperscript{rd}, 40\textsuperscript{th}, 55\textsuperscript{th} and 72\textsuperscript{nd} weeks of age</td>
</tr>
<tr>
<td>2 (rHVT-ND</td>
<td>live(1)&amp;killed)</td>
<td>rHVT-ND s.c., apathogenic live vaccine i.o.</td>
<td>Killed vaccine s.c. (15)</td>
</tr>
<tr>
<td>3 (rHVT-ND</td>
<td>live(1)&amp;live(2)&amp;killed)</td>
<td>rHVT-ND s.c., apathogenic live vaccine i.o.</td>
<td>Lentogenic live vaccine spray (6) killed vaccine s.c. (15)</td>
</tr>
<tr>
<td>4 (control)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\(a\) I.o. (intra-ocular) application was used for the conventional live ND vaccine and s.c. (subcutaneous) application for the vectored ND vaccine on the day of hatch.

\(b\) This challenge was dedicated to the measurement of egg drop in the vaccinated groups after challenge.

\(c\) Group 3 was challenged only at two dates: at 10 weeks of age (4 weeks post-vaccination) and at 55 weeks of age.

\(d\) Challenges when shedding measurement was included.

with the same dose of NDV to validate the challenge system. Egg production was followed daily for 13 days in the challenged groups 1–2 and the non-challenged control group. Effect of NDV challenge was evaluated by the comparison of egg production data during the pre-challenge period (from the 2nd to the 8th day of acclimatization period) with the ones egg production during the post-challenge observation period (from the 1st to the 13th day post-challenge) for each group.

2.5. rHVT-ND vaccine virus detection

Vaccine-take of rHVT-ND was checked from individual spleen samples by HVT specific real-time PCR method. Only groups 1 and 2 were sampled (group 2 was omitted from the samplings at 6 and 10 weeks of age). Number of birds included was 10/group at 2, 3, 4, 15, 19, 42 and 57 weeks of age, while at 74 weeks of age 20 hens in group 1 and 12 hens in group 2 were tested. At 6 and 10 weeks of age only 5 chickens from group 1 were tested. Samples from 42 weeks of age onwards were performed post-challenge from the survived birds. Approximately 1 cm\(^3\) of spleen was homogenized by TissueLyser II (Qiagen) after the addition of 1 ml sterile PBS. DNA was purified from the organ homogenate by QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. HVT qPCR was performed by Rotor-Gene Probe PCR Kit (Qiagen) using 2 μl purified DNA, 1 μl HVT sorf 1 F3 5’-GGG TCG TCG ACT TGG AGT TT-3’, 1 μl R3 5’-GTG TAT TTT GGC ACG GAG AT-3’ primers and 0.5 μl HVT sorf 1 probe 3’- (FAM)-TCA CAG GTG TTC CAT AGC GGG GA-BHQ1)-3’ in 20 μl final volume. Amplification and detection of specific products were done using Rotor-Gene Q real-time PCR machine (Qiagen) with the following cycle profile: 1 cycle of 95 °C for 10 min and 40 cycle of 95 °C for 15 s and 60 °C 45 s.

2.6. Serology

Serum samples were collected to measure the decay of MDA and the humoral immune response to vaccination at day-old, at 2 weeks of age and from 3 weeks of age at each challenge dates (pre-challenge samples). In case of the challenge performed at 33 weeks of age to check the effect on egg production, pre-challenge sampling was performed at the beginning of acclimatization period at 32 weeks of age.

To detect antibodies to NDV in the serum samples, both ELISA (Newcastle Disease Antibody Test kit, BioChek B.V., Holland) and haemagglutination inhibition test (HI) were used. Haemagglutination inhibition (HI) test was performed using standard method against 4 haemagglutinating unit (HAU) of LaSota antigen. Positivity limit of HI titre was set to equal or higher than 2 log\(_2\).

