AVIAN DISEASES 56:913–922, 2012

Efficacy of rHVT-AI Vector Vaccine in Broilers with Passive Immunity Against Challenge with Two Antigenically Divergent Egyptian Clade 2.2.1 HPAI H5N1 Strains

F. Rauw,[AE] V. Palya,[B] Y. Gardin,[C] T. Tatar-Kis,[B] K. Moore Dorsey,[D] B. Lambrecht,[A] and T. van den Berg[A]

[A]Avian Virology and Immunology Unit, Veterinary and Agrochemical Research Centre (VAR), Groeselenberg 99, 1180 Ukkel, Brussels, Belgium
[B]CEVA Phylaxia, Szallas utca 5, H-1107 Budapest, Hungary
[C]CEVA Santé Animale, BP 126, 33501 Libourne, France
[D]Ceva Biomune, 8901 Rosehill Road, Lenexa, KS 66215-3513

Received 11 April 2012; Accepted 8 June 2012; Published ahead of print 11 June 2012

SUMMARY. In countries where avian influenza has become endemic, early vaccination of layer pullets or broilers with classical inactivated vaccines at the hatchery is no longer an option because of interference with passive immunity indirectly induced by the necessary vaccination of the breeders. On the other hand, injection of thousands of chicks from 7 to 10 days old on farms has been determined to be unreliable and, therefore, poorly efficacious. For these reasons, interest has arisen regarding a newly developed live recombinant vector vaccine based on a turkey herpesvirus (HVT) expressing the H5 gene from a clade 2.2 H5N1 highly pathogenic avian influenza virus (HPAIV) strain (rHVT-H5), which in theory is capable of breakthrough passive immunity to both the vector (HVT) and the insert (H5) and is consequently applicable at the hatchery. The objectives of this trial were to evaluate the impact of maternally derived antibodies (MDAs) specific to H5N1 on the immunity and the efficacy (protection and virus shedding) of different vaccination programs including rHVT-H5 and inactivated H5N1 and H5N2 vaccines applied alone or in combination. Therefore, broilers carrying MDAs against both HVT and Asian H5N1 HPAIV were vaccinated on the first day of age with rHVT-H5, with or without boosting vaccination by an inactivated vaccine after 10 days. The different groups were challenged with two antigenically highly divergent Egyptian clade 2.2.1 H5N1 HPAIVs at 4 wk of age. Protection against challenge was compared with unvaccinated birds or vaccinated birds without MDAs. Between 70% and 90% clinical protection could be observed in the vaccinated groups possessing MDAs, indicating no or very low interference of MDAs with vaccination. Results regarding clinical protection, humoral, cell-mediated, and mucosal immunity, as well as re-excretion of challenge virus are presented and discussed.

RESUMEN. Eficacia de una vacuna recombinante rHVT-AI en pollos de engorde con inmunidad pasiva contra el desafío con dos cepas egipcias del virus de la influenza aviar altamente patógena H5N1antigénicamente divergentes clade 2.2.1.

En los países donde la influenza aviar se ha convertido en endémica, la vacunación temprana de las pollitas de postura o de pollos de engorde, con vacunas inactivadas clásicas en la incubadora ya no es una opción debido a la interferencia con la inmunidad pasiva, inducida indirectanente por la vacunación de los reproductores. Por otro lado, la inyección de miles de pollos de 7 a 10 días de edad en las granjas se ha determinado ser poco confiable y, por lo tanto ineficaz. Por estas razones, se ha suscitado interés con respecto al desarrollo de una vacuna de virus vivo recombinante con un vector de herpesvirus de pavo (HVT) que expresa el gene H5 de un virus de la influenza aviar de alta patogenicidad H5N1 clado 2.2 (rHVT-H5), que en teoría es capaz de sobrepasar la inmunidad pasiva tanto para el vector (HVT) y para el inserto (H5) y en consecuencia, se puede aplicar en la incubadora. Los objetivos de este estudio fueron evaluar el impacto de los anticuerpos maternales especificos para el subtipo H5N1 en la inmunidad y en la eficacia (protección y la eliminación del virus) de programas de vacunación diferentes, incluyendo las vacunas rHVT-H5 y las vacunas inactivadas contra los subtipos H5N1 y H5N2 aplicadas solas o en combinación. Por lo tanto, los pollos que poseen anticuerpos maternos contra el virus y contra el subtipo asiático H5N1 del virus de la influenza aviar altamente patógeno fueron vacunados en el primer día de edad con rHVT-H5, con o sin una vacunación de refuerzo con una vacuna inactivada después de los 10 días. Los diferentes grupos se desafiaron a las cuatro semanas con dos virus de la influenza aviar de alta patogenicidad subtipo H5N1 egipcios clados 2.2.1 antigénicamente muy divergentes. La protección contra la exposición se comparó con las aves no vacunadas o con las aves vacunadas sin anticuerpos maternos. Entre un 70% y un 90% de protección clínica que observó en los grupos vacunados que poseían anticuerpos maternos, lo que indica se no existe interferencia de los anticuerpos maternos con la vacunación o esta es muy baja. Se presentan y discuten los resultados relativos a la protección clínica, la inmunidad humoral, la inmunidad mediada por células, de las mucosas, así como re-excreción del virus de desafío.

Key words: avian influenza, recombinant turkey herpesvirus, immunity, vaccination, protection

Abbreviations: AIV = avian influenza virus; CMI = cell-mediated immunity; EID = egg infective dose; ELISA = enzyme-linked immunosorbent assay; HI = hemagglutination inhibition; HPAI(V) = highly pathogenic avian influenza (virus); LPAI(V) = low pathogenic avian influenza (virus); MDA = maternally derived antibody; MDV = Marek's disease virus; ND = Newcastle disease; rHVT = recombinant turkey herpesvirus; S.I. = stimulation index

H5N1 highly pathogenic avian influenza (HPAI) virus emerged in Egypt in early 2006, and then spread very rapidly within the whole country, causing a huge number of outbreaks both in backyard and intensively reared poultry (3). The main approaches for controlling the outbreaks have been the culling of all poultry suspected of harboring the virus and the vaccination of domestic birds (8,22). Initially, emergency vaccination was used to protect grandparent and parent flocks. However, stamping-out policies and targeted vaccination were insufficient to control the spreading of the disease, and the country was declared endemic in July 2008. Vaccination of domestic poultry against H5N1 has been used on a large-scale in Egypt since the veterinary authorities made the decision to vaccinate all commercial flocks starting in May 2007, followed by vaccination

[E]Corresponding author. E-mail: fabienne.rauw@coda-cerva.be

of household poultry. More than 1.3 billion doses of different types of inactivated H5 vaccines, mainly based on Eurasian and American H5N2 and Eurasian H5N1 subtype viruses, were applied (2).

