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An ELISA for detection of antibodies against influenza A nucleoprotein in humans and various animal species

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Summary. A double antibody sandwich blocking ELISA, using a monoclonal antibody (MAb) against influenza A nucleoprotein (NP) was developed to detect antibodies against influenza. Collections of serum samples were obtained from human and various animal species. All influenza A subtypes induced antibodies against hemagglutinins and NP. A close correlation between titers of the hemagglutination inhibition (HI) test and the NP-ELISA was seen. Antibodies against influenza NP were demonstrated in serum samples from humans, ferrets, swine, horses, chickens, ducks, guinea pigs, mice, and seals. The serum samples were collected at intervals during prospective epidemiological studies, from experimental and natural infections, and vaccination studies. The decline of maternal antibodies was studied in swine and horses. The NP-ELISA enables rapid serological diagnosis and is suited for influenza A antibody screening, especially in species which harbor several influenza subtypes. The HI and neuraminidase inhibition tests, however, must still be used for subtyping.

Introduction

Influenza serotypes A, B and C are defined by the antigenic differences of their nucleoprotein (NP) and matrix protein. In animal species, influenza A viruses are most prevalent and are further grouped into different subtypes according to their specific hemagglutinin (H) and neuraminidase (N) surface proteins. The NP of influenza viruses was thought to be stable, but studies with monoclonal antibodies (MAb) have detected point mutations and reassortment of the NP genes of different influenza A strains [5, 19, 27]. The genomic variability may explain differences in host specificity between strains of the same HN subtype

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[3, 20]. Comparison of genomic NP sequences of H10 strains recovered from mink and chickens, however, showed constant regions [15].

Previously we described a double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) for influenza A antigen detection using one MAb directed to a conserved antigenic determinant of NP of influenza A viruses [21]. Using the same MAb, we developed a blocking DAS-ELISA to detect antibodies raised by influenza A NP in various animal species (NP-ELISA).

In influenza diagnostics by ELISA, plates are usually coated with antibodies against hemagglutinins and the conjugates are directed against immunoglobulins [1, 4, 6, 8, 12, 22, 23, 25]. We used one MAb directed against influenza NP for coating and for preparing the conjugate. Serum samples collected from humans, ferrets, swine, horses, chickens, ducks, mice and seals were used to compare the NP-ELISA with the hemagglutination inhibition (HI) test. The serum samples were collected at intervals during prospective epidemiological studies, experimental and natural infections, and vaccination studies. In addition, samples were collected from mares and sows and their offspring to study the decline of maternal antibodies.

Materials and methods

Serum samples

Ferrets

15 ferrets were intranasally infected with various strains of influenza serotypes A and B, described in Table 1. Five ferrets were not infected and served as negative controls. Serum samples were collected 2 to 3 weeks post infection.

Humans

55 human volunteers were intramuscularly vaccinated with three commercial trivalent vaccines containing A/Leningrad/360/86 (H3N2), A/Singapore/6/86 (H1N1), and B/Ann Arbor/1/86. A subunit (Duphar), a whole virus (Duphar), and a split-virus vaccine (Rhône Mérieux) were compared. Serum samples were collected before and 3 weeks post vaccination.

Swine

Two specified-pathogen-free (SPF) pigs were twice intranasally infected with A/swine/Netherlands/25/80 (H1N1) and another two with A/swine/Netherlands/1/85 (H3N2). Serum samples were collected before inoculation and at 11, 18, 22 and 29 days post infection, and again at 7 and 21 days after the booster infection.

During a prospective study, serum samples were collected at 3-week intervals in a closed farm with breeding stock and fattening pigs. A group of 16 fattening pigs of this farm was tested by NP-ELISA after the HI test had revealed a subclinical infection with an H3N2 subtype.

Six groups of 29 conventional pigs were twice vaccinated with six different experimental vaccines, which contained different concentrations of purified influenza A whole virus antigen of subtype H1N1 (A/swine/Netherlands/25/80), 8 to 12 µg hemagglutinin, and subtype H3N2 (A/Philippines/2/82 and A/Port Chalmers/1/73), respectively 4 to 6 µg hemagglutinin. The experimental vaccines were all formulated as an oil-in-water emulsion. The HI and ELISA titers were compared 2 weeks post vaccination. A sow which was

vaccinated twice with a vaccine containing the same H1N1 and H3N2 subtypes, received a booster vaccination 2 weeks before parturition. Serum samples of her litter of twelve piglets were collected at 2, 7, 8, and 10.5 weeks of age.

Horses

Ten foals were serologically monitored by HI test and NP-ELISA from the day of parturition until 5.5 months of age. Their dams had been routinely vaccinated twice a year with inactivated vaccines, containing A/equi/Prague/1/56 (H1N7), A/equi/Miami/1/63 (H3N8), and A/equi/Kentucky/81(H3N8) and had received the same vaccine 4 to 6 weeks before parturition.

Chickens, ducks, guinea pigs, mice, and seals

Nine SPF chickens were twice intramuscularly infected with $10^4 EID_{50}$ A/duck/Ukraine/1/63 (H3N8), at day 1 and at day 35. Serum samples were collected two weeks after the first (day 14) and three weeks after the second vaccination (day 56).

65 sera were collected from wild ducks during the hunting season and from materials submitted to the Regional Animal Health Centre in Velp and our laboratory. Sera were screened in the NP-ELISA at dilution of 1 : 2.

16 guinea pigs were vaccinated twice at 3-week intervals with eight experimental vaccines to be used in horses. These vaccines were of three different types: 5 whole virus, 2 subunit, and one iscom (immunostimulating-complex) vaccine, which all contained viral components of the subtypes H7N7 and H3N8. The guinea pigs received 1% of the dosage that is given to horses. The HI and ELISA titers were compared at 2 weeks after the second vaccination.

To produce MAb against influenza NP, four BALB/c mice were intraperitoneally inoculated at 4-week intervals with 100 μg density gradient-purified A/chicken/Netherlands/85 (H1N1) in incomplete Freund's adjuvant, 50 μg A/Hongkong/68 (H3N2) in complete Freund's adjuvant, and intravenously with 50 μg A/duck/Ireland/84 (H5N8). Serum samples were collected 10 weeks after the second booster.

We received a large collection of serum samples collected from seals and sealions: 757 serum samples were collected from seals in the North Sea during the 1988 epidemic attributed to a Morbilli virus [13], and 338 serum samples were collected from seals and sealions in the Beringsea between 1978 and 1988. The sera were screened in the NP-ELISA in a dilution of 1 : 20. Serum samples that were positive in the NP-ELISA were tested in the HI test after treatment with Vibrio Cholerae filtrate and kaolin.

Serological techniques

ELISA

Monoclonal antibody, conjugate, and substrate. A hybridoma cell line that produces MAb against a conserved antigenic NP determinant of influenza A was obtained from the American Type Culture Collection (ATCC No.: HB65, H16-L10-4R5). This hybridoma was originally established by Yewdell et al. [28] who used influenza A/WSN/33 (H1N1) and A/PR8/34 (H1N1) to immunize BALB/c mice. This MAb was used for coating of the ELISA plates (Costar Cat. No. 3590) and for preparing the conjugate with horseradish peroxidase; 3,3, 5,5 tetramethylbenzidine (TMB) (Sigma Cat. No. T 2885) was used as substrate. The techniques used for these manipulations have been described before [21].

Antigen. Influenza A/Japan/305/57 (H2N2) was grown in 11-day-old embryonated eggs of SPF chickens. After 4 days, the allantoic fluid was harvested and centrifuged at 2000 rpm for 10 min. To disrupt the virus particles, the clarified allantoic fluid was treated with equal amounts of 1% Nonidet P40 in phosphate-buffered saline (PBS) for 1 h at 37 °C.

NP-ELISA

Polystyrene 96-well microtiter plates were coated with MAb HB65 directed against NP of influenza A in a dilution of 1 : 2,000 in 100 µl coating buffer for 18 h at 37 °C. The coating buffer was prepared by adding Na_2CO_3, 5.28 g/l to $NaHCO_3$, 4.2 g/l until a pH of 9.6 was obtained. The plates were washed two times with 0.05% Tween 80 in tap-water and were sealed after 100 µl of 87% glycerol was added. The plates could be stored at 4 °C for several months.

The NP-ELISA was performed as follows. After washing of the plates, 50 µl of the undiluted test serum was mixed in the test wells with 50 µl of antigen diluted 1 :10 in ELISA buffer. The ELISA buffer contained per liter: 59 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.05% Tween 80, and 4% calf serum (Gibco Ltd., Cat. No. 021-06010 M). After incubation at 37 °C for 1 h, 100 µl horseradish peroxidase conjugate, diluted 1 :1000 in ELISA buffer, was added to each well and plates were further incubated at 37 °C for 1 h. After two washing steps, 100 µl of TMB substrate [21] was added and incubated at room temperature for 10 min. The reaction was stopped by adding 100 µl H_2SO_4 2M. The extinctions were measured at 450 nm with a micro ELISA reader (Multiscan, Flow). A reduction of the extinction greater than 50% of the maximum extinction was considered positive. The maximum extinction was determined as the mean extinction of eight wells that were filled with ELISA buffer instead of serum. An equine hyperimmune serum was used as positive control.

Hemagglutination inhibition test

The HI tests were performed in three laboratories, basically according to standard procedures. The HI test used to detect antibodies in human and ferret sera was described in detail previously [11a]. Before being tested in the HI test, all sera were treated with Vibrio Cholerae filtrate to reduce false-positive reactions. The serum samples from seals and sealions were also treated with kaolin.

Results

The NP-ELISA detected antibodies against influenza A nucleoprotein in all ten ferrets that were infected with human isolates of subtypes H1N1 and H3N2. Serum samples from influenza B-infected ferrets were negative in the ELISA. Hemagglutinating antibodies were detected in all infected ferrets at 2 to 3 weeks post infection (Table 1). In a group of 19 human volunteers, mean NP-ELISA titers (mean = 83) were not increased by vaccination with hemagglutinin subunit vaccine, unlike mean HI antibody titers, which were. Vaccination with whole virus and split virus vaccines, however, resulted in an increase of NP-ELISA titers, from 77 to 107 and from 80 to 136, within a 3-week period. The split virus vaccine yielded the strongest antibody response as measured by both the HI test and NP-ELISA (Table 2).

In pigs, both types of antibodies were induced after experimental infection with H1N1 and H3N2 subtypes (Fig. 1) and after natural exposure to H3N2 (Fig. 2). The HI response against experimental infection with H3N2 was stronger than against the H1N1 subtype. The NP-ELISA titers increased slowly after booster vaccination with both subtypes of swine influenza virus. The increase of NP-ELISA antibodies during the subclinical infection was as slow as after experimental exposure. The first indication of subclinical infection was HI

Table 1. Detection of antibodies against influenza A by HI test and NP-ELISA in serum samples from ferrets

Ferret no.	Infected with[a]	HI		NP-ELISA titer[c]
		subtype	titer[b]	
1	A/Brazil/11/78	H1N1	768	⩾256
2	A/Netherlands/198/80	H3N2	8,689	⩾256
3	A/Netherlands/204/80	H1N1	2,172	128
4	A/Netherlands/216/81	H3N2	4,344	⩾256
5	A/India/6263/80	H1N1	1,086	64
6	A/Netherlands/252/82	H3N2	17,375	⩾256
7	A/Netherlands/249/82	H3N2	7,741	⩾256
8	A/Netherlands/290/83	H1N1	543	64
9	A/Bangkok/1/79	H3N2	15,482	⩾256
10	A/Netherlands/224/81	H1N1	4,344	128
11	B/Netherlands/200/80		272	<2
12	B/Netherlands/201/80		1,086	<2
13	B/Netherlands/223/81		2,172	<2
14	B/Hong Kong/1/83		136	<2
15	B/Netherlands/318/84		4,344	<2
16–20	None (controls)		<9	<2

[a] Ferrets were twice intranasally infected with an interval of 4 weeks
[b] Reciprocal of HI titer against homologous antigens at 2–3 weeks p.i.
[c] Reciprocal of NP-ELISA titer at 2–3 weeks p.i.

Table 2. Influenza A antibodies as detected by HI test and NP-ELISA in serum samples from 55 human volunteers before and after vaccination with three types of influenza vaccine

	Type of vaccine					
	subunit (19[a])		whole virus (19)		split virus (17)	
	Titer[b]					
	pre	post[c]	pre	post	pre	post
HI						
A/Leningrad/360/86 (H3N2)	25	335	22	604	19	589
A/Singapore/6/86 (H1N1)	17	115	8	205	9	420
B/Ann Arbor/1/86	74	371	84	344	41	557
NP-ELISA	83	86	77	107	80	136

[a] No. of individuals vaccinated with an influenza subunit vaccine
[b] Reciprocal of geometrical mean titer (GMT)
[c] Serum samples were collected after 3 weeks

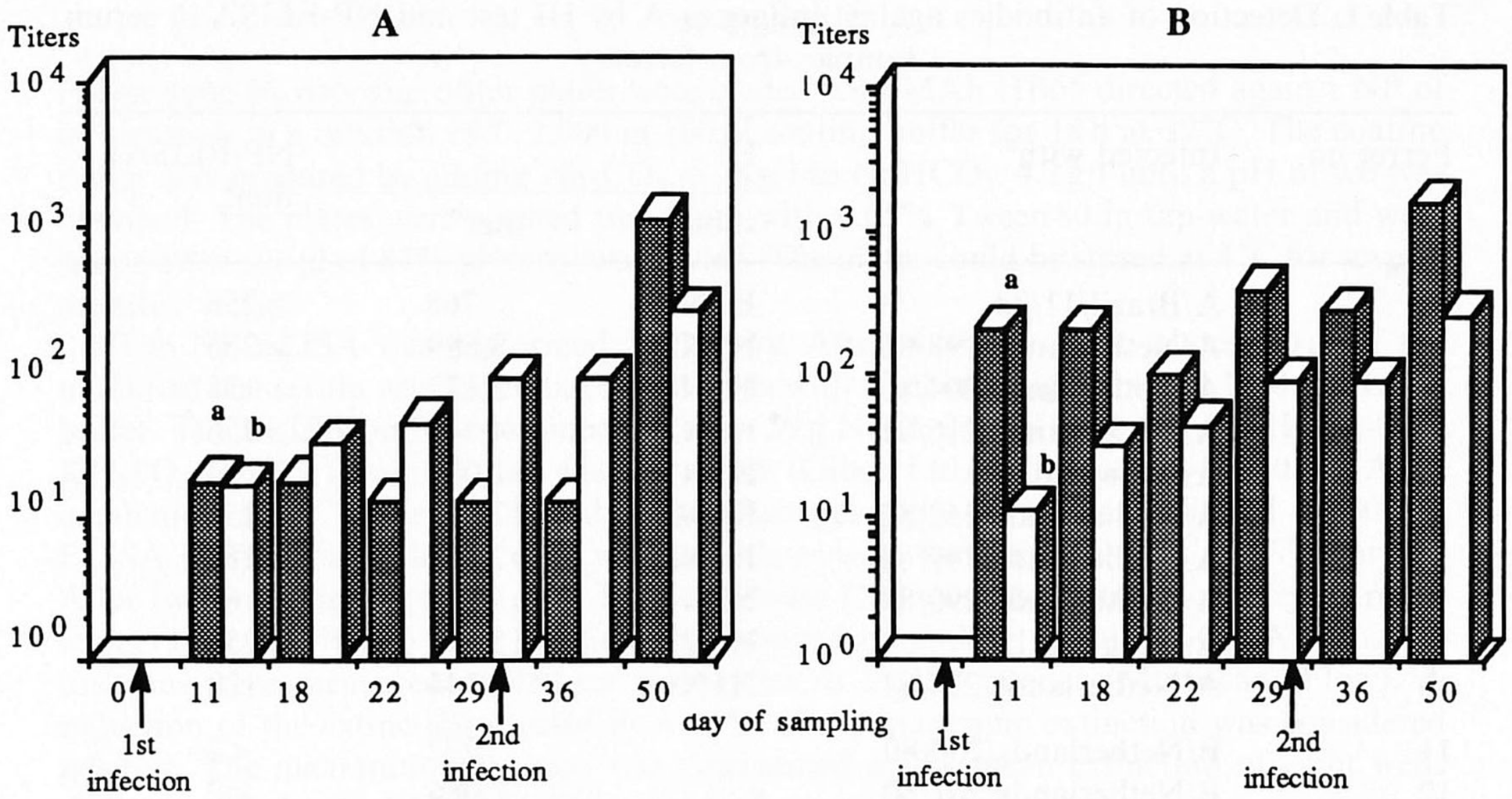

Fig. 1. Influenza antibodies detected by HI test and NP-ELISA after intranasal infection of 2 pigs with A/swine/Netherlands/80 (H1N1) **(A)** or of another with A/swine/Netherlands/85 (H3N2) **(B)**. *a* Reciprocal of mean HI titer against H1N1 (**A**) or H3N2 (**B**); *b* reciprocal of mean NP-ELISA titer

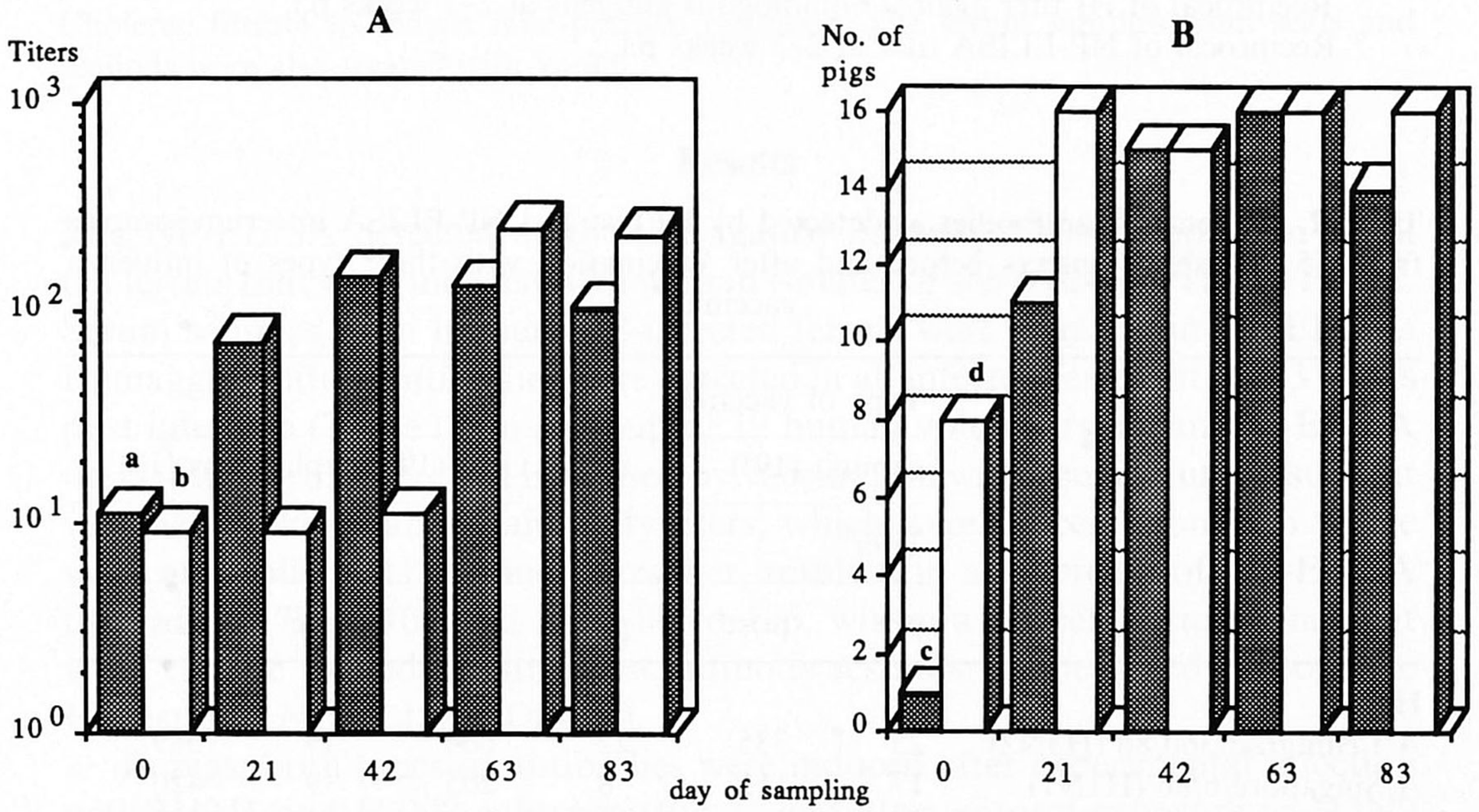

Fig. 2. Natural infection with influenza A subtype H3N2 in 16 fattening pigs in a closed farm. **A** *a* Reciprocal of geometric mean HI titer against H3N2, *b* reciprocal of geometric mean NP-ELISA titer. **B** *c* No. of pigs with HI antibodies, *d* no. of pigs with antibodies against NP