2.7. Challenge virus shedding measurement

Virus shedding was tested by one step RT-real-time PCR. Swab samples were vortex mixed for 10 min to improve the elution of virus containing material from the swab. Resulted samples were clarified by centrifugation (900 × g for 3 min at room-temperature). 200 μl of the supernatant was used for RNA extraction by QIAxtractor Virus kit (Qiagen) according to the manufacturer’s instructions. Two microliter of RNA was used as a template for the real-time one-step RT-PCR, amplifying a fragment of M gene (TaqMan\textsuperscript{®} NDV reagents and controls, LifeTechnologiesTM; Wise et al., 2004). Internal positive control supplied by the manufacturer was included. Ct value of the positive control in the different runs was set within a narrow range to ensure similar cut off in case of separate runs. Titre equivalent unit was calculated by extrapolation from sample Ct to the Ct of standard (total RNA extracted from tenfold dilution series of the challenge virus strain used in the trial with known titre determined by titration in embryonated SPF eggs). Results were expressed on log\(_{10}\) titre equivalent unit values (log\(_{10}\) ELD\(_{50}\) of 0.1 ml).

2.8. Statistical analysis

Statistical analysis was performed using Statgraphics Centurion software (StatPoint Technologies, Inc.). The following analyses were done: (i) HVT vaccine detection rate: number of birds positive and number of birds negative in groups 1 and 2 obtained at the different sampling dates was compared by Chi-square test; (ii) comparison of serological results obtained at the same age among the different groups: log\(_{10}\) ELISA titres and log\(_{2}\) HI titres were used for comparison of means by ANOVA, then 95% LSD test was...
used to find statistically homogeneous groups; (iii) pairwise comparison of serological results within the same group at two consecutive sampling dates: Mann–Whitney test was used; (iv) clinical protection: distribution of individuals among the different categories (protected or not protected) was compared by Fisher’s exact test; (v) challenge virus shedding: log_{10} titre equivalent units in the same type of swabs were compared between the different groups sampled at the same date by Kruskal–Wallis test (homogenous groups were found using pair-wise comparisons); (vi) pre- and post-challenge egg production within the same groups was compared by Mann–Whitney test. Trend of post-challenge egg production in the vaccinated groups was compared with the mock-challenged control group by comparison of regression lines (ANOVA for the slopes).

All statistical analyses were done at 95% confidence level (p < 0.05).

3. Results

3.1. Detection of rHVT-ND vaccine virus replication in the spleen

All tested non-vaccinated control animals proved to be negative, with no detectable virus DNA. In the ratio of positives in the vaccinated groups 1 and 2 there was no significant difference (p = 0.252). The overall ratio of positives (groups 1 and 2 together) showed fluctuation in the range of 70–100% (except a single occasion at 3 weeks of age in group 1). Although the highest positivity ratio was achieved between 4 and 19 weeks of age, there was no clear tendency of decrease in the detection rate throughout the tested period. Results are summarized in Table 2.

3.2. Humoral immune response to vaccination

Serological results obtained are summarized in Figs. 1 and 2.

3.2.1. HI test results

Level of anti-NDV MDA measured at day-old was 5.5 ± 2.6 log_{2}, which decayed to a low level by 3 weeks of age and dropped below the positivity limit of the HI test in the majority of control chickens by 4 weeks of age. No HI antibodies could be detected in the control birds from 6 weeks of age onwards. rHVT-ND vaccine alone induced low level of antibodies during the first 6 weeks of life. Although the HI test detected elevated antibody level in this group compared to the controls at 4 and 6 weeks of age, but the mean antibody level was very low, and some birds were still sero-negative at six weeks of age. However, all of these birds proved to be protected against NDV challenge (at 6 weeks of age) indicating protective immunity without the presence of detectable HI antibodies. Strong increase of HI titres was observed between 6 and 10 weeks of age and the mean HI titre exceeded 5 log_{2} by 10 weeks of age. The HI antibody titre remained at very similar level throughout the experiment up to 72 weeks of age with minor fluctuation.