However, the impact of avian influenza (AI) vaccination on disease control remains quite limited in Egypt, i.e., outbreaks are still reported regularly despite continuous vaccination of poultry, and spillover to humans remains a permanent risk. The shortcomings of the vaccination strategy may be attributed to several factors (14), including limitations of inactivated vaccines, which are playing an important role. Indeed, inactivated vaccines must be administered individually by the parental route, which is laborious, time consuming, and harbors the risk of transmission of the field virus by the vaccination crews. Moreover, after several rounds of routine vaccination of the breeders, high levels of maternally derived antibodies (MDAs) are transferred to the progeny. MDAs bring some protection during the early life of the chicks, when immunocompetence is not yet fully developed, but they also interfere with successful vaccination of these young animals because of their capacity to neutralize, at least partially, the vaccine virus and increase the clearance of the vaccine antigens, thereby preventing optimal exposure to the immune system (1,6,12,15). Therefore, early vaccination of layer pullets or broilers with classical inactivated vaccines at the hatchery is no longer a reliable option in countries where AI has become endemic. Their use at a later age has been investigated, but depending on the level of MDAs, different levels of interference with vaccine still occur, decreasing the efficacy of the vaccination (1,6,12). Even a prime/boost vaccination regimen at 1 and 12 days of age with inactivated vaccines was not sufficient to afford satisfactory protection to chickens against recent ''variant'' clade 2.2.1 Egyptian HPAI field strains (2,19). Finally, extensive vaccination with inactivated vaccine has been suspected to induce the emergence of vaccine escape mutants in Mexico (11) and might be responsible for a similar situation with H5N1 in Egypt (4,9). Since genetic drifts occur in countries and regions experiencing immune pressure from enzootic infection or extensive vaccination, future successes of inactivated vaccines will depend largely on how close the vaccine seed virus matches with the field virus. In response to MDA interference and to antigenic variants, the reverse genetic system and biotechnologies for producing vectored vaccines might allow easier and more rapid development and testing of new vaccine strains that will be more adapted to the field situation. In this context, the studied turkey herpesvirus (HVT) vector vaccine with an HA insert from the recent clade 2.2 HPAI H5N1 strain appears to be very promising to use as a single rHVT-H5 vaccination for day-old chicks against recent H5N1 strains circulating in Egypt (19).

Here, we evaluated the efficacy of different vaccination programs, including the live rHVT-H5 vaccine and two inactivated vaccines, based either on the Chinese reverse genetics H5N1 or the low pathogenic avian influenza (LPAI) A/Chicken/Mexico/232/1994 H5N2 virus strains, applied alone or in combination, in broiler chickens with or without MDA to H5. The immunity and the protection against challenge with the classic and variant 2.2.1 lineage HPAI H5N1 strains, which are currently circulating in Egypt, were examined.

MATERIALS AND METHODS

Chickens. Commercial Hubbard F15-type broiler chickens with MDAs to avian influenza virus (AIV) and Marek's disease virus (MDV) were hatched from broiler breeders vaccinated twice before the start of lay with inactivated H5N1 Re-1 AIV vaccine at 3-wk intervals at Prophyl Ltd., Mohacs, Hungary. Broilers without MDA were hatched from unvaccinated breeders of the same origin. Breeders received the classical vaccination schedule, including the combination of HVT and Rispens against Marek's disease at 1 day old. After hatching, all birds were kept in biosecurity level 3 (BSL-3) isolators, and animal experiments were conducted under the authorization and supervision of the Biosafety and Bioethics Committees at the Veterinary and Agrochemical Research Institute (Belgium), following national and European regulations.

Vaccines and challenge strains. The Cevac Flu Kem H5N2 vaccine (inact-H5N2) was produced by Ceva (Cuarnavaca, Mexico) and is an oil-emulsion inactivated vaccine containing H5N2 LPAI virus (A/Chicken/Mexico/232/1994 strain). The Reassortant-1 (Re-1) H5N1 vaccine (inact-H5N1) was produced by Harbin Weike Biotechnology Development Co. (Harbin, China) and is an oil-emulsion inactivated vaccine manufactured using the seed virus of the A/Harbin/Re-1/2003/H5N1 reverse-genetic strain, which contains six internal genes from high-growth A/Puerto Rico/8/1934 H1N1 virus and the HA and NA genes of A/Goose/Guangdong/1/96 H5N1 strain. The cryopreserved cell-associated rHVT-H5 vaccine (Vectormune® HVT-AIV, Acc #359-07) was developed by CEVA-Biomune Co. Ltd. (Lenexa, KS). It was constructed with the FC-126 strain of HVT as backbone and the HA gene from the H5N1 clade 2.2 HPAI A/Swan/Hungary/4999/2006 strain as insert. Vaccines were administrated subcutaneously in the neck using a dose of 4000 pfu in 200 µl at 1 day old for the rHVT-H5 vaccine or using one dose of 500 µl or 300 µl at 10 days of age for the inactivated H5N2 and H5N1 vaccines, respectively.

The two HPAI H5N1 viruses selected for the challenge were isolated in poultry farms located in Egypt at the end of 2007 and beginning of 2008. The A/Chicken/Egypt/1709-1VIR08/2007 and A/Chicken/Egypt/1709-6/2008 strains belong to the genetic clade 2.2.1 but cluster separately, as previously described (19). Chickens were challenged at 4 wk of age by the oculonasal route with 100 µl (50 µl in the eye, 50 µl in the nostril) of inoculum containing 10^6 egg infective dose that kills 50% of eggs (EID_{50}) of HPAI H5N1 virus.

Mitogens and AIV antigens. The mitogens phorbol 12-myristate 13-acetate (PMA) and ionomycin (Iono) were purchased from Sigma (Belgium). AIV recall antigens were prepared from the LPAI H5N2 strain A/Chicken/Belgium/150VB/99, as previously described (16), and contained an extract of all H5N2 proteins (herein designated ''prot-H5N2'').

Viral excretion. Quantification of the AIV challenge strain in the oropharyngeal swabs was determined by quantitative real-time reverse transcription–polymerase chain reaction (QRT-PCR) targeting the influenza A matrix (M) gene, as previously described (19). The results were expressed as the number of M gene copies per milliliter of swab samples. Moreover, the quality of the sample and the RNA extraction procedure was validated using β-actin as housekeeping gene, as previously described (24).

AIV-specific humoral and digestive antibody-mediated immunity. AIV-specific humoral immunity was evaluated by hemagglutination inhibition (HI) test and AIV-specific IgG/IgM/IgA enzyme-linked immunosorbent assays (ELISAs). Digestive antibody-mediated immunity to AIV was measured on supernatant of *ex vivo* duodenum tissue culture as previously described (18). The HI tests were conducted as previously described (19) with four units of HA antigens to check for cross-reactivity of the serums toward the different strains used in our study, i.e., the two antigenically divergent Egyptian HPAI H5N1 strains used for challenge (A/Chicken/Egypt/1709-1VIR08/2007 and A/Chicken/Egypt/1709-6/2008); the Hungarian HPAI H5N1 strain (A/Duck/Hungary/4571/2006), which is genetically and antigenically very close to the HPAIV strain (A/Swan/Hungary/4900/2006) and which provided the HA gene for the construction of the rHVT-H5 vaccine; the AIV H5N1 Re-1 antigen included in the inact-H5N1 vaccine; and the Mexican LPAI H5N2 antigen (A/Chicken/Mexico/232/94) included in the inact-H5N2 vaccine. The HI geometric mean titers were expressed as reciprocal $\log_2$, and inhibition of hemagglutination at a dilution of $>2^3$ was considered as specific to AIV.

AIV-specific IgG/IgM/IgA ELISAs were performed as previously described (18), except that MaxiSorp Nunc-Immuno F96 microwell

Table 1. Vaccination and challenge schedule.

Exp.	Group	Vaccination		Challenge strain
		Vaccine	Age (day)	
I	Negative (MDA−)	None		H5N1 Egypt 2008
	Negative (MDA+)	None		H5N1 Egypt 2008
	rHVT-H5 (MDA−)	rHVT-H5	1	H5N1 Egypt 2008
	rHVT-H5 (MDA+)	rHVT-H5	1	H5N1 Egypt 2008
	Inact-H5N1 (MDA−)	Inact-H5N1	10	H5N1 Egypt 2008
	Inact-H5N1 (MDA+)	Inact-H5N1	10	H5N1 Egypt 2008
	rHVT-H5/Inact-H5N1 (MDA−)	rHVT-H5	1	H5N1 Egypt 2008
		Inact-H5N1	10	
	rHVT-H5/Inact-H5N1 (MDA+)	rHVT-H5	1	H5N1 Egypt 2008
		Inact-H5N1	10	
II	Negative (MDA+)	None		H5N1 Egypt 2007
	Negative (MDA+)	None		H5N1 Egypt 2008
	rHVT-H5 (MDA+)	rHVT-H5	1	H5N1 Egypt 2007
	rHVT-H5 (MDA+)	rHVT-H5	1	H5N1 Egypt 2008
	rHVT-H5/Inact-H5N1 (MDA+)	Inact-H5N1	10	H5N1 Egypt 2007
	rHVT-H5/Inact-H5N1 (MDA+)	Inact-H5N1	10	H5N1 Egypt 2008
	rHVT-H5/Inact-H5N2 (MDA+)	rHVT-H5	1	H5N1 Egypt 2007
		Inact-H5N1	10	
	rHVT-H5/Inact-H5N2 (MDA+)	rHVT-H5	1	H5N1 Egypt 2008
		Inact-H5N1	10	

plates were coated overnight at 4 C with prot-H5N2 diluted at 2 μg/ml in carbonate/bicarbonate pH 9.6 buffer.

AIV-specific cell-mediated immunity. The AIV-specific cell-mediated immunity (CMI) was assessed by the ChIFNγ production after *ex vivo* antigenic recall activation of lymphocytes from the spleen and the peripheral blood, as previously described (16). Briefly, splenocytes and peripheral blood lymphocytes (PBL) were activated by mitogens (1 μg/ml PMA/Iono), as positive control of their *ex vivo* activability, and by AIV recall antigens (1 μg/ml). ChIFNγ production was measured by capture ELISA. Cellular responses were expressed as a stimulation index (S.I.) that was calculated for each bird by dividing the optical density (O.D.) values of mitogen- and antigen-activated cells by the O.D. of nonactivated lymphocytes, and the S.I. per group was calculated. Chickens having an O.D. value <0.1 or S.I. <2 after mitogen-activation were excluded from further antigen-activation analysis. An O.D. value ≥ 0.1 and S.I. ≥ 2 for AIV-activation in the inoculated but not in the negative group were considered as evidence of a significant AIV-specific CMI.

Experimental design. The experimental design of both conducted studies is summarized in Table 1.

In the first trial, chickens with (MDA+) and without (MDA−) maternally derived antibodies specific to H5N1 were both divided into four groups of 20 birds. Animals were vaccinated either at 1 day old with the rHVT-H5 vaccine or at 10 days of age with the inact-H5N1 vaccine. The third group was primed at 1 day old with the rHVT-H5 vaccine and boosted at 10 days of age with the inact-H5N1 vaccine. The last group was left unvaccinated. Sera were taken ($n = 5$) at 3 and 4 wk of age to assess humoral immunity. The challenge was performed at 4 wk of age with the 2008 Egyptian H5N1 strain on 10 of the remaining vaccinated chickens.

In the second trial, only MDA+ chickens were used, and they were divided into four groups of 30 birds. The first group was vaccinated at 1 day old with the rHVT-H5 vaccine. The second group was primed at 1 day old with the rHVT-H5 vaccine and boosted at 10 days of age with the inact-H5N1 vaccine, while the third group was boosted with the inact-H5N2 vaccine. The last group was left unvaccinated and used as control. Spleen, blood, serum, and duodenal tissues were taken ($n = 5$) at 3 and 4 wk of age to assess humoral, CMI, and mucosal antibody-mediated immunity. Each of the four groups was further divided into two groups of 10 birds for the challenge at 4 wk of age with the 2007 and 2008 Egyptian H5N1 strains.

In both experiments, each chicken was identified individually. After challenge, birds were monitored daily for clinical symptoms (prostration, diarrhea, respiratory distress, and for neurologic signs like loss of balance, torticollis, and incoordination). Birds showing severe clinical signs were humanely euthanatized. To monitor viral excretion, oropharyngeal swabs were taken at 2, 4, and 7 days postinfection (dpi), as previously described (19), and stored at −80 C before processing. To assess immunity, spleen, blood, serum, and duodenal tissues were taken at 3 and 4 wk of age. Serum samples were taken at the end of experiment, at 2 wk postinfection, and stored at −20 C until further analysis.

Statistical analysis. The ELISA, HI, and challenge virus shedding data were analyzed using Minitab 13 software (statistical programs for Windows 2000), and differences were considered as significant at $P < 0.05$. After checking the validity hypotheses (homogeneity of the within-groups variances using the Levene test, and normality of the distribution of the criterion for each group using the Ryan-Joiner test), a one-way ANOVA was carried out on this criterion in order to compare the different groups. In case of a significant global group effect, pairwise comparisons were carried out using Tukey tests. When normality or homogeneity of variance tests failed, the nonparametric Kruskal-Wallis test was used. In case of group effect, pairwise comparisons were carried out using the Kruskal-Wallis test, with the Bonferroni method to adjust the risk α (adjusted risk $\alpha' = \alpha/c$, where c = number of pairwise comparisons). Similar statistical analyses were performed on the HI titers in order to compare the results obtained by the different HA antigens for the same serum sample.

The qualitative criterions ''mortality'' and ''excretion'' were analyzed using STATA 10 software. Because of the small value of expected frequencies for cells of the contingency table, a Fisher exact test was performed. In the case of global significant difference between groups, pairwise comparisons were carried out by creating new contingency tables and using Fisher exact tests. The risk was adjusted using the Bonferroni method (adjusted risk $\alpha' = \alpha/c$, where c = number of pairwise comparisons).

RESULTS

Protection against morbidity and mortality. In the first trial, unvaccinated chickens without MDA showed typical signs of HPAI, including depression, ruffled feathers, and nervous signs at 2 days after challenge with the H5N1 Egypt 2008 strain. Birds showed acute signs of disease from 2 dpi, started dying on 2 dpi, and all

Table 2. Protection of vaccinated broiler chickens with or without MDA specific to H5N1 against mortality after challenge at 4 wk of age with H5N1 Egypt 2007 and 2008 strains.

Exp.	Group	MDA	Challenge strains (%)	
			H5N1 Egypt 2007	H5N1 Egypt 2008
I	Negative	−	N.D.[A]	0
		+	N.D.	0
	rHVT-H5	−	N.D.	90
		+	N.D.	100
	Inact-H5N1	−	N.D.	70
		+	N.D.	40
	rHVT-H5/Inact-H5N1	−	N.D.	90
		+	N.D.	80
II	Negative	+	0	0
	rHVT-H5	+	90	70
	rHVT-H5/Inact-H5N1	+	80	90
	rHVT-H5/Inact-H5N2	+	90	80

[A]N.D. = not determined.

animals were dead by the 3 dpi, thus validating the challenge (Table 2). Mortality was slightly delayed in the unvaccinated group with MDA, but all the chickens were also dead by 6 dpi. In the groups where the birds had been vaccinated with the rHVT-H5 vaccine, 90% and 100% of animals without and with MDA survived, respectively. When the chickens were boosted with the inact-H5N1 vaccine, the protection was 90% and 80% in the MDA− and MDA+ groups, respectively. The inact-H5N1 vaccine used alone protected only 70% and 40% of birds without or with MDA, respectively.

In the second trial, which was only conducted on chickens with MDA, unvaccinated birds started dying at 2 days after the challenge with H5N1 Egypt 2008 strain, and all animals were dead at 4 dpi (Table 2). The course of mortality was extended to 6 dpi in the unvaccinated group that was challenged with the H5N1 Egypt 2007 strain. Within the rHVT-H5 vaccinated group, 70% and 90% of animals were protected against H5N1 Egypt 2008 and 2007 challenge, respectively. The protection afforded by the prime/boost rHVT-H5/inact-H5N1 and rHVT-H5/inact-H5N2 vaccination schedules reached 80%–90% whatever the strain used for challenge.