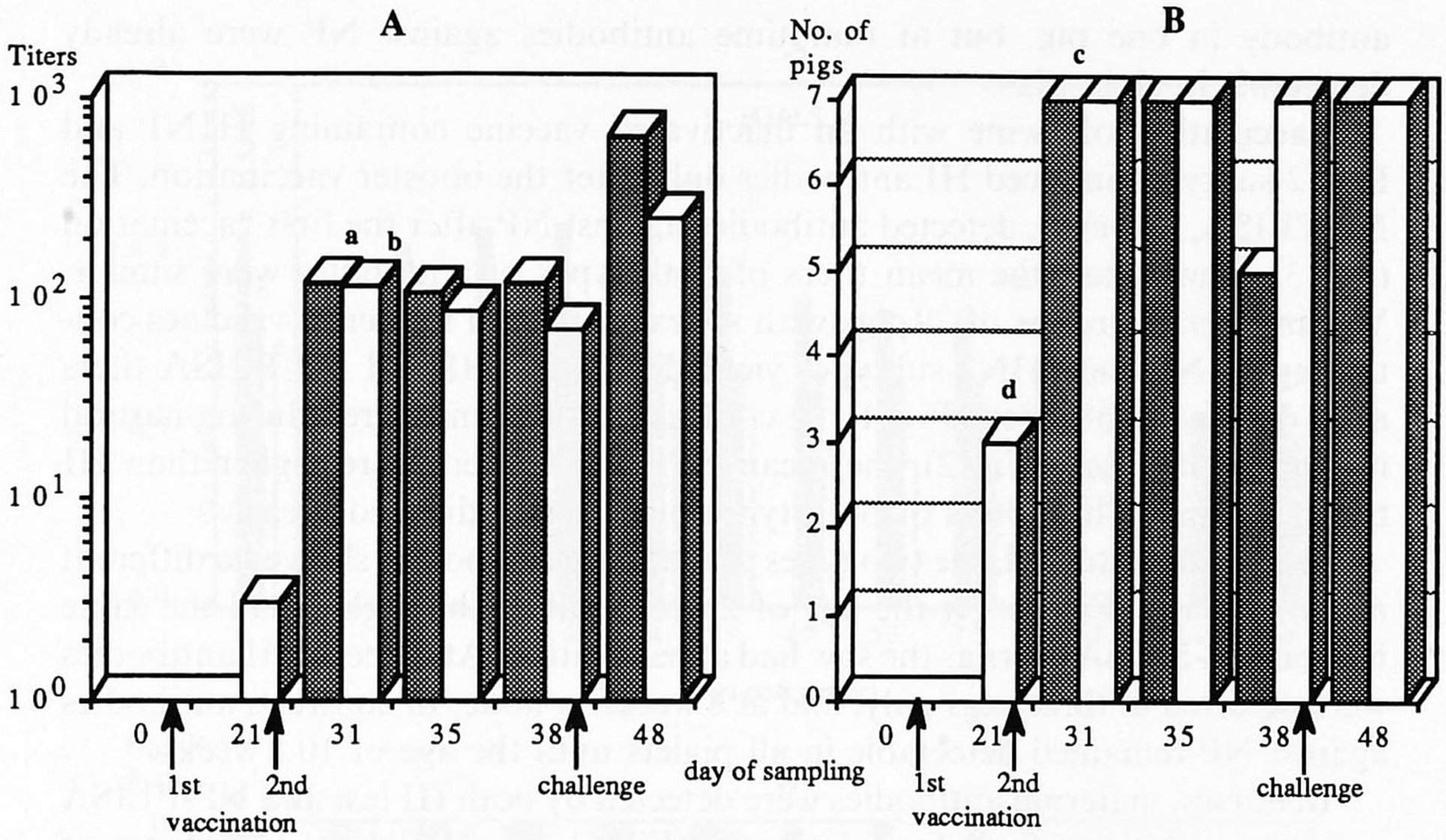

Fig. 3. Seven pigs were twice vaccinated with an inactivated influenza A vaccine, containing subtypes H1N1 and H3N2, and were challenged with A/swine/Netherlands/80 (H1N1) at 17 days after the second vaccination. **A** *a* Reciprocal of geometric mean HI titer against H1N1, *b* reciprocal of geometric mean NP-ELISA titer. **B** *c* No. of pigs with HI antibodies, *d* no. of pigs with antibodies against NP

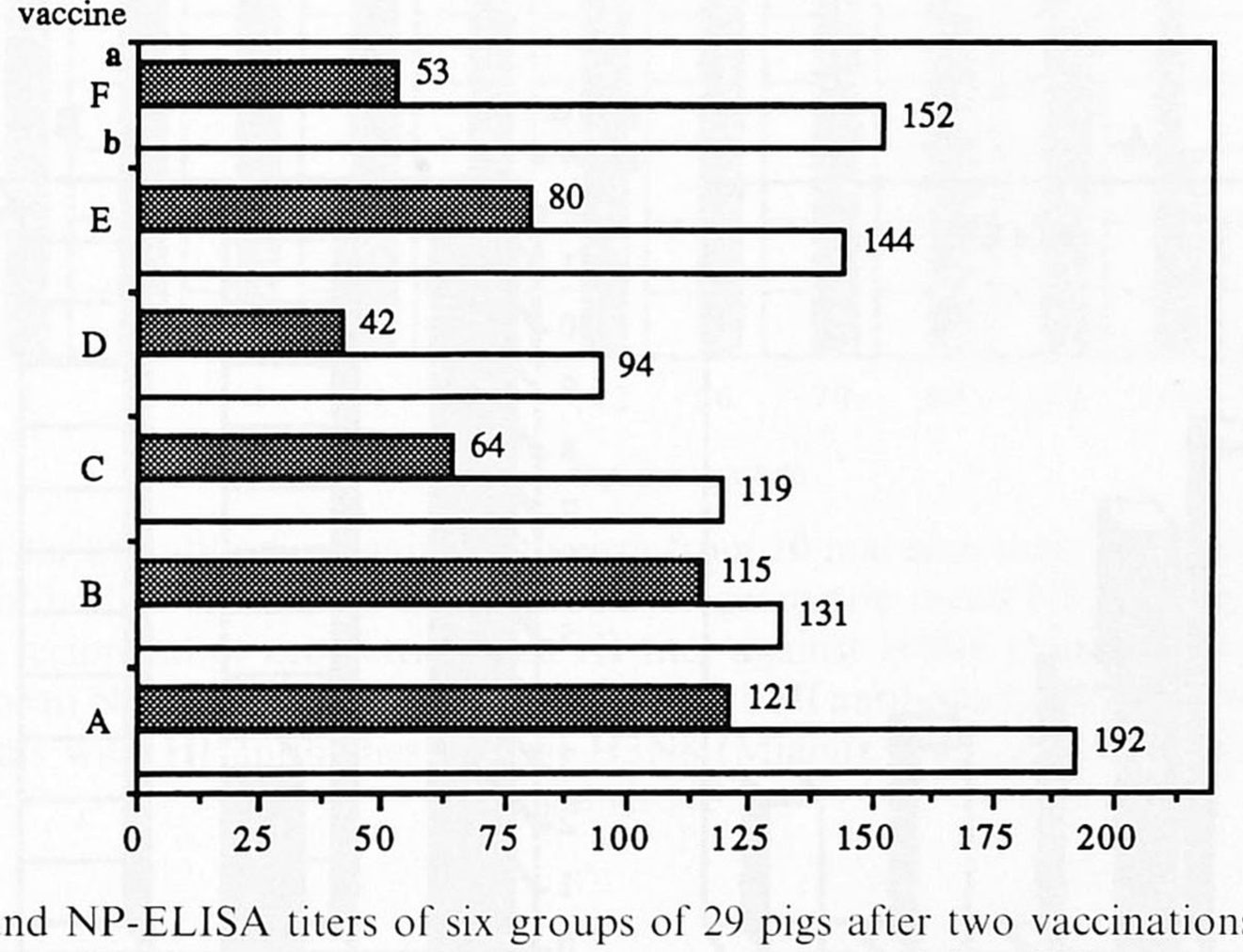

Fig. 4. HI and NP-ELISA titers of six groups of 29 pigs after two vaccinations, with a 3-week interval, with six experimental influenza vaccines, containing different hemagglutinin concentrations of H1N1 and H3N2 subtypes. The hemagglutinin concentrations of H1N1 vaccines ranged from 8 to 12 µg per dosis, those of H3N2 vaccines from 4 to 6 µg. Influenza A/swine/Netherlands/25/80 (H1N1) and A/Philippines/2/82 and A/Port Chalmers/1/73 (H3N2) were used. *a* Geometric mean HI titer of serum samples of 29 pigs at 2 weeks after the 2nd vaccination, *b* geometric mean NP-ELISA titer of serum samples of 29 pigs at 2 weeks after the 2nd vaccination

antibody in one pig, but at that time antibodies against NP were already detectable in eight pigs.