Co-application of conventional live ND vaccine with rHVT-ND at day-old resulted in higher HI titres at 3, 4 and 6 weeks of age compared to the group vaccinated with rHVT-ND only. This effect was transient, at 10 and 15 weeks of age no positive effect of the conventional live vaccine co-administration at day-old was detected on the humoral immune response.

Booster vaccination with LaSota strain vaccine at 6 weeks of age increased significantly the antibody level, however this effect was transient; no difference among the vaccinated groups could be detected at 15 weeks of age (9 weeks p.v.).

Vaccination of groups 2 and 3 with killed ND vaccine at 15 weeks of age resulted in significant booster effect in both groups. Group 3 which received 2 booster vaccinations (live and killed) showed significantly higher titre increase compared to group 2. All booster vaccinated groups had significantly higher level of HI titres than the group vaccinated with rHVT-ND alone throughout the laying period. HI titres remained on the same level with some fluctuations, but no decreasing tendency up to the latest sampling date (55 weeks of age in group 3 and 72 weeks of age in groups 1 and 2) could be detected.

3.2.2. ELISA results

Non-vaccinated controls proved to be negative from 3 weeks of age onwards.

Humoral immune response induced by rHVT-ND alone could be first detected at 4 weeks of age; although the titres were far below the positivity limit of the test kit, they were significantly higher than in the non-vaccinated controls. At 6 weeks of age mean titre approached the positivity limit of ELISA, but a high percentage of birds was still sero-negative based on the positivity threshold set by the ELISA kit manufacturer. From 10 weeks of age, the vast majority of the vaccinated chickens found to be positive.

Application of live conventional vaccine together with the rHVT-ND had significant effect on the humoral immune-response during the first 6 weeks of life. The geometric mean ELISA titre of group 2 which received both the rHVT-ND and conventional live ND vaccine at day-old differed significantly from the one of group 1 that was vaccinated only with the rHVT-ND vaccine. From 10 weeks to 15 weeks of age, difference between the groups immunized with rHVT-ND only or with the combination of rVTM-ND and live vaccine at day old became negligible, statistically not significant. Booster vaccination with the live vaccine at 6 weeks of age, resulted in significantly higher antibody level in the rHVT-NDlive(1)&live(2) group, compared to the other ones, however this difference was short-lived and was not significant already at 15 weeks of age. Booster vaccination with the killed vaccine at 15 weeks of age induced significant titre increase. The increase was significantly higher in group 3 which received booster vaccination with conventional live vaccine at 6 weeks of age.

Level of ELISA titres reached the plateau by 10–15 weeks of age in the rHVT-ND alone vaccinated group and by 25 weeks of age in the booster vaccinated groups, then it remained stable throughout the rest of the laying period, with slight fluctuation.
Table 2
Detection of HVT vaccine virus by PCR in spleen samples collected at different ages.

<table>
<thead>
<tr>
<th>Group</th>
<th>HVT vaccination</th>
<th>Ratio of HVT positive spleen samples at different ages (age in weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>rHVT-ND</td>
<td>7/10</td>
</tr>
<tr>
<td>2</td>
<td>rHVT-ND&amp;live(1)&amp;killed</td>
<td>9/10</td>
</tr>
<tr>
<td>4 (control)</td>
<td>No</td>
<td>NT</td>
</tr>
</tbody>
</table>

Number of positive samples/number of tested samples.

Fig. 1. Detection of humoral immune response to different vaccination regimes based on HI test results. Same letters indicate statistically homogeneous groups (comparison of different groups sampled at the same date) above the graph. * indicates statistically significant difference between two consecutive sampling dates of the same group. Live boosted group was tested from 10 weeks of age (same results for this group are shown as the ones of rHVT-ND&live group from 3 to 6 weeks of age, since before the live booster these two groups were treated in the same way).

3.3. Efficacy against NDV challenge

3.3.1. Clinical protection

Level of clinical protection (i.e., prevention of ND specific clinical signs and mortality) measured during the consecutive challenges from 3 to 72 weeks of age is shown in Table 3. SPF control birds were included for validation of the challenge system at early challenge dates only (3 and 4 weeks of age). Effect of maternally derived antibodies on the outcome of challenge was detectable only at 3 weeks of age, resulting in 20% clinical protection in the non-vaccinated control group. All SPF controls died at
4–5 days post-challenge validating the challenge system. From 4 weeks of age onwards ND specific mortality in the commercial layer control group was 100% (4–7 days post-challenge).

Protective immunity induced by vaccination was detected as soon as the first challenge at 3 weeks of age. Similar level of protection (74–75%) was observed in all vaccinated groups. Difference in the level of protection between the vaccinated groups and the control group proved to be highly significant.

After the challenge at 4 weeks of age, only 1 bird died among the vaccinated chickens, in the group receiving rHVT-ND vaccine alone. Except this single bird, all other chickens were clinically protected in the vaccinated groups (95–100%). The bird that died had an HI titre <1:2 and very low ELISA titre (88). There were 3 further chickens in this group with similar antibody level that were completely protected against the challenge. This indicates that complete clinical protection can be achieved without detectable humoral immune response to NDV (with the serological methods used in the study).

Following the challenges performed at 6, 10, 15, 25, 33, 40, 55 and 72 weeks of age, all vaccinated birds receiving the different vaccination regimes were clinically protected against NDV challenge (group 3, receiving live booster at 6 weeks of age was challenged only at 10 and 55 weeks of age). No additional positive effect of the live vaccines (application at day-old or at day-old and 6 weeks of age) could be detected regarding clinical protection, since the rHVT-ND vaccine alone provided 100% protection.

3.3.2. Protection against egg-drop after velogenic NDV challenge

Efficacy of vaccination against egg-drop induced by NDV challenge was checked at 33 weeks of age at the peak of egg production. Results obtained during the acclimatization and post-challenge observation period are shown in Table 4 and Fig. 3. Egg production in the different groups during the acclimatization period was different (mean egg production in this period ranged between 81% and 100%) but after challenge this difference among the groups became smaller indicating different level of transportation induced stress for the birds. Since the number of hens was small and the rearing conditions were not the ones used for commercial flocks, such differences are acceptable. Effect of challenge infection was analyzed by comparison of pre- and post-challenge egg production within the same group to overcome the pre-existing differences.

### Table 3
Clinical protection against NDV challenges performed at different ages.

<table>
<thead>
<tr>
<th>Group</th>
<th>ND vaccination</th>
<th>Clinical protection at different ages (age in weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>rHVT-ND</td>
<td>74(^\text{b})</td>
</tr>
<tr>
<td>2</td>
<td>rHVT-ND&amp;live(1)&amp;killed</td>
<td>75(^\text{b})</td>
</tr>
<tr>
<td>3</td>
<td>rHVT-ND&amp;live(1)&amp;live(2)&amp;killed</td>
<td>NT</td>
</tr>
<tr>
<td>4 (control)</td>
<td>No</td>
<td>20(^a)</td>
</tr>
<tr>
<td>SPF control</td>
<td>No</td>
<td>0(^\text{b})</td>
</tr>
</tbody>
</table>

NT: not tested.
Different superscript letter indicate statistically different groups (p < 0.05).

### Table 4
Egg production during the pre-challenge acclimatization and the post-challenge observation periods.

<table>
<thead>
<tr>
<th>Group</th>
<th>Egg production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-challenge(^a)</td>
</tr>
<tr>
<td>1 (rHVT-ND)</td>
<td>81(^\text{a})</td>
</tr>
<tr>
<td>2 (rHVT-ND&amp;live(1)&amp;killed)</td>
<td>100(^\text{a})</td>
</tr>
<tr>
<td>4 (negative control)</td>
<td>92(^\text{a})</td>
</tr>
</tbody>
</table>

\(^a\) Eight days long acclimatization period was included before the challenge infection.

\(^b\) All groups contained the same number of birds (30). Post-challenge observation period was 13 days long. Negative control group was mock-challenged with PBS.

No tendency of decrease in egg production as an effect of challenge virus replication could be detected in any of the challenged groups. Comparison of egg production before and after challenge in the vaccinated groups revealed no significant reduction Result of Mann–Whitney test for group 1 was \(p = 0.001\), indicating significant increase of egg production, while for group 2 was \(p = 0.241\) indicating no significant difference between the production levels before and after challenge. By comparing the trend of production levels of the vaccinated challenged groups with the one of the mock-challenged negative control group no significant difference could be shown (ANOVA for the slope of regression lines, \(p = 0.577\)). 100% of the non-vaccinated controls succumbed to challenge.

### Fig. 3
Daily egg production during the pre-challenge acclimatization and the post-challenge observation period. First day of acclimatization was excluded due to the strong effect of previous transportation. Negative control group was mock infected at the date of challenge of vaccinated groups (on the 8th day of acclimatization).
3.3.3. **Reduction of challenge virus replication, protection against shedding**

Challenge virus replication was measured after the challenges at different ages, excluding the ones at 15, 25 and 33 weeks of age. Swab samples were taken from the choanal slit (“oro–nasal swab”) and from the cloaca at 3 and 7 dpch of the chickens which were alive, and on the day of mortality from the dead birds. Effect of vaccination was analyzed in comparison to the challenged controls at 3 dpch. Since only a few control birds survived until 7 dpch, reduction of challenge virus replication at 7 dpch is shown in comparison with the results obtained from the dead birds at 5–6 dpch in the challenged control groups (usually all control birds died from 4 to 7 dpch). There was no significant difference between the results obtained at 5 or 6 dpch from dead control birds, therefore results obtained from them at 5–6 dpch were combined for the evaluation.

Statistical analysis was performed on the basis of medians to reduce the effect of great individual heterogeneity at certain samplings and the presence of outliers. Results are shown in Figs. 4a, 4b, 5a and 5b.

Significant reduction of challenge virus replication was achieved by all vaccination programmes as early as 3 weeks of age, even when the clinical protection was still not complete. The only exception was the cloacal swabs taken 3 days after the 1st challenge, when the difference between the vaccinated groups and the control group did not prove to be statistically significant, due to the high heterogeneity of individual results. After the subsequent challenges up to 72 weeks of age the effect of vaccination(s) was more evident. Oro-nasal replication of challenge virus was suppressed less efficiently compared to the cloacal shedding by all vaccination programmes tested. Effect of vaccination with rHVT-ND alone (group 1) or in combination with live conventional vaccine at day old and boosted with killed vaccine before the start of the laying period (group 2) on the level of challenge virus excretion proved to be similar after the challenges.
performed between 3 and 25 weeks of age. However after the challenges carried out at 40 or 55 weeks of age the oro-nasal replication of the challenge virus was significantly higher in group 1 compared to group 2. Vaccination programme comprising rHVT-ND&live(1)&live(2)&killed vaccines (group 3) resulted in significantly reduced challenge virus replication at the oro-nasal site compared to the single vaccination with rHVT-ND. Difference between group 2 and 3 was smaller than the difference existing between groups 1 and 3, being significant only at 7 dpch. Comparison of results obtained during the consecutive challenges revealed stronger reduction in challenge virus excretion following the vaccination programmes that included the use of live and killed vaccines along with rHVT-ND.

Cloacal shedding was not detectable or was negligible from 6 weeks of age in all vaccinated groups, which indicates efficient immunity against viraemia and generalized replication of the challenge virus. There was no significant difference among the vaccinated groups at any sampling date regarding cloacal shedding.