Viral shedding after challenge. Unvaccinated chickens challenged with either of the two Egyptian HPAI H5N1 strains shed large amounts of AI virus before dying, as measured by real-time RT-PCR (Table 3). Vaccination of chickens without MDA either with the rHVT-H5 alone or with the rHVT-H5/inact-H5N1 vaccine in a prime-boost vaccination schedule significantly reduced ($P < 0.05$) the shedding of the H5N1 2008 Egypt challenge virus, as revealed by the statistical comparison of the number of excreting birds and the average gene copy number with the unvaccinated group. The inact-H5N1 vaccine reduced the excreted titer but not

Table 3. Challenge virus shedding by the oropharyngeal route after challenge at 4 wk of age with 2007 and 2008 Egyptian HP H5N1 strains in MDA+ and MDA− vaccinated chickens.

Exp.	Groups	MDA	Days after challenge					
			2		4		7	
H5N1 Egypt 2008 strain[A]								
I	Negative	−	8.63 ± 0.29a[B]	6/6a[C]	S.M.[D]		S.M.	
		+	5.26 ± 2.23b	9/10a	7.86 ± 0.75a	6/6a	S.M.	
	rHVT-H5	−	0.49 ± 1.54d	1/10b	0.00 ± 0.00d	0/10b	0.00 ± 0.00a	0/9a
		+	0.35 ± 1.10d	1/10b	2.07 ± 2.24c	5/10ab	0.00 ± 0.00a	0/10a
	Inact-H5N1	−	3.12 ± 2.20c	7/10a	4.46 ± 1.87b	9/10a	0.95 ± 2.50a	1/7a
		+	3.06 ± 2.20c	7/10a	4.38 ± 1.99b	9/10a	1.87 ± 3.23a	2/7a
	rHVT-H5/inact-H5N1	−	0.52 ± 1.65d	1/10b	1.08 ± 2.28cd	2/10b	0.00 ± 0.00a	0/9a
		+	1.77 ± 2.32cd	4/10ab	4.36 ± 1.69b	9/10a	0.00 ± 0.00a	0/7a
II	Negative	+	8.98 ± 0.84a	10/10a	S.M.		S.M.	
	rHVT-H5	+	6.26 ± 2.36ab	9/10a	6.79 ± 1.36a	10/10a	2.65 ± 3.40a	3/7a
	rHVT-H5/inact-H5N1	+	3.89 ± 3.42b	6/10a	6.09 ± 2.54a	9/10a	2.63 ± 3.39a	4/9a
	rHVT-H5/inact-H5N2	+	5.82 ± 3.28ab	8/10a	7.04 ± 1.06a	9/9a	4.01 ± 3.01a	5/7a
H5N1 Egypt 2007 strain								
II	Negative	+	8.40 ± 1.04a	10/10a	10.09 ± 0.38a	6/6a	S.M.	
	rHVT-H5	+	7.36 ± 1.22a	10/10a	4.93 ± 3.43b	7/10a	0.91 ± 2.11a	2/9a
	rHVT-H5/inact-H5N1	+	5.56 ± 3.09a	8/10a	6.86 ± 1.45ab	9/9a	3.90 ± 3.38a	6/9a
	rHVT-H5/inact-H5N2	+	5.60 ± 3.90a	7/10a	6.76 ± 2.85ab	9/10a	2.80 ± 3.36a	4/9a

[A]Data are determined by QRRT-PCR on 1 ml of swabs taken at specific time after challenge. Different lowercase letters indicate a significant ($P < 0.05$) difference between the groups (per column). The total numbers of chickens tested were reduced with time because of specific mortality.

[B]Data represent mean ± standard deviation of matrix gene copies in milliliters of swabs ($\log_{10}$). Means ± standard deviations at time points with no common letter differ significantly ($P < 0.05$).

[C]Frequency (number of positive/total tested chicken) of virus detection in 1 ml of swabs.

[D]S.M. = specific mortality.

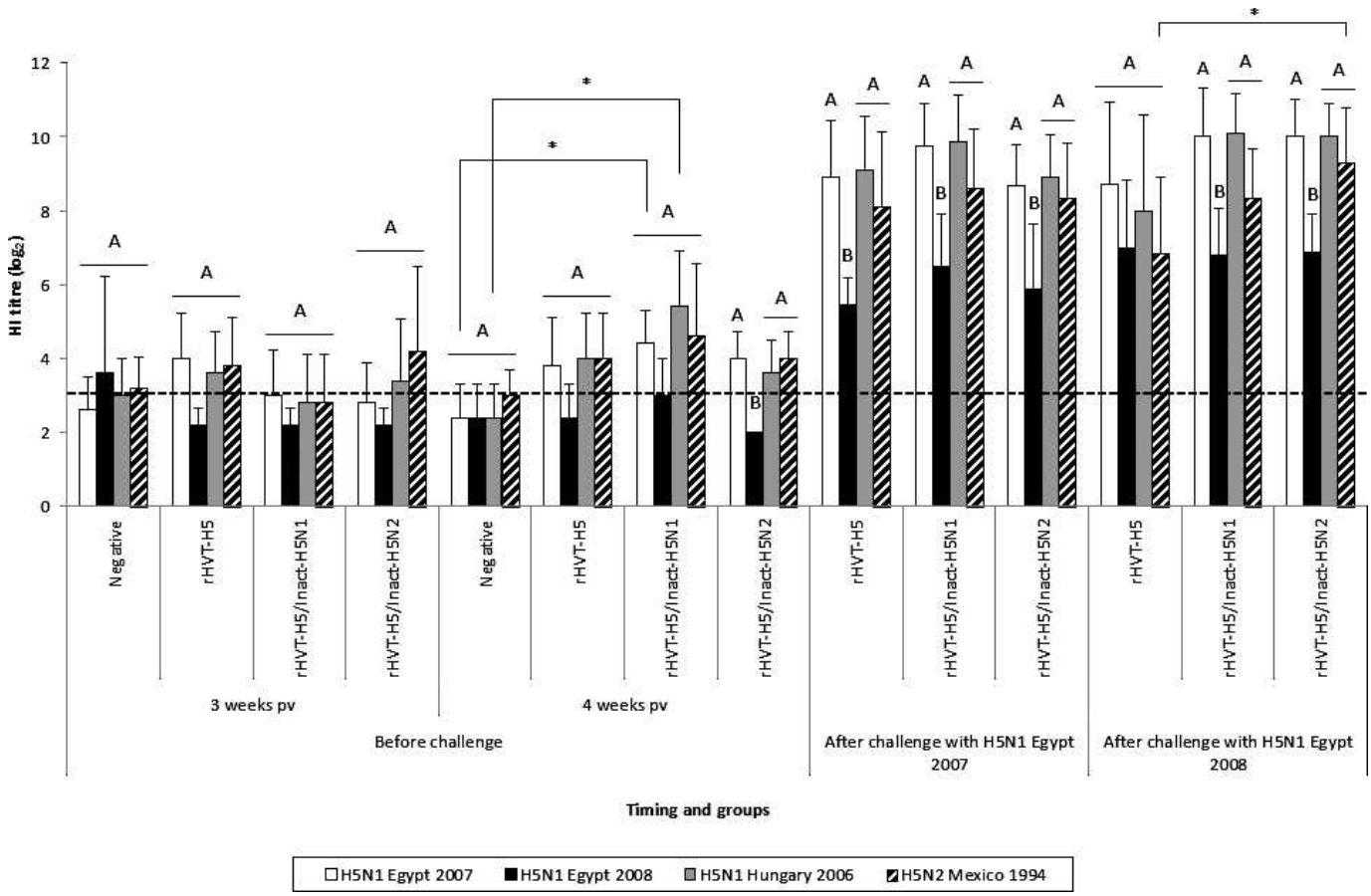

Fig. 1. HI titers of MDA+ chickens vaccinated at 1 day old with the rHVT-H5 in combination at 10 days old with the inact-H5N1 or the inact-H5N2 vaccines. Ten birds per group were challenged at 4 wk of age with 10^5 EID_{50} H5N1 Egypt 2007 or 2008 strains by oculonasal route (trial II). Data represent mean ± standard deviation of HI titer (log_2), which corresponds to the last dilution showing an inhibition of hemagglutination of 4 hemagglutination units of AIV strains. The HI geometric mean titers were expressed as reciprocal log_2, and titers >3 log_2 were considered positive (this cutoff is indicated by the dotted line). Means ± standard deviations at time points with no common letters or with asterisk superscript differ significantly ($P < 0.05$) by used vaccine or HA antigen, respectively.

the number of excreting animals. The reduction of challenge virus shedding was significantly ($P < 0.05$) higher in the chickens that received either the rHVT-H5 vaccine alone or the combination of rHVT-H5/inact-H5N1 vaccines compared to those that received the rHVT-H5/inact-H5N2 vaccination schedule. The same observation could be noted in the chickens with MDA in the first trial. In the second trial, only the rHVT-H5/inact-H5N1 vaccination program resulted in significantly reduced ($P < 0.05$) shedding, when compared to the unvaccinated group, while the reduction in the groups that were vaccinated either with the rHVT-H5 alone or with the rHVT-H5/inact-H5N2 vaccines was not significant.

