Development and Optimization of Immunoassays for the Characterization of Candidate Vaccines to different Clades of Influenza Virus H5N1

Diploma Thesis

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1. INTRODUCTION

1.1. Genetic Structure of H5N1 Influenza Virus

H5N1 virus is a subtype of the species influenza A virus and belongs to the Influenzavirus A genus of the *Orthomyxoviridae* family. Like all other influenza A subtypes, the H5N1 virus is an RNA virus.
H5N1 has a segmented genome that consists of 8 single strands of negative-sense RNA. Viruses are classified by antigenic differences in their nucleoprotein (NP) and matrix protein (M1) into A, B and C groups. Type A viruses like H5N1, are further sub-typed, based on the variations of the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). Currently, there are 16 HA (H1-H16) and 9 NA (N1-N9) identified [11;12;13].

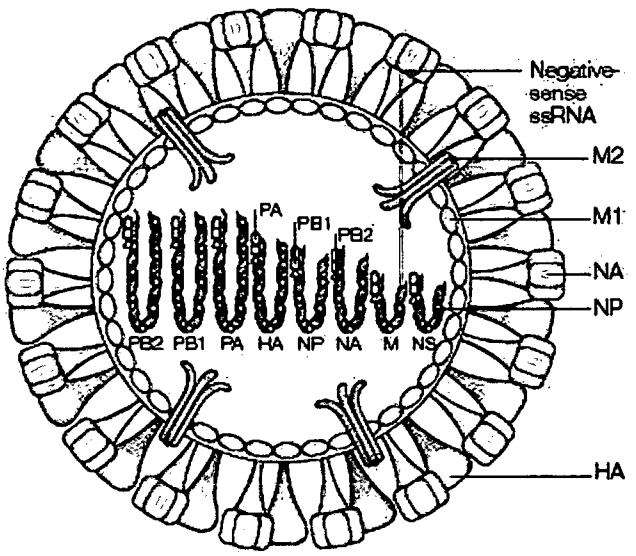


Figure 1.1: structure of influenza A virus (image taken from Horimoto, 2005; [11])

During the viral replication cycle, 11 proteins from the 8 segments of the RNA genome are generated: haemagglutinin (HA), neuraminidase (NA), nucleocapsid protein (NP), polymerase (PB1, PB2 and PA), matrix proteins (M1 and M2) non-structural (NS), and nuclear export (NEP) genes. The location of NEP in the virion is unknown (figure 1.1) [18;27;25].

Influenza A virus particles have a lipid envelope derived from the host cell membrane. The 3 surface glycoproteins (HA, NA and M2) are embedded in the lipid-bilayer of the viral envelope. HA is responsible for attachment of viral particles to host cell surface receptors and facilitates fusion between the viral envelope and the endosomal membrane. