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Protective efficacy of several vaccines against highly pathogenic H5N1 avian influenza virus under experimental conditions

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Summary Although several vaccines have been developed to protect against highly pathogenic avian influenza of subtype H5N1 'Asia' their efficiency has primarily been assessed individually. Thus, a direct comparison of their performance is still lacking. The following study was conducted to compare the protective efficacy of three commercially available inactivated vaccines based on influenza virus strains of subtypes H5N2 (vaccine A), H5N9 (vaccine B), and H5N3 (vaccine C), as well as two hemagglutinin expressing experimental vector vaccines (modified vaccinia virus Ankara-H5 and Newcastle disease virus-H5) against a lethal dose of highly pathogenic H5N1 avian influenza virus in chickens. To assess their potential as emergency vaccines, a single immunisation was performed for all vaccines, despite the recommendation of a double-vaccination schedule for commercial vaccines B and C. Overall, all vaccines induced clinical protection against challenge infection 3 weeks after immunisation. No mortality was observed in chickens immunised with vaccine A and viral shedding could not be detected. Immunisation with NDV-H5, vaccine C and MVA-H5 conferred also protection against lethal challenge. However, viral RNA was detected by real-time RT-PCR in swabs of 10%, 20% and 50% of animals, and 0%, 10% and 30% of animals, respectively, shed infectious virus. Immunisation with vaccine B was less protective since 50% of the vaccinated animals shed infectious virus after challenge and 20% of the chickens succumbed to disease. These results indicate that the NDV-H5 vectored vaccine is similarly effective as the best inactivated vaccine. Considering the advantage of live

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NDV which can be administered via spray or drinking water as well as the potential use of this H5 expressing vector vaccine for an easy DIVA (differentiating infected from vaccinated animals) strategy, NDV-H5 could represent an alternative for extensive vaccination against avian influenza in chickens.

Introduction

Highly pathogenic avian influenza A virus (HPAIV) of subtype H5N1 caused outbreaks in poultry in many Asian, European and African countries. In attempts to control the disease, millions of birds have been destroyed. Despite these efforts, HPAIV H5N1 has become endemic in several regions in domestic and wild birds [1,2]. This situation represents a constant threat to poultry and wild birds worldwide. The imminent danger of introduction of HPAIV into domestic poultry led to implementation of vaccination in an increasing number of countries. However, vaccination as a tool to combat HPAIV is a contentious issue. The most convincing argument against vaccination is the possibility of undetectable AIV spread under vaccination coverage within poultry resulting in endemicity rather than in eradication. Continuous circulation of AI virus in vaccinated birds may then result in antigenic drift as has been reported from Mexico [3]. However, vaccination may also serve as a tool for reduction of viral load in the environment, thus decreasing the risk of transmission within poultry and, in consequence, to humans.

To date, with the exception of a fowlpox vectored AI H5 vaccine [4], commercially available vaccines against avian influenza are exclusively adjuvanted, inactivated whole virus preparations, which have been shown experimentally to be capable to interrupt transmission of HPAI viruses [5,6]. However, in most countries vaccination is still prohibited or only allowed with extensive restrictions, since vaccination may interfere with detection of infected animals and, thus, could endanger a virus-free status obtained by immediate stamping out of diseased flocks. To overcome this problem, several different strategies have been applied to generate vaccines that allow the differentiation between infected and vaccinated animals providing the benefits of vaccination while still permitting an easy and reliable identification of infected flocks.

One DIVA strategy is based on the use of inactivated vaccines specifying the same hemagglutinin (HA) but a different neuraminidase (NA) subtype compared to circulating field viruses [7]. In this situation, field virus infection will induce an anti-NA humoral response which can be differentiated from vaccine derived NA antibodies. With the advent of plasmid-based reverse genetics systems for influenza virus [8—10] recombinant influenza viruses with desired HA and NA combinations have been constructed. H5N3 reassortants have been shown to be efficacious against HPAIV H5N1 viruses [11—13]. However, application of this strategy may be problematic, especially when multiple AIV subtypes circulate in the field. Furthermore, the diagnostic procedure to differentiate NA antibodies is based on NA inhibition and/or indirect immunofluorescence antibody tests, which are labour intensive and, therefore, not suited for mass serological surveillance.

Alternatives to whole influenza virus vaccines may be vectored vaccines typically expressing the protective HA protein, enabling a DIVA strategy based on the presence or absence of antibodies against conserved AIV proteins such as nucleoprotein (NP). ELISA tests for the detection of NP-specific serum antibodies have been developed [14,15], which represent simple assays for mass screening. Whereas whole virus influenza vaccines have to be applied in inactivated form due to safety concerns, vectored vaccines provide more safety allowing live virus vaccination at low cost, since lower amounts of antigen are needed to confer protection. Moreover, depending on the vector, mass application techniques may be feasible, which is of utmost importance in poultry to replace the individual handling of masses of birds for injection of inactivated vaccines. Several live virus vectored vaccines based on fowlpox virus [4,16,17], Newcastle disease virus [18,19], infectious laryngotracheitis virus [20] and adenovirus [21,22] expressing an H5 hemagglutinin have been shown to be effective against HPAIV in poultry.

Several avian influenza vaccines have been developed and reported to protect poultry against lethal HPAIV H5N1 challenge infection, clinical disease and, in part, also virus shedding. However, the results are difficult to interpret in comparison due to the use of different bird species and/or of different ages, varying immunisation schemes and different strains and doses of challenge viruses. Furthermore, the sensitivity of AIV detection is also relevant for a valid comparative assessment.

Thus, we performed a comparative animal trial to evaluate in parallel in a standardized experimental set-up the protective efficacy of three commercially available inactivated whole virus vaccines and two H5 expressing recombinant viruses against H5N1 HPAIV after a single immunisation in chickens.

Materials and methods

Viruses

The HPAIV isolates A/chicken/Vietnam/P41/2005 (H5N1) and A/duck/Vietnam/TG24-01/2005 (H5N1) were kindly provided by P. Song Lien (National Centre for Veterinary Diagnosis, Donga, Vietnam). The HPAIV isolate A/chicken/Italy/8/98 (H5N2) was kindly provided by I. Capua (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy). The viruses were propagated in the allantoic cavity of 10-day-old embryonated specific pathogen-free (SPF) chicken eggs. Allantoic fluid of the first egg passage was used for RNA preparation and amplification of the HA gene (AIV A/chicken/Vietnam/P41/2005 (H5N1)) and for

AIV challenge infection (AIV A/duck/Vietnam/TG24-01/2005 (H5N1)), respectively. Allantoic fluid of the second egg passage of AIV A/chicken/Italy/8/98 (H5N2) was used as antigen in hemagglutination inhibition test.

Inactivated and recombinant vaccines

Inactivated vaccines

All inactivated vaccines were adjuvanted, commercially available whole virus preparations obtained from manufacturers in Europe (Fort Dodge Animal Health, United Kingdom; Intervet International B.V., The Netherlands; Merial Italia S.p.A., Italy). They are based on field viruses of subtypes H5N2 and H5N9, or a recombinant H5N3 virus derived by genetic engineering. The latter contains a modified HA gene of A/chicken/Vietnam/C58/04 (H5N1), the neuraminidase gene of A/duck/Germany/1215/73 (H2N3) and the internal genes of A/PR/8/34 (H1N1) [12].

Modified vaccinia virus Ankara (MVA)-H5

Modified vaccinia virus Ankara is a highly attenuated strain of vaccinia virus undergoing extensive development as third generation vaccine against human smallpox. It is currently evaluated as a replication-deficient viral vector vaccine for prophylaxis of various infectious diseases including influenza [23,24, for review see 25].

Here, we used vaccine preparations based on a recombinant MVA expressing the hemagglutinin gene of influenza virus (H5N1) A/Vietnam/1194/04 (MVA-H5) [26]. Briefly, MVA-H5 was amplified in primary chicken embryo fibroblasts (CEF), purified by ultracentrifugation through sucrose, plaque-titrated in CEF, reconstituted in 1 mM Tris—HCl pH 9.0 buffer, and stored at −80 °C. Finally, MVA-H5 was administrated intramuscularly at a dose of 10^8 plaque forming units (PFU) diluted in 100 μl saline.

Newcastle disease virus (NDV)-H5

Recombinant NDV-H5 expressing the HA gene of AIV A/chicken/Vietnam/P41/2005 (H5N1) is based on the lentogenic NDV vaccine strain Clone 30 [27] and was constructed according to recombinant NDV-H5m [19]. In brief, the generation was associated with the following adaptations. Viral RNA was isolated from allantoic fluid of eggs infected with HPAIV A/chicken/Vietnam/P41/2005 (H5N1) using the QIAamp Viral RNA Mini Kit (Qiagen). The HA open reading frame (ORF) was amplified by reverse transcription polymerase chain reaction (RT-PCR) using primers Bm5sHA5hp (5′-TAT TCG TCT CAG GGA GCA AAA GCA GGG GTC TAA TCT GTC AAA ATG GAG AAA ATA GTG CTT CTT CTT GC-3′) and Bm3asHA5hp (5′-ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT GTT TTT AAT TAA AAT CTG AAC TCA CAA TTT TAA ATG CAA ATT C-3′), and subsequently cloned into vector pGem-T Easy (Promega). A transcription termination like sequence within the HA ORF was removed by mutagenesis using primers MPH5VF (5′-GAA ATA GCC CTC AAA GAG AGA GGA GGA AGA AGA GAG GAT TAT TTG GAG C-3′) and MPH5VR (5′-GCT CCA AAT AAT CCT CTC TTC TTC CTC CTC TCT CTT TGA GGG CTA TTT C-3′) (Quik Change II XL site directed mutagenesis kit, Stratagene) without altering the protein. Subsequently, primers PH5F2 (5′-CCT TCC ATG GAG AAA ATA GTG CTT C-3′) and PH5VR (5′-CCT CCT TAA GTA TAA TTG ACT TTA AAT GCA AAT TCT GCA TTG TAA CGA CC-3′) were used to generate artificial restriction sites for NcoI and AflII (underlined) flanking the HA ORF by PCR. Finally, using these restriction sites the HA ORF was inserted within the intergenic region between the genes coding for the fusion (F) and hemagglutinin-neuraminidase (HN) protein of NDV. Infectious virus was recovered as described [27,19] and propagated in 10-day-old embryonated SPF chicken eggs. The mean embryo infectious dose (EID_{50}) was calculated by the method of Kaerber [28].

Sequence analyses

Sequencing of the cloned HA ORF of AIV isolate A/chicken/Vietnam/P41/2005 (H5N1) was performed with vector-specific primers on a 3130 genetic analyzer (ABI) and analyzed using the GCG software package version 11.1.3-UNIX (Accelerys Inc., San Diego, CA).

Animal experiment

Sixty SPF white leghorn chickens (Lohmann Tierzucht GmbH, Cuxhaven, Germany) were assigned randomly to 6 groups of 10 animals. At 3 weeks of age chickens were immunised intramuscularly with 0.5 ml of either vaccine A or C, or 0.3 ml of vaccine B, or 10^8 PFU of MVA-H5, or oculonasally with 10^6 EID_{50} of NDV-H5, respectively. For the commercial vaccines, the dosage was as recommended in the manufacturer's instructions. We note, however, that for commercial vaccines B and C a double-vaccination schedule is recommended for chickens. However, we applied a single vaccination schedule to better uncover differences in efficacy between the vaccines, in particular under emergency vaccination conditions which may not allow to rely on a second vaccination 2—3 weeks after the first injection.

Three weeks after immunisation the vaccinated groups and non-immunised control birds were challenged oculonasally with 10^6 EID_{50} per animal of HPAIV A/duck/Vietnam/TG24-01/05 (H5N1). After challenge infection, the birds were observed daily for clinical signs and scored as follows: healthy (0), reduced activity (0.25), slightly ill (listlessness or slight respiratory signs: occasional coughing, gasping or sneezing (0.5)), ill (one of the following signs: dyspnoea, depression, diarrhoea, cyanosis, oedema, nervous signs (1)), severely ill (severe or more than one of the signs mentioned above (2)), or dead (3). A clinical index was calculated which represents the mean value of all chickens per group for the indicated period. Oropharyngeal and cloacal swabs were collected on days 2, 4, 7, 10 and 14 after AIV challenge infection for analysis of viral shedding. To determine the presence of AIV-specific antibodies, blood samples were obtained before immunisation, at days 10 and 20 post-immunisation, and at the end of the experiment (14 days post-challenge).

Analysis of viral shedding by real-time RT-PCR and virus isolation

The swabs were placed in 1 ml minimum essential medium containing 5% fetal calf serum (Life Technologies),

1 mg/ml enrofloxacin (Bayer), 50 μg/ml gentamicin (Gibco), and 1 mg/ml lincomycin (Ceva Tiergesundheit GmbH, Düsseldorf, Germany), and stored at −70 °C until use. RNA was extracted from swab samples on a Tecan instrument using the Nucleospin Multi 96 Virus kit (Macherey-Nagel). For detection of viral RNA real-time RT-PCR was performed based on amplification of the matrix protein gene [29] as duplex assay using a heterologous internal control [30] for verification of RNA quality and absence of inhibitory factors as described [19]. Real-time RT-PCR positive swabs were further analyzed by virus reisolation in 10-day-old embryonated SPF chicken eggs. To this end, the swab media were serially diluted, inoculated into the allantoic cavity of three eggs per dilution and incubated for 5 days or until death of the embryo, respectively. Allantoic fluids lacking hamagglutinating activity were subjected to a second egg passage. The EID_{50} was calculated by the method of Kaerber [28]. Since samples with threshold cycle (Ct) values higher than 35 often failed to yield infectious virus after egg inoculation (19, and unpublished results), only C_t values lower than 35 were counted as indicative for the presence of infectious virus.

Hemagglutination inhibition (HI) test and NP-based enzyme-linked immunosorbent assay (ELISA)

Sera were subjected to HI test according to the standard procedure described in the Commission of the European Communities Council Directive using 4 HA units [31] of AIV A/chicken/Italy/8/98 (H5N2). HI titers $\geq 3 \log_2$ were considered positive. For determination of antibodies against AIV NP sera collected at 20 days post-immunisation (p.i.) and 14 days post-challenge (p.c.) were tested in an indirect in-house ELISA as described [19] and in a commercially available ELISA (Institut Pourquier, Montpellier, France) as recommended by the manufacturer.

Statistical analyses

Data sets were analyzed by Fisher's exact test for significant differences ($p < 0.05$). The statistical tests were performed using the R Foundation for Statistical Computing (2005), R 2.1.1 (URL http://www.r-project.org) and StatSoft Inc. (2001), STATISTICA for Windows (Software-system for data analyses) Version 6.0 (URL http://www.statsoft.com).

Results

Generation of recombinant NDV-H5

Sequence analysis of the amplified HA ORF of HPAIV A/chicken/Vietnam/P41/05 (H5N1) revealed nine nucleotide (nt) differences to the sequence of this isolate given in GenBank (acc. no.: AM18672) which result in 2 amino acid (aa) substitutions (aa 8: F → L; aa 567: L → I). These two aa differences at the 5′- and 3′-ends of the HA ORF had been introduced artificially with the primers used for amplification and should not interfere with immunogenicity. Furthermore, an NDV gene end signal-like sequence was detected within the H5 ORF (nts 1023—1031). This potential transcription termination sequence was altered by silent mutagenesis since previous studies showed increased amounts of H5 full-length transcript after elimination of corresponding structures in other NDV-H5 recombinants [19]. The modified HA ORF was inserted into a full-length clone of the NDV Clone 30 genome within the intergenic region between the NDV F and HN genes flanked by non-coding regions derived from the NDV HN gene, and NDV-specific gene start and gene end signal sequences. Infectious virus was generated by transfection of the NDV-H5 full-length plasmid and helper plasmids in T7-BSR cells, which constitutively express RNA polymerase of bacteriophage T7 [32], and propagated in embryonated chicken eggs. Allantoic fluid of the second egg passage was used for determination of the EID_{50}, and subsequently for immunisation.

Protective efficacy against HPAIV H5N1 in chickens

For direct comparison of the protective efficacy of the vaccines A—C, MVA-H5 and NDV-H5, each of ten chickens per group received a single immunisation at the recommended dose and route of vaccination, even though for vaccines B and C a double vaccination schedule was recommended.

Clinical observations

None of the chickens showed any signs of illness after vaccination. Challenge infection was performed 3 weeks later with HPAIV A/duck/Vietnam/TG24-01/05 (H5N1). A high dose (10^6 EID_{50} per animal) of challenge virus was used which reproducibly induced 100% mortality of naive chickens within 2 days. Thus, as expected, all naive control animals succumbed to disease within this period (Fig. 1). In the immunised groups the majority of chickens were also

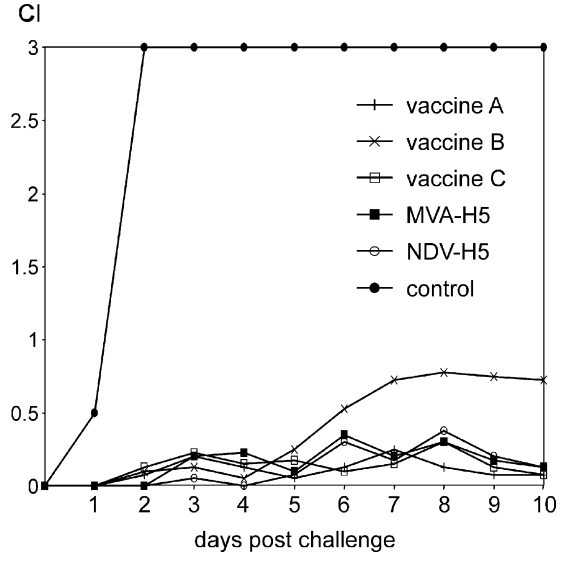

Figure 1 Daily clinical scores after challenge infection with HPAIV H5N1. The animals were observed daily for a period of 10 days for clinical signs and scored as followed: healthy (0), reduced activity (0.25), slightly ill (0.5), ill (1), severely ill (2), or dead (3). A daily clinical index (CI) was calculated which represents the mean value of all chickens per group for the given day.

Table 1 Clinical data after AIV challenge infection

Group	Mortality	Morbidity	Clinical score[a]	AIV shedding rRT-PCR/VI[b]						H5 homology (aa)[c] (%)
	2–8 days p.c.[d]	1–10 days p.c.	1–10 days p.c.	2 days p.c. (%)		4 days p.c. (%)		Total (%)		
Inact. vaccine A	0/10	7/10	0.11	0	0	0	0	0	0	84.6
Inact. vaccine B	2/10	9/10	0.40	50	20	50	30	70	50*	88.7
Inact. vaccine C	0/10	9/10	0.14	20	10	0	0	20	10	98.8
MVA-H5	0/10	10/10	0.17	30	20	50	30	50	30	99.5
NDV-H5	0/10	9/10	0.13	0	0	10	0	10	0	99.5
Control	10/10*	10/10	2.75*	†[e]	†	†	†	†	†	

(*) denotes a significant difference to the other groups ($p < 0.05$).
[a] Clinical score (0, healthy; 0.25, reduced activity; 0.5, slightly ill; 1, ill; 2, severely ill; 3, dead).
[b] AIV shedding determined by real-time RT-PCR (rRT-PCR) and virus isolation (VI).
[c] Amino acid (aa) homology of H5 genes of vaccines and challenge virus.
[d] Days post-challenge.
[e] Death of the chickens.

affected to some extent (Table 1). However, in most cases no classical symptom of HPAI infection but only a reduced activity and mild listlessness was observed mainly between days 3 and 8 after challenge. Mild respiratory signs or mild depression were observed in two to four animals per group. Consequently, the mean daily clinical indices (Fig. 1) as well as mean clinical indices for a 10 days period of 0.11–0.17 (Table 1) were rather low for the groups immunised with vaccines A or C, MVA-H5 and NDV-H5. However, two vaccine B immunised chickens developed severe depression and diarrhoea and died on day 7 and 8 p.c., respectively. Consequently, this group yielded a higher clinical index of 0.4 (Table 1). In comparison, the non-vaccinated control animals showed a mean clinical index of 2.75 (Table 1). Thus, in our experimental setting, vaccines A and C, as well as MVA-H5 and NDV-H5 induced similar clinical protection. Due to the low number of animals which were used for animal welfare reasons, the apparently lower protection afforded by vaccine B could not be substantiated by formal statistical evaluation.

Serological analyses

All prevaccination sera were negative for H5 antibodies in the HI test. Although the viral antigen used in the HI test carries a heterologous H5 to all vaccines (Table 2) and comparability may thus be restricted in terms of absolute titers induced by immunisation, the results are useful to show a tendency with regard to onset and level of humoral immunity. Therefore, we did not statistically analyse differences in H5-specific antibody levels, but simply give the results.

Whereas all MVA-H5 and NDV-H5 immunised chickens had developed H5-specific antibodies already 10 days after immunisation, vaccination with vaccine C had not induced any detectable levels of H5-specific antibodies at this time point. After immunisation with vaccines A and B about half of the chickens had detectable H5-specific antibodies (Table 2). The mean titers of 4.4 and 4.9 $\log_2$ of MVA-H5 and NDV-H5 immunised animals were higher than the mean titers in sera of chickens immunised with inactivated vaccines A and B of 3.3 and 3.0 $\log_2$, respectively (Table 2). However, at 20 days p.i. the situation changed, since in sera of birds

Table 2 H5-specific antibodies after immunisation and challenge infection

Group	HA-specif. Ab HI-titer		H5 homology (aa)[a] (%)	HA-specif. Ab HI titer
	10 days p.i.[b]	20 days p.i.		14 days p.c.[b]
Inact. vaccine A	6/10 $2^{3.3}$	10/10 $2^{7.3}$	89.1	10/10 $2^{8.7}$
Inact. vaccine B	5/10 $2^{3.0}$	10/10 $2^{6.1}$	92.7	8/8 $2^{6.5}$
Inact. vaccine C	0/10 –	10/10 $2^{4.6}$	93.5	10/10 $2^{5.7}$
MVA-H5	10/10 $2^{4.4}$	10/10 $2^{4.3}$	94.2	10/10 $2^{7.2}$
NDV-H5	10/10 $2^{4.9}$	9/10 $2^{4.9}$	93.8	10/10 $2^{4.9}$
Control		0/10 –		†

[a] Amino acid (aa) homology of H5 genes of vaccines and isolate AIV A/chicken/Italy/8/98 (H5N2) used as antigen in HI test.
[b] The time scale differentiates between days post-immunisation (p.i.) and post-challenge (p.c.).

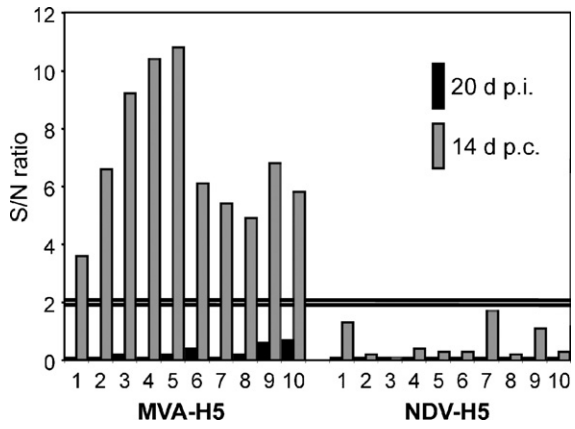

Figure 2 Serological examinations by an indirect NP-ELISA. The obtained S/N (optical density of sample/optical density of negative control) ratios were plotted for sera of chickens immunised with MVA-H5 and NDV-H5 before and after challenge infection (20 days p.i. and 14 days p.c., respectively). The cut-off value of 2.0 is marked by a double line.