Vaccination of swine with an inactivated vaccine containing H1N1 and H3N2 subtypes induced HI antibodies only after the booster vaccination. The NP-ELISA, however, detected antibodies against NP after the first vaccination (Fig. 3). Thereafter, the mean titers of both types of antibodies were similar. Vaccination of groups of 29 pigs with six experimental influenza vaccines containing H1N1 and H3N2 subtypes yielded different HI and NP-ELISA titers at 35 days post infection (Fig. 4). In contrast to titers measured during natural influenza infections (Fig. 2), the mean NP-ELISA titers were higher than HI titers, but individual titers of both types of antibody differed greatly.

In 12 piglets studied, the two types of maternal antibodies showed a different rate of decline (Fig. 5). At the age of 2 weeks, all of the piglets had the same HI and NP-ELISA titers as the sow had at parturition. At 7 weeks, HI antibodies were detected in three pigs only, and at 8 weeks in none. In contrast, antibodies against NP remained detectable in all piglets until the age of 10.5 weeks.

In horses, maternal antibodies were detected by both HI test and NP-ELISA and persisted in ten foals for 5.5 months (Fig. 6). The decline of both types of maternal antibodies was parallel. The correlations between HI and NP-ELISA titers for subtypes H7N7 and H3N8 were 93% and 91%.

All nine chickens that were twice infected with influenza A/duck/Ukraine/1/63 (H3N8) developed antibodies as detected by both HI test and NP-ELISA (Table 3).

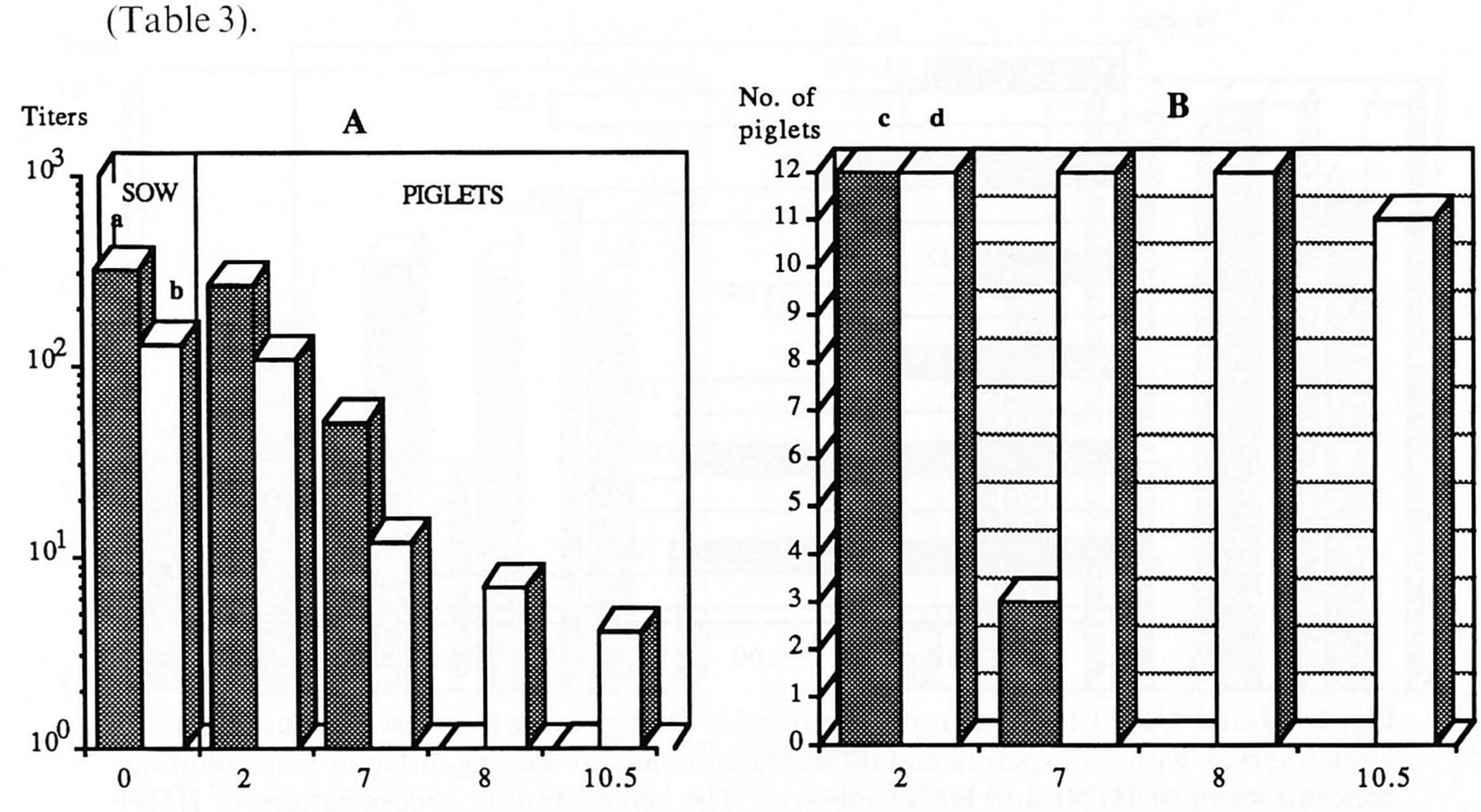

Fig. 5. Transfer of antibodies from a sow to her 12 piglets and decline of maternal antibodies in piglets. **A** *a* Reciprocal of geometric mean HI titer against H1N1, *b* reciprocal of geometric mean NP-ELISA titer. **B** *c* No. of piglets with HI antibodies, *d* no. of piglets with antibodies against NP