A significant reduction of the viral excretion was also observed in birds with MDA at 4 dpi in the rHVT-H5 vaccinated group ($P < 0.05$) after the H5N1 Egypt 2007 challenge.

AIV-specific humoral immunity. *Passive immunity*. At 1 day old and before challenge, the HI titers of all unvaccinated chickens without MDA in trial I were below the threshold of positivity ($\leq 2^3$), regardless of the HA antigen used, and were therefore considered as negative (data not shown). The titers of day-old unvaccinated chickens with MDA averaged $2^{6.6}$ and $2^{7.5}$ against the H5N1 Re-1 antigen, $2^{4.7}$ and $2^{4.5}$ against the H5N2 Mexican antigen, and $2^{6.7}$ and $2^{8.7}$ against the H5N1 Hungarian antigen in trials I and II, respectively. The HI titers of the vaccinated chickens in trials I and II are represented in Figure 1 and Table 4, respectively.

HI test results using Egyptian challenge strains as antigens. In the first trial, when the 2008 H5N1 Egypt isolate was used as HA antigen, all rHVT-H5/inact-H5N1 vaccinated chickens without MDA showed positive HI titers at 3 wk of age. All the birds without MDA from the inact-H5N1 group turned positive only 1 wk later, namely at 4 wk of age, while only 40% of rHVT-H5 vaccinated animals showed seroconversion to this strain. An HI titer equal to 2^5 was observed from the third week of age after the prime/boost rHVT-H5/inact-H5N1 and the inact-H5N1 vaccination regimens, while it averaged $2^{3.5}$ in the rHVT-H5 group at 4 wk. In chickens with MDA, a seroconversion was observed in only 40% of the inact-H5N1 and rHVT-H5/inact-H5N1 vaccinated chickens and 20% of the rHVT-H5 vaccinated group at 4 wk of age. No residual MDA could be detected in the unvaccinated chickens at 3 and 4 wk of age.

In the second trial, similar to the first one, a seroconversion was measured only in the rHVT-H5 and rHVT-H5/inact-H5N1 vaccinated chickens, reaching 20% and 40%, respectively, by 4 wk of age, when the 2008 H5N1 Egypt isolate was used as HA antigen. By contrast, the rHVT-H5/inact-H5N2 group remained negative. All birds in the rHVT-H5/inact-H5N1 vaccinated group developed measurable HI titers by 4 wk of age against the 2007 H5N1 Egypt isolate, while 80% and 40% seroconverted in the rHVT-H5/inact-H5N2 and rHVT-H5 vaccinated groups, respectively. The HI titers using the 2007 H5N1 Egypt isolate as the HA antigen were about

Table 4. HI titers of MDA− and MDA+ chickens vaccinated at 1 day old with the rHVT-H5 alone or in combination at 10 days old with the inact-H5N1 vaccine and challenged at 4 wk of age with 10^5 EID_{50} H5N1 Egypt 2008 strains by oculonasal route (trial I).

		HA antigen			
Groups	MDA	H5N1 Egypt 2008		H5N1 Hungary 2006	
4 wk of age					
Negative	−	<3.0c[A]	0/5[B]	<3.0e	0/5
	+	<3.0c	0/5	<3.0e	0/5
rHVT-H5	−	3.5 ± 1.3b	2/5	7.3 ± 2.5b	5/5
	+	<3.0b	1/5	3.8 ± 1.6d	2/5
Inact-H5N1	−	5.4 ± 0.6a	5/5	6.8 ± 0.5bc	5/5
	+	4.0 ± 1.4ab	2/5	4.5 ± 1.7cd	3/5
rHVT-H5/Inact-H5N1	−	5.4 ± 0.9a	5/5	9.8 ± 1.6a	5/5
	+	4.0 ± 2.5b	2/5	4.6 ± 3.0cd	4/5
2 wk postinfection					
Negative	−	S.M.[C]		S.M.	
	+	S.M.		S.M.	
rHVT-H5	−	6.7 ± 1.7b	8/9	11.4 ± 1.0a	9/9
	+	5.6 ± 1.7c	9/10	9.7 ± 1.6b	10/10
Inact-H5N1	−	7.0 ± 1.0b	7/7	12.0 ± 0.0a	7/7
	+	6.3 ± 0.5bc	4/4	7.0 ± 0.8c	4/4
rHVT-H5/Inact-H5N1	−	8.7 ± 1.3a	9/9	11.9 ± 0.3a	9/9
		6.4 ± 0.8b	7/7	10.0 ± 1.9b	7/7

[A]Data represent means ± standard deviations of HI titer (log_2), which correspond to the last dilution showing an inhibition of hemagglutination of 4 hemagglutination units of AIV strains. The HI geometric mean titers are expressed as reciprocal log_2, and titers >3 log_2 were considered as positive. Means ± standard deviations at time points with no common letters differ significantly ($P < 0.05$) by used vaccine.
[B]Frequency (number positive/total tested chickens) of positive HI titer.
[C]S.M. = specific mortality.

one log higher than those obtained using the 2008 H5N1 Egypt isolate as HA antigen in the vaccinated groups.

HI test results using the Mexican LPAI H5N2 vaccine strain as antigen. The H5N2 Mexico 1994 HA antigen was not used in the first trial because no chickens received the inact-H5N2 vaccine. In the second trial, the prime/boost rHVT-H5/inact-H5N1 and rHVT-H5/inact-H5N2 vaccination regimens resulted in 80% seroconversion against the Mexican H5N2 HA antigen by 4 wk of age. In the rHVT-H5 group, only 60% of the animals seroconverted, while all the unvaccinated chickens became seronegative by 4 wk of age.

HI tests using the Hungarian clade 2.2 H5N1 strain as antigen. Vaccination of birds without MDA with the rHVT-H5 or rHVT-H5/inact-H5N1 or inact-H5N1 vaccine resulted in seroconversion in all chickens against the homologous H5N1 Hungary HA antigen by 4 wk of age, as shown in the first trial. A significantly higher HI titer ($P < 0.05$) was measured in the rHVT-H5/inact-H5N1 vaccinated chickens, reaching $2^{9.8}$, compared to the other groups; in groups with MDA, the seroconversion was reduced to 80% in the rHVT-H5/inact-H5N1, to 60% in the inact-H5N1, and to 20% in the rHVT-H5 vaccinated groups. No residual MDA was detected in unvaccinated chickens.

AIV-specific IgG/IgM/IgA. The profile of AIV-specific IgG in unvaccinated chickens indicated a decline of passive maternally derived immunity until the fourth week of age in trials I (Fig. 2A) and II (Fig. 2B). Both inact-H5N1 and rHVT-H5/inact-H5N1 vaccination schedules induced significant IgG titers ($P < 0.05$) as early as 3 wk of age in chickens without MDA, when compared to unvaccinated chickens. In chickens with MDA, only the inact-H5N1 vaccination induced active IgG response at this early age, while the detection of specific IgG was delayed to the fourth week in the rHVT-H5/inact-H5N1 vaccinated group. The rHVT-H5 and rHVT-H5/inact-H5N2 groups remained negative.