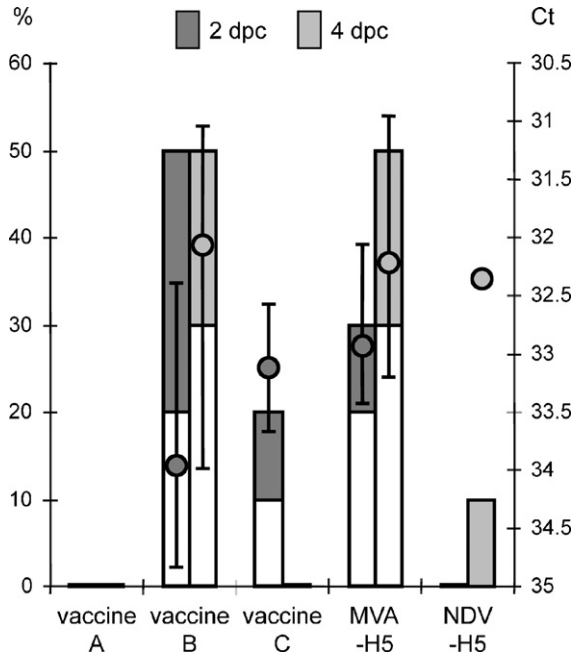

Figure 3 Analyses of viral shedding by real-time RT-PCR and virus reisolation. Left scale: the percentages of viral RNA positive swabs per group are given for day 2 p.c. (dark grey) and day 4 p.c. (light grey). The fraction of swabs containing infectious virus are marked in white. Right scale: the mean threshold cycles (C_t) of positive swabs per group as relative values of viral shedding are given for day 2 p.c. (dark grey circle) and day 4 p.c. (light grey circle). The maximum and minimum C_t values per group and day are given.

immunised with inactivated vaccines mean titers reached a comparable level (4.6 $\log_2$, vaccine C) or exceeded (7.3 $\log_2$, vaccine A and 6.1 $\log_2$, vaccine B) the titers of MVA-H5 and NDV-H5 vaccinated animals (Table 2). Despite exhibiting the lowest aa homology to the used H5 antigen, vaccines A and B induced relatively high H5-specific antibody titers. With respect to early onset of immunity, the live vaccines were apparently superior to the inactivated preparations.

A further increase of the mean H5-specific antibody titers after challenge infection was observed in surviving animals of the groups immunised with vaccines A and C as well as MVA-H5 (Table 2). The most pronounced increase in antibody titer after challenge was observed in MVA-H5 immunised birds (4.3—7.2 $\log_2$, Table 2). HI titers in sera of chickens immunised with vaccine B increased only slightly (Table 2). It is notable that in this group two animals succumbed to disease, which, thus, did not contribute to the mean HI titer post-challenge. Only a minimal increase was seen in NDV-H5 immunised chickens with a constant mean HI titer of 4.9 $\log_2$, which was present in 9 of 10 chickens before and in all 10 chickens after challenge infection (Table 2).

Serological investigations were also performed to determine AIV NP-specific antibodies before and after challenge infection. As expected, NP-specific antibodies were detectable in sera of all chickens vaccinated with whole virus preparations already after immunisation (data not shown). In contrast, NP-specific antibodies were absent in MVA-H5 and NDV-H5 immunised chickens (Fig. 2). After challenge infection all MVA-H5 immunised birds developed NP-specific antibodies. Surprisingly, none of the NDV-H5 immunised chickens showed detectable levels of NP antibodies. The data were analyzed by an in-house ELISA (Fig. 2) and reconfirmed by a commercially available ELISA system.

Analyses of viral shedding

RNAs extracted from swab samples were analyzed by real-time RT-PCR for presence of viral RNA. Birds, which had been immunised with vaccine A, did not yield any positive result from the collected swabs. In contrast, viral RNA could be detected in 10% of NDV-H5 immunised chickens at 4 days p.c., in 20% of vaccine C immunised birds at 2 days p.c., and in 50% of MVA-H5 and 70% of vaccine B immunised chickens until day 4 post-challenge (Fig. 3, Table 1). At later time points no viral RNA could be observed in any of the immunised animals. Under ideal conditions, in real-time RT-PCR the amount of amplicon increases about 10-fold every three cycles [33]. Since the mean Ct values observed ranged between 32.1 and 34.0, all groups shed virus at a comparable level. Whereas in NDV-H5 immunised chickens viral RNA was detected in a single cloacal swab, in the other groups viral RNA could be observed only in oropharyngeal swabs.