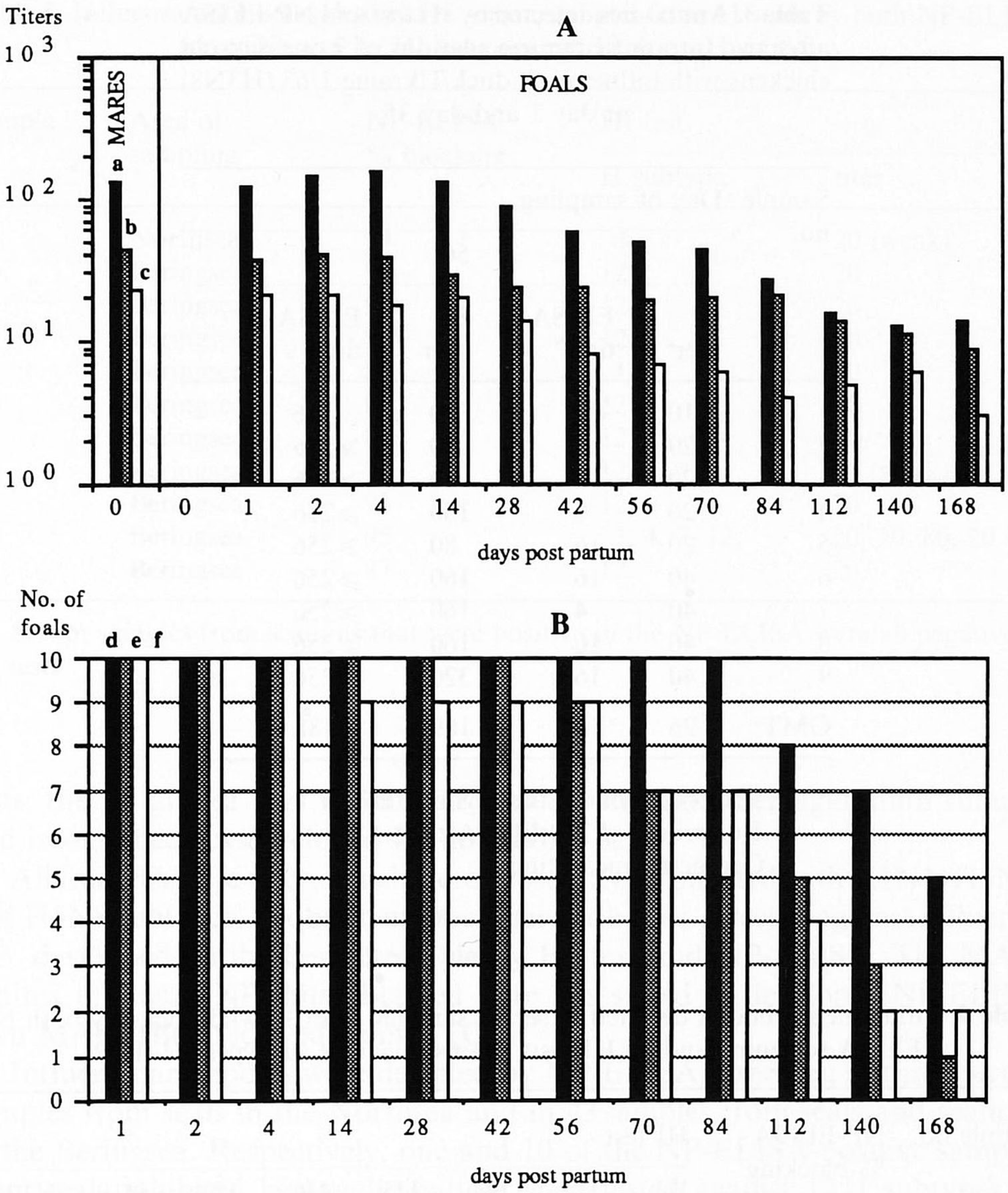

Fig. 6. Transfer of antibodies against influenza from 10 mares to their foals and decline of maternal antibodies in foals. **A** *a* Reciprocal of geometric mean HI titer against H7N7 (Prague), *b* reciprocal of geometric mean HI titer against H3N8 (Miami), *c* reciprocal of geometric mean NP-ELISA titer. **B** *d* No. of foals with HI antibodies against H7N7 (Prague), *e* no. of foals with HI antibodies against H3N8 (Miami), *f* no. of foals with antibodies against NP

In wild ducks antibodies were detected by NP-ELISA in 15 out of 63 serum samples. 14 of these samples were also positive in the HI test. The following influenza H subtypes were detected by HI tests: H1, H2, H4, H5, H6, H9, H10, and H12 (Table 4).

The ten guinea pigs, which had been twice vaccinated with five experimental vaccines containing H7N7 and H3N8 subtypes, had a seroresponse in both

Table 3. Antibodies detected by HI test and NP-ELISA after two intramuscular inoculations of 9 one-day-old chickens with influenza A/duck/Ukraine/1/63 (H3N8) at day 1 and day 35

Sample no.	Day of sampling			
	14		56	
	HI titer[a]	ELISA titer[b]	HI titer	ELISA titer
1	< 10	< 2	20	16
2	20	4	80	⩾ 256
3	20	8	80	⩾ 256
4	20	8	160	⩾ 256
5	20	16	80	⩾ 256
6	40	16	160	⩾ 256
7	40	4	160	⩾ 256
8	40	16	160	⩾ 256
9	40	16	320	⩾ 256
GMT[c]	26	10	109	188

[a] Reciprocal of HI titer against H3N8
[b] Reciprocal of NP-ELISA titer
[c] Geometric mean titer

Table 4. Influenza antibodies detected in serum samples from 14 wild ducks by both NP-ELISA screening and by HI tests against avian H subtypes H1–H12