Specific IgM was detected at 3 wk of age in both the inact-H5N1 and rHVT-H5/inact-H5N1 vaccinated groups without MDA. In chickens with MDA to AIV, only the rHVT-H5/inact-H5N1 induced IgM response by 4 wk of age. No specific IgA was observed at any tested times.

AIV-specific CMI. CMI was only assessed in the second trial, and the results obtained at the different sampling time points after splenocyte activation with PMA/Iono mitogens showed that all chickens were similarly immunocompetent (O.D. > 0.1 and S.I. > 2), validating the spleen cells activability (data not shown). An AIV-specific CMI was measurable in 80% and 60% of rHVT-H5/inact-H5N1 vaccinated chickens at 3 and 4 wk of age, respectively (Table 5), while in the rHVT-H5 and rHVT-H5/inact-H5N2 groups, 60% and 40%, respectively, were positive at both tested times points.

The PBL activation with PMA/Iono mitogens remained negative at 3 wk of age, indicating that birds were not able to elicit peripheral white blood cells activability at this age or a limit in sensibility of the test. Animals having an O.D. value <0.1 or an S.I. <2 for mitogen activation were excluded from further PBL AIV-activation analysis. No AIV-specific peripheral CMI was noted in any vaccinated groups at 4 wk of age (data not shown).

AIV-specific mucosal antibody-mediated immunity in the digestive tract. In comparison to the unvaccinated birds, local antibody-mediated immunity in the digestive tract increased significantly ($P < 0.05$) only in the rHVT-H5/inact-H5N1 vaccinated chickens from the fourth week of age (Fig. 3). The specific IgG levels in this group were significantly higher than those measured in the other vaccinated groups. Specific IgM was detected in the digestive tract of some of the vaccinated birds and, at significant level ($P < 0.05$) in the rHVT-H5/inact-H5N1 group. No specific IgA could be measured in any animal.

DISCUSSION

Vaccination has been implemented in Egypt since 2006, together with culling, quarantine, improved biosecurity, and surveillance.

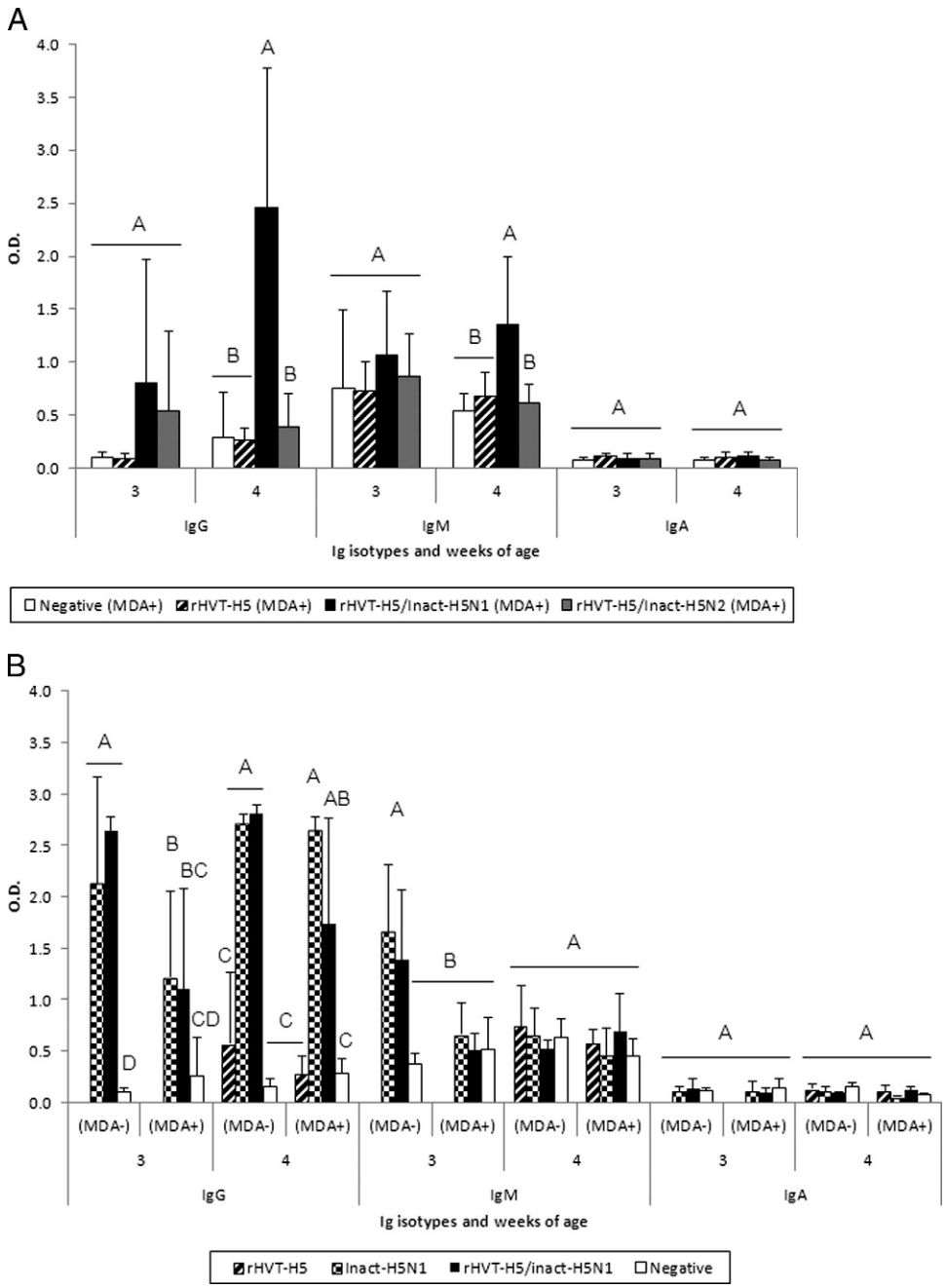

Fig. 2. AIV-specific IgG, IgM, and IgA detection by ELISA on sera from MDA− and MDA+ chickens vaccinated with the rHVT-H5 and the inact-H5N1/H5N2 vaccines according to different vaccination schedules. Data represent mean ± standard deviation of absorbance values determined on serum diluted 1:100 by ELISA at specified time of age ($n = 5$) in trials I (A) and II (B). Means ± standard deviations with no common superscript differ significantly ($P < 0.05$).

During the first large-scale vaccination campaign, AI-inactivated vaccines based on LPAI virus strain A/Chicken/Mexico/232/1994 were used. These vaccines were then gradually replaced by reverse genetics Chinese H5N1 inactivated vaccines (9) and consequently, day-old chicks possess MDA against H5N2 and/or H5N1 AIV. Although this passive immunity has been shown to afford some partial protection to young chickens, it has also been shown to interfere with vaccination (1,6). Indeed, even repeated vaccination using a commercial inactivated H5N2-based vaccine did not provide sufficient protection against a heterologous variant 2.2.1 lineage HPAI H5N1 challenge (2,19), and its extensive use has been suspected to be responsible for the emergence of vaccine escape mutants (4,9).

The goal of the present study was to evaluate the influence of MDA against H5N1 on the efficacy of different vaccination programs, including rHVT-H5 and reverse genetics Chinese H5N1 and LPAI H5N2 A/Chicken/Mexico/232/1994 inactivated vaccines applied alone or in combination, against challenge with two antigenically divergent clade 2.2.1 HPAI H5N1 strains, which have been currently circulating in Egypt.

Challenge performed at 4 wk of age resulted in 100% mortality in broiler chickens hatched from both unvaccinated and vaccinated breeders, confirming the short half-life and limited value of passive immunity for protection against Asian H5N1 HPAIV (1,6,12). However, there was a delay in the onset of clinical symptoms and

Table 5. AIV-specific splenic CMI of vaccinated chickens with MDA (trial II).