Subsequently, swab samples, which tested positive in the real-time RT-PCR, were subjected to virus reisolation in embryonated chicken eggs. Out of the five, two and three viral RNA positive swabs in birds immunised with vaccines B, C and MVA-H5, respectively, in two, one and two swabs infectious virus could be detected on day 2 p.c. (Fig. 3, Table 1). At 4 days p.c., challenge virus was reisolated in three out of five RT-PCR positive swabs from birds immunised with vaccine B or MVA-H5 (Fig. 3, Table 1). No infectious virus could be reisolated from the single RNA positive swab of a bird vaccinated with NDV-H5 (Fig. 3, Table 1). The mean EID_{50}/ml of all positive swabs per group ranged at both days in all groups between $10^{0.9}$ and $10^{1.4}$. The highest value could be observed on day 2 p.c. in a swab of a bird immunised with vaccine B ($10^{1.5}$ EID_{50}/ml), whereas infectious virus could be

detected after a second egg passage in one swab of a bird immunised with MVA-H5 at 4 days p.c. In summary, the viral titers observed were rather low and within the same range in birds of all groups. The higher amount of swab samples that contain viral RNA as compared to the recovery of infectious virus is most likely due to the higher sensitivity of real-time RT-PCR analyses.

Taken together, there was no significant difference between groups regarding the magnitude of viral shedding, although the number of birds that shed virus differed. In keeping with the results from clinical protection, the group immunised with vaccine B exhibited a significantly higher number of birds shedding challenge virus (Table 1).

The combined results of protective efficacy based on clinical index (CI) and percentage of animals per group shedding infectious virus (s/g) resulted in the following ranking: vaccine A (CI: 0.11; s/g: 0%), NDV-H5 (CI: 0.13; s/g: 0%), vaccine C (CI: 0.14; s/g: 10%), MVA-H5 (CI: 0.17; s/g: 30%) and vaccine B (CI: 0.4; s/g: 50%). However, with the exception of a statistically significant higher percentage of viral shedders in the vaccine B group, no significant differences of protective efficacy could be demonstrated by formal statistical analyses between the other immunised groups.

Discussion

The objective of this study was to evaluate in a comparative setting the protective efficacy of several H5 avian influenza vaccines in chickens against H5N1 HPAIV. In this study we included three commercially available inactivated whole virus vaccines, and the experimental MVA-H5 and NDV-H5 as prototypes of H5 expressing vectored vaccines. Although vaccines B and C are recommended to be administered twice, to follow our standardized protocol we performed a single vaccination schedule. However, we followed the routes and doses as recommended by the manufacturers. This serves to highlight differences in efficacy and to assay for early onset of protection which is of utmost importance in emergency situations. After challenge infection with a high dose of HPAIV A/duck/Vietnam/TG24-01/2005 (H5N1) protective efficacy was evaluated based on clinical observations and the magnitude of viral shedding. From our results the tested vaccines can be ranked in the order vaccine A, NDV-H5, vaccine C, MVA-H5 and vaccine B. However, the differences are not evident by formal statistical analyses with exception of a higher percentage of viral shedders in the vaccine B group. Furthermore, it would be of interest, if challenge infection with alternative H5N1 strains would confirm our ranking.

From the tested vaccines, vaccines A and C, MVA-H5 and NDV-H5 were basically equivalent as concerns protection from clinical disease after challenge since all animals survived the challenge infection and the mean clinical scores over a 10 days period varied only between 0.11 and 0.17. Furthermore, these low clinical scores are due to unspecific general signs such as reduced activity rather than typical symptoms of HPAI. However, since there was no indication for other causes of these health problems, we considered them as effects of the challenge infection. In contrast, two animals, which had been immunised with vaccine B, succumbed to the challenge.

The highest overall protection was obtained by vaccine A, the only vaccine where neither virus shedding nor viral RNA could be detected in swabs of immunised birds under our experimental conditions. Remarkably, the HA of vaccine A exhibited the lowest homology to the HA of the challenge virus. However, the HA homology was calculated based on partial sequence data only, and therefore might be somewhat inaccurate. Although vaccine A exhibits also the lowest HA homology to the antigen used in the HI test, immunised chickens showed a mean H5-specific antibody titer of 7.3 $\log_2$ at the time of challenge infection, which indicates an efficient induction of the humoral immune response. However, we note, that a direct comparison of absolute levels of HI titers may not be valid on the basis of the heterologous antigen used in HI test and the heterogeneity of H5 proteins in the different vaccines.

Vaccine B also exhibits a rather low HA homology (88.7%) to the challenge virus. Despite the induction of mean H5-specific antibody titers of 6.1 $\log_2$, two of ten immunised chickens did not survive the AIV challenge. Vaccine C with a high HA homology of 98.8% to the challenge virus protected chickens from lethal challenge, although only a low mean H5-specific antibody titer of 4.6 $\log_2$ was induced after immunisation which indicates that mean HA-specific antibody titers should not be the only criterion for estimating protection. Thus, although a good match between vaccine and field virus HA is certainly beneficial, as can be deduced from the comparison between vaccines B and C, the fact that the highest level of protection was conferred by vaccine A supports that other factors contribute to the ability to confer protection. Important factors are, e.g., the antigenic mass and/or the quality of the adjuvant used [34,35]. Thus, the observations support previous results indicating that a high HA homology to field viruses is not the sole decisive factor for inactivated avian influenza vaccines [36,37].