Sample no.	NP-ELISA % blocking	HI test							
		H1	H2	H4	H5	H6	H9	H10	H12
1	85	–	–	–	–	9	–	–	–
2	77	–	–	–	–	–	36	–	–
3	66	–	–	–	–	9	–	–	–
4	73	–	–	–	–	–	9	–	9
5	89	–	–	–	–	–	9	–	–
6	89	–	9	–	–	9	–	–	–
7	92	36	–	–	–	18	–	36	–
8	81	–	–	–	–	18	9	–	–
9	68	–	–	–	–	9	–	–	–
10	63	–	–	–	–	–	9	–	–
11	92	18	36	–	18	36	–	–	–
12	93	–	18	–	–	9	–	–	–
13	84	–	–	9	–	–	36	–	–
14	62	–	–	–	–	36	–	–	–

Table 5. Influenza antibodies detected in serum samples from 11 seals by both NP-ELISA screening and by HI tests against 12 avian H subtypes

Sample no.	Area of sampling	NP-ELISA % blocking	HI test	
			H subtype	titer
1	Northsea	74	4	20 (weak)
2	Beringsea	93	12	20
3	Beringsea	92	12	20
4	Beringsea	93	12	20
5	Beringsea	87	1	40
6	Beringsea	89	12	40
7	Beringsea	81	12	40
8	Beringsea	77	12	20 (weak)
9	Beringsea	93	12	20
10	Beringsea	95	3, 4, 7, 12	20, 20, 80, 20
11	Beringsea	87	12	20

Serum samples from sealions that were positive in the NP-ELISA were all negative in HI tests

tests; the six guinea pigs vaccinated with experimental hemagglutinin subunit and iscom vaccines developed HI antibodies only.

All four BALB/c mice, which were subsequently infected with H1N1, H3N2, and H5N8 subtypes, to obtain monoclonal antibodies directed against influenza NP, developed antibodies detectable by HI test and NP-ELISA. The MAbs against influenza NP thus obtained were less suited to develop a NP-ELISA than MAb HB65 received from ATCC.

Influenza antibodies were detected by NP-ELISA screening in three serum samples from seals in the Northsea and in 43 samples from seals and sealions in the Beringsea. Respectively, one and 10 of the NP-ELISA-positive samples from seals inhibited hemagglutination when tested against 12 H subtypes of influenza virus. The HI antibodies were directed against subtypes H1, H3, H4, H7, H12 (Table 5). HI antibodies were not detected in the NP-ELISA-positive sera from sealions.

Discussion

The NP-ELISA detected antibodies induced by influenza A infections in humans, ferrets, swine, horses, chickens, ducks, guinea pigs, mice, and seals. A MAb directed against a conserved influenza A nucleoprotein epitope also reacted with numerous influenza A subtypes from various sources [21] and was very useful in developing a broad-spectrum blocking DAS-ELISA to detect antibodies. Because we used the same MAb to coat ELISA plates and prepare the conjugate, a high specificity was achieved [14]. A high sensitivity was

obtained because we used disrupted antigens of a human subtype that showed the strongest binding in DAS-ELISA [21].

In general, antibodies detected by the HI test and NP-ELISA appeared in association with each other. After natural influenza virus infections in ferrets, swine, and chickens, antibody titers detected in the HI test were higher than those directed against NP. The titers of the two types of antibody cannot be compared, however, because the tests detect different types of antibody and have different sensitivities. Vaccination of swine and chickens, however, induced a more consistent and earlier antibody response against NP than against hemagglutinins. As a result of vaccine preparation NP antigens apparently become better exposed. The differences between the six swine influenza vaccines (Fig. 4) were probably caused by the different amounts of influenza antigens incorporated into them and to the various preparation techniques.

In the human population frequent influenza infections cause a natural booster effect by repeated stimulation of antibodies directed against proteins which during evolution remained conserved among the various strains of influenza A viruses. The humoral immune response against nucleoproteins seems not affected by antigenic shift or drift. Antigen presentation of the NP is improved in the split virus vaccines because the influenza virus is disrupted during the preparation of the vaccine. This improvement was demonstrated by higher NP-ELISA titers in those human volunteers that were vaccinated with the split virus vaccine. Of course, the NP-ELISA titers were unaltered after vaccination with hemagglutinin subunit vaccine, but these were increased by vaccination with whole virus and split virus vaccines. These titers, however, increased only minimally, probably because of the presence of pre-existing antibodies directed against NP.

Recently, Rott and Klenk reviewed the prevalence of the different H subtypes of influenza A viruses in humans and animals [17]. They reported that subtype H3 had been found in humans, swine, horses, and birds; H12 was found only in birds. By using the NP-ELISA for screening and the HI test for subtyping, we not only confirmed the prevalence of various avian influenza subtypes in seals [7, 9, 18, 26], but we also observed for the first time that influenza subtype H12 circulates in seals. The discrepancy between NP-ELISA and HI results suggests that more than the known 13 H subtypes are prevalent in nature.

It has only recently been established that cytotoxic T lymphocytes are sensitized by NP in the immune response against influenza virus infection. The cytotoxic T lymphocyte response promotes the clearance of virus and recovery, but does not prevent infection [24, 28, 30]. Cytotoxic T cells may recognize both subtype-specific and cross-reactive determinants of NP. Immunization of mice with NP purified from the recombinant influenza virus X31 protected them against the PR8 strain [29]. Transfer studies showed that stimulated cytotoxic T cells protect animals against lethal challenge, but that in vivo-activated cells fail to move to the lungs [2]. It is likely that B-cell immunity against hemagglutinin is enhanced by T-helper functions stimulated by NP.

Pigs with maternal antibodies against NP had a weaker HI response than those without. It is of interest to study whether the antibody responses against different structural proteins of influenza virus are somehow associated. This might be of importance for the timing of the first vaccination in foals and piglets.

In conclusion, like the DAS-ELISA for antigen detection [21], the NP-ELISA can be used to rapidly differentiate between influenza and Aujeszky's disease in swine, and between influenza and Newcastle disease in fowl. The NP-ELISA is particularly suited to epidemiological screening of animal species that harbor various influenza A subtypes and for the diagnosis in those species to which no vaccines are routinely administered. The HI and neuraminidase inhibition tests, however, which require pre-treatment to eliminate nonspecific reactions, are still necessary for subtyping.

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