Groups	Weeks of age[A]			
	3		4	
Negative	1.1 ± 0.6a[B]	0/5[C]	0.8 ± 0.3a	0/5
rHVT-H5	2.2 ± 1.1a	2/5	3.0 ± 1.1a	3/5
rHVT-H5/inact-H5N1	6.0 ± 4.6a	4/5	6.4 ± 5.4a	3/5
rHVT-H5/inact-H5N2	5.5 ± 6.4a	2/5	4.0 ± 4.5a	2/5

[A]Different lowercase letters indicate a significant ($P < 0.05$) difference between the groups (per column).
[B]Data represent means ± standard deviations of S.I. that was calculated for each bird by dividing the O.D. values of antigen-activated splenocytes by the O.D. of nonactivated splenocytes. An S.I. value equal to or greater than 2 was considered as evidence of specific antigen activation.
[C]Data represent frequency (number antigen-positive/mitogen-positive chickens) of splenic cellular response to antigen activation.

mortality in MDA+ birds in comparison to MDA− birds, as previously shown (1,6,12). Surprisingly, MDA was no longer detectable by HI test at the age of 4 wk, but it was still measurable by ELISA-IgG test, indicating the presence of limited amount of antibodies and the low sensitivity of the HI test for detection of serum antibodies, where HI detected antibodies to HA only while ELISA detected antibodies to all viral proteins. The clinical protection obtained in the MDA+ chickens was higher in the group vaccinated with the rHVT-H5 vaccine used alone at 1 day old or in combination with the inactivated vaccine at 10 days, whatever the H5N2 or H5N1 seed strain used for boost, than in the MDA+ chicks that received only the inactivated vaccines. In addition, under our experimental conditions, the prime/boost rHVT-H5/inact-H5N1 vaccination schedule was the most efficient with regard to the reduction of excretion after challenge.

Our study further indicates that AI protection against mortality and virus shedding is dependent on the intensity of the systemic humoral immune response. Indeed, the HI titers measured against the 2008 Egyptian H5N1 challenge strain in the rHVT-H5/inact-H5N1 vaccinated chickens were higher than those obtained with the other vaccinated schedules. This homologous prime/boost vaccination induced also a high humoral immunity against the 2006 Hungarian H5N1 strain that donated the HA gene for the rHVT-H5 vaccine, and which was shown to have 97.9% and 95.8% similarity at nucleotide and amino acid levels, respectively, with the 2008 Egyptian H5N1 strain (19). Nevertheless, protection cannot be explained by humoral immunity only. Indeed, our experiment showed that the single rHVT-H5 vaccinated chickens could be protected without detectable antibody levels against either homologous or heterologous HA antigens. This could be explained by the poor sensitivity of the HI test and/or by the demonstration of the induction of AI-specific CMI in rHVT-H5 vaccinated birds after *ex vivo* antigen recall activation of splenocytes in the second trial. In addition, the high humoral immunity induced by the inact-H5N1 vaccine used alone was not sufficient to prevent mortality in the vaccinated chickens. Even though it was not tested in the first trial, the CMI induced by this vaccine was probably weak, since inactivated vaccines are recognized as poor inducers of CMI (10). It is thus reasonable to speculate that the higher cellular and humoral immunity observed after the application of the prime-boost rHVT-H5/inact-H5N1 vaccination regimen reflects the advantages of combining the two different types of vaccine in a vaccination program, as previously shown for vaccination against Newcastle disease (ND) (17). The inactivated vaccines are known to induce

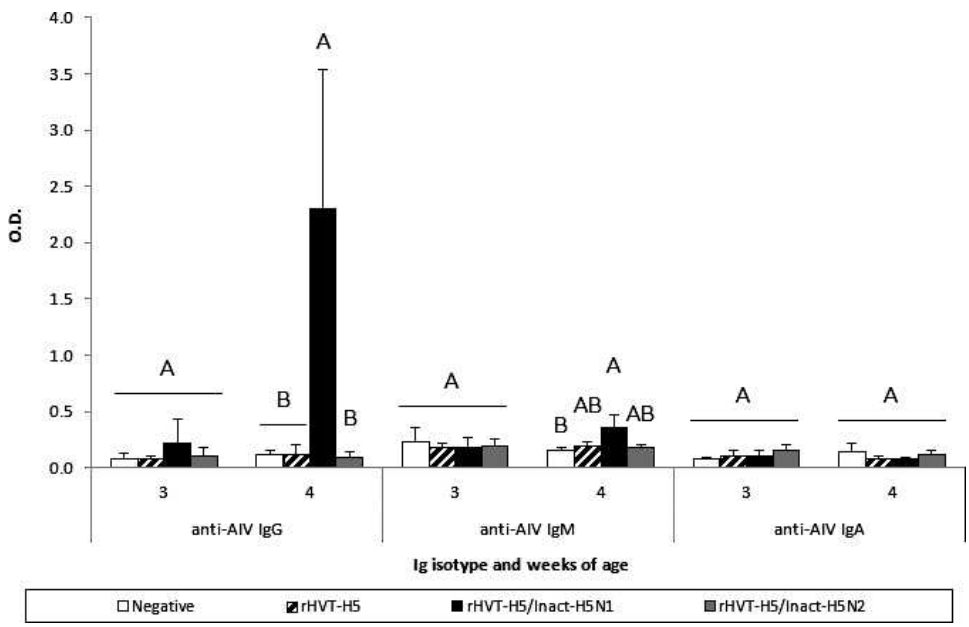

Fig. 3. AIV-specific IgG, IgM, and IgA detection by ELISA on supernatant of *ex vivo* duodenal tissues culture from MDA+ chickens vaccinated with the rHVT-H5 and the inact-H5N1/H5N2 vaccines according to different vaccination schedules. Data represent mean ± standard deviation of absorbance values determined on samples diluted 1:2 by ELISA at specified time of age ($n = 5$) in trial II. Means ± standard deviations with no common superscript differ significantly ($P < 0.05$).

mostly humoral immunity (5,10,13), and their application at early age is known to be sensitive to the interference of MDA (1,6,12). To the contrary, because the HVT vaccines replicate in a highly cell-associated manner in lymphocytes, this delivery system might be stimulating a high degree of CMI (7) and a slower humoral response, as shown previously for an rHVT-ND vaccine (17). This cell-associated form is also well known to be hardly sensitive to interference with MDA. Finally, HVT establishes a persistent viremia in chickens for at least 8 wk following vaccination (20,21), offering the advantage of delivering foreign antigens to the immune system of vaccinated birds during an extended period of time (23), and it is therefore expected to induce a longer-lasting immunity.

In comparison with the rHVT-H5/inact-H5N2 regime, the rHVT-H5/inact-H5N1 prime/boost vaccination program induced a higher level of humoral and mucosal IgG and IgM as well as a stronger CMI. This suggests a better effect that is probably explained by a higher homology between the H5 included in the rHVT-H5 and the inact-H5N1 vaccines. Despite these observations, the theoretical potential interest of producing a tailor-made rHVT-H5 vaccine to possibly increase the level of protection could be still worth investigating. However, one has to realize that this may not be the optimal solution because of the antigenic variety and continuous variations of the AIV strains simultaneously present in a country where the disease is endemic, and that probably partially explains the failures recorded with classical inactivated vaccines and the persistence of the infection. The other major obstacle to this approach lies in the delay, the difficulties, and the cost of the registration process of such specific vaccines, which would still be considered by licensing authorities as totally new products. This would annihilate the interest and probably the potential increase in efficacy of such an approach.

In conclusion, the rHVT-H5 vaccine appeared very promising for use in day-old chickens possessing MDA to AIV. The prime/boost rHVT-H5/inact-H5N1 schedule vaccination could increase the immunity and could therefore be recommended in areas with high infection pressure for optimal protection. The HVT offers the advantage of delivering foreign antigens to the immune system of vaccinated birds during an extended period of time; however, further studies with commercial chickens are required to investigate the onset and the duration of immunity induced by this rHVT-H5 vaccine.

REFERENCES

1. Abdelwhab, E. M., C. Grund, M. M. Aly, M. Beer, T. C. Harder, and H. M. Hafez. Influence of maternal immunity on vaccine efficacy and susceptibility of one day old chicks against Egyptian highly pathogenic avian influenza H5N1. Vet. Microbiol. 155(1):13–20. 2012.

2. Abdelwhab, E. M., C. Grund, M. M. Aly, M. Beer, T. C. Harder, and H. M. Hafez. Multiple dose vaccination with heterologous H5N2 vaccine: immune response and protection against variant clade 2.2.1 highly pathogenic avian influenza H5N1 in broiler breeder chickens. Vaccine 29(37):6219–6225. 2011.

3. Aly, M. M., A. Arafa, and M. K. Hassan. Epidemiological findings of outbreaks of disease caused by highly pathogenic H5N1 avian influenza virus in poultry in Egypt during 2006. Avian Dis. 52:269–277. 2008.

4. Balish, A. L., C. T. Davis, M. D. Saad, N. El-Sayed, H. Esmat, J. A. Tjaden, K. C. Earhart, L. E. Ahmed, M. Abd El-Halem, A. H. Ali, S. A. Nassif, E. A. El-Ebiary, M. Taha, M. M. Aly, A. Arafa, E. O'Neill, X. Xiyan, N. J. Cox, D. Ro, and A. I. Klimov. Antigenic and genetic diversity of highly pathogenic avian influenza A (H5N1) viruses isolated in Egypt. Avian Dis. 54(Suppl. 1):329–334. 2010.

5. Bermudez, A. J., and B. Stewart-Brown. Disease prevention and diagnostic. In: Disease of poultry, 11th ed. Y. M. Saif, ed. Iowa State University Press. pp. 17–55. 2003.

6. De Vriese, J., M. Steensels, V. Palya, Y. Gardin, K. Dorsey Moore, B. Lambrecht, S. Van Borm, and T. van den Berg. Passive protection afforded by maternally-derived antibodies in chickens and the antibodies' interference with the protection elicited by avian influenza–inactivated vaccines in progeny. Avian Dis. 54(Suppl. 1):246–252. 2010.

7. Heller, E. D., and K. A. Schat. Enhancement of natural killer cell activity by Marek's disease vaccines. Avian Pathol. 16(1):51–60. 1987.

8. Kapczynski, D. R., and D. E. Swayne. Influenza vaccines for avian species. Curr. Top. Microbiol. 333:133–152. 2009.

9. Kim, J. K., G. Kayali, D. Walker, H. L. Forrest, A. H. Ellebedy, Y. S. Griffin, A. Rubrum, M. M. Bahgat, M. A. Kutkat, M. A. A. Ali, J. R. Aldridge, N. J. Negovetich, S. Krauss, R. J. Webby, and R. G. Webster. Puzzling inefficiency of H5N1 influenza vaccines in Egyptian poultry. Proc. Nat. Acad. Sci. USA 107(24):11044–11049. 2010.

10. Lambrecht, B., M. Gonze, G. Meulemans, and T. van den Berg. Assessment of the cell-mediated immune response in chickens by detection of chicken interferon-gamma in response to mitogen and recall Newcastle disease viral antigen stimulation. Avian Pathol. 33:343–350. 2004.

11. Lee, C. W., D. A. Senne, and D. L. Suarez. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J. Virol. 78(15):8372–8381. 2004.

12. Maas, R., S. Rosema, D. van Zoelen, and S. Venema. Maternal immunity against avian influenza H5N1 in chickens: limited protection and interference with vaccine efficacy. Avian Pathol. 40(1):87–92. 2011.

13. Marangon, S., and L. Busani. The use of vaccination in poultry production. Rev. Sci. Tech. Off. Int. Epiz. 26(1):265–274. 2006.

14. Peyre, M., G. Fusheng, S. Desvaux, and F. Roger. Avian influenza vaccines: a practical review in relation to their application in the field with a focus on the Asian experience. Epidemiol. Infect. 137:1–21. 2009.

15. Poetri, O., A. Bouma, I. Claassen, G. Koch, R. Soejoedono, A. Stegeman, and M. van Boven. A single vaccination of commercial broilers does not reduce transmission of H5N1 highly pathogenic avian influenza. Vet. Res. 42(1):74–86. 2011.

16. Rauw, F., S. Anbari, T. van den Berg, and B. Lambrecht. Measurement of systemic and local respiratory cell-mediated immunity after influenza infection in chickens. Vet. Immunol. Immunopathol. 143:27–37. 2011.

17. Rauw, F., Y. Gardin, V. Palya, S. Anbari, S. Lemaire, M. Boschmans, T. van den Berg, and B. Lambrecht. Improved vaccination against Newcastle disease by an *in ovo* recombinant HVT-ND combined with an adjuvanted live vaccine at day-old. Vaccine 28:823–833. 2010.

18. Rauw, F., Y. Gardin, V. Palya, S. Van Borm, M. Gonze, S. Lemaire, T. van den Berg, and B. Lambrecht. Humoral, cell-mediated and mucosal immunity induced by oculo-nasal vaccination of one-day-old SPF and conventional layer chicks with two different live Newcastle disease vaccines. Vaccine 27:3631–3642. 2009.

19. Rauw, F., V. Palya, S. Van Borm, S. Welby, T. Tatar-Kis, Y. Gardin, K. Moore Dorsey, M. M. Aly, M. K. Hassan, M. A. Soliman, B. Lambrecht, and T. van den Berg. Further evidence of antigenic drift and protective efficacy afforded by a recombinant HVT-H5 vaccine against challenge with two antigenically divergent Egyptian clade 2.2.1 HPAI H5N1 strains. Vaccine 29:2590–2600. 2011.

20. Reddy, S. K., J. M. Sharma, J. Ahmad, D. N. Reddy, J. K. McMillen, S. M. Cook, M. A. Wild, and R. D. Schwartz. Protective efficacy of a recombinant herpesvirus of turkeys as an *in ovo* vaccine against Newcastle and Marek's diseases in specific-pathogen-free chickens. Vaccine 14(6): 469–477. 1996.

21. Sharma, J. M., and B. R. Burmester. Resistance to Marek's disease at hatching in chickens vaccinated as embryos with the turkey herpesvirus. Avian Dis. 26(1):134–149. 1982.

22. Swayne, D. E. Principles for vaccine protection in chickens and domestic waterfowl against avian influenza: emphasis on Asian H5N1 high pathogenicity avian influenza. Ann. NY. Acad. Sci. 1081:174–181. 2006.

23. Tsukamoto, K., S. Saito, S. Saeki, T. Sato, N. Tanimura, T. Isobe, M. Mase, T. Imada, N. Yuasa, and S. Yamaguchi. Complete, long-lasting

protection against lethal infectious bursal disease virus challenge by a single vaccination with an avian herpesvirus vector expressing VP2 antigens. J. Virol. 76(11):5637–5645. 2002.

24. Van Borm, S., M. Steensels, H. L. Ferreira, M. Boschmans, J. De Vriese, B. Lambrecht, and T. van den Berg. A universal avian endogenous real-time reverse transcriptase–polymerase chain reaction control and its application to avian influenza diagnosis and quantification. Avian Dis. 51:213–220. 2007.

ACKNOWLEDGMENTS

The study was financially supported by CEVA Santé Animale. We thank M. Aly and G. Cattoli for providing the Egyptian strains. We gratefully acknowledge E. Ngabirano for qualified technical work (serologic and molecular tests) and C. Delgrange and M. Vandenbroeck for qualified technical assistance in the bird experiments (animal handling and sampling assistance).