The experimental vector vaccines MVA-H5 and NDV-H5 conferred solid protection against lethal challenge, even though only rather low H5-specific antibody titers of 4.3 and 4.9 $\log_2$, respectively, were induced in immunised chickens. In contrast to MVA-H5 immunised animals, no shedding of infectious virus was observed in NDV-H5 immunised birds. This could be due to local and/or cellular immunity induced by oculonasal application of NDV-H5. In addition, there is little experimental experience on the use of recombinant MVA vaccines in chicken and the impact of dosage and replication deficiency of MVA-H5 still needs to be evaluated in more detail. Whereas the role of local immunity in chickens has been studied for respiratory pathogens like NDV [38,39], there is a lack of knowledge about the local immune response to HPAI viruses. However, it can be assumed that mucosal immunity plays an important role in the defense against infection, as the mucosal tissue of the respiratory tract is the main site of entry for influenza viruses. The failure of detection of viral shedding in NDV-H5 immunised chickens and the detection of viral RNA in only a single cloacal swab in comparison to the other groups, in which challenge virus could be detected exclusively in oropharyngeal swabs, also argues for protective local immunity in the upper respiratory tract. Interestingly, there was no indication of seroconversion in NDV-H5 immunised animals after challenge, as there was no amnestic response based on HI titers post-challenge. However, there was also no obvious

amnestic response in chickens immunised with vaccine B, although half of these chickens shed challenge virus. Thus, also the post-challenge HI titers have to be interpreted with caution. However, absence of productive virus replication in NDV-H5 immunised animals was supported by the failure to detect antibodies against the AIV nucleoprotein. Insufficient sensitivity of the in-house ELISA used was excluded by verifying the absence of NP-specific antibodies by a second, commercially available ELISA. The absence of seroconversion to AIV NP in NDV-H5 immunised birds indicates a very high protective efficacy in this experimental setting. The efficiency of the NDV-H5 recombinant may be due to a close HA sequence homology of 99.5% between vaccine and challenge virus, since NDV-H5 recombinants expressing H5 with lower homology of about 94% were less capable to prevent mortality (unpublished results). In contrast to inactivated adjuvanted vaccines, high HA sequence homology between vaccine and field virus appears to be important for protection conferred by HA expressing vectored vaccines. Similar positive correlations between H5 homology of vaccine and challenge virus and protective efficacy have been reported also for other H5 expressing vector vaccines, based on fowlpox and infectious laryngotracheitis virus, respectively [4,20].

The present study did not consider duration of immunity, another important aspect of vaccine efficacy. An inactivated H5N1 vaccine has been shown to prevent clinical disease and to reduce virus excretion after challenge for up to 10 months [40], which has also been postulated for a fowlpox vectored vaccine [41]. However, the advantage of live virus vaccination resides in its capability to induce a faster onset of immunity, since both live vectored vaccines used in this study induced higher humoral antibody levels than the inactivated preparations at an early time point after vaccination. This is particularly important in an emergency vaccination.

Besides identifying vaccine A as the most immunogenic and effective vaccine in our trial, the data also demonstrate the efficacy of the experimental NDV-H5 vector vaccine. Thus, the NDV-H5 recombinant could represent an alternative when extensive vaccination against avian influenza is intended in chickens, since the NDV-based vectored vaccine is suitable for mass application via aerosol or drinking water and the parental strain Clone 30 (which has been derived from the lentogenic strain La Sota) is already in widespread use for a long time to protect chickens against ND. A similar H5 expressing NDV recombinant is supposedly already in use in China, and a recombinant fowlpox-AI vaccine is licensed in several countries and widely used in Mexico. It has been shown that protection of fowlpox virus vectored vaccine varied significantly after application to chickens which had previously been exposed to the vector virus [42]. Whether this also applies to NDV-H5 in NDV preimmunised chickens has to be determined. Prime-boost schemes using different viral vectors suitable for mass application, such as NDV and ILTV could overcome this problem if it arises.

Nevertheless, NDV-H5 could represent a potent bivalent vaccine to be used in prophylactic vaccination programs against avian influenza and ND. Recently, a live attenuated as well as a vector virus-based H5 vaccine has been shown to be suitable for *in ovo* immunisation [22,43], representing a new promising approach for vaccination of poultry against avian influenza. However, all these vaccines have advantages and disadvantages. Thus, a spectrum of effective vaccines is highly desirable and licensing of available vaccines should be promoted to supplement and expand current intervention strategies against avian influenza consistent under different epidemiological situations.

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