4. Discussion

Commercial light brown layers with moderate level of MDA were vaccinated at day old s.c. with rHVT-ND vaccine alone, or rHVT-ND s.c. and conventional live ND vaccine by eye drop. A third group of chickens received the same vaccination at day old, but was boosted with a lentogenic live ND vaccine at 6 weeks of age. Both groups that received the conventional live ND vaccine(s) were boosted with a killed ND vaccine at 15 weeks of age.

Single application of the rHVT-ND vaccine resulted in excellent clinical protection against challenge with a recent Asian NDV isolate belonging to genotype VII from 4 weeks of age up to 72 weeks of age (95% at 4 weeks of age and 100% at all later challenges). The immunity induced by this rHVT-ND vaccine was so strong, that from 6 weeks of...
age the clinical protection against NDv challenge was not further improved by the inclusion of conventional live or inactivated ND vaccines in the vaccination programme.

Vaccination with rHVT-ND alone efficiently reduced the cloacal shedding to very limited or sometimes non-detectable level, which means 6–7 log_{10} reduction compared to the non-vaccinated controls. This result indicates very good systemic immunity which could very efficiently reduce or prevent viremic infection by the challenge virus. Oro-nasal replication of the challenge virus was also significantly reduced, however based on this parameter the efficacy of vaccination with rHVT-ND alone was weaker, resulting in detectable virus replication in the majority of vaccinated birds and 3–5 log_{10} reduction (although there were some birds at each challenge occasion in which no challenge virus replication could be detected). Local replication of the challenge virus at the oro nasal mucosa was further reduced by the booster vaccination(s): best efficacy against oro-nasal shedding was found in the group that received vaccination with both conventional live and killed ND vaccines in addition to the day-old rHVT-ND vaccination. The added value of conventional live ND vaccine administration at the time of rHVT-ND vaccination compared to the rHVT-ND-only vaccination was moderate.

Antibodies induced by the rHVT-ND vaccination alone could be detected by HI test from 4 weeks and by ELISA kit from 6 weeks of age. The antibody level elicited by the single application of rHVT-ND vaccine reached its maximum by 10 weeks of age and remained stable for 72 weeks of age covering a usual laying period. Application of live conventional vaccine at day old together with the HVT-ND significantly increased the humoral immune-response during the first 6 weeks of life, indicating a synergic effect on the stimulation of immune system by the two vaccines, which may contribute to an earlier onset of immunity to ND. Similar effect could be seen after the application of the inactivated ND vaccine at 15 weeks of age.

The rHVT-ND vaccine virus could be detected by PCR throughout the tested period (from 2 to 74 weeks of age) verifying life-long persistence of the virus in vaccinated birds and thereby ensuring strong and long lasting immunity to ND through the continuous antigen stimulus. Efficacy results obtained in this study in layer-type chickens together with the ones already published in broilers (Palya et al., 2012) indicate, that the strong immunity elicited by the rHVT-ND vaccine expressing the F gene from a genotype I NDV strain is able to provide full clinical protection along with efficient reduction of shedding even after a single application at day-old against different recent genotypes of NDV strains. The level of immunity induced by the rHVT-ND vaccine – considering antibody response, resistance to challenge as well as reduction of challenge virus shedding – was further increased by the concomitant application of a live ND vaccine at day-old, as well as by the application of a booster vaccination with live lentogenic vaccine (given by spray at 6 weeks of age) and by a killed ND vaccine administered at 15 weeks of age. The possibility of optimally controlled mass application of the rHVT-ND vaccine in ovo or s.c. to day-old chicks at the hatchery in combination with the live apathogenic ND vaccine and its characteristic to induce efficient immunity even when applied in face of maternally derived antibodies makes this type of ND vaccination programme a good choice and a promising tool for the prevention of ND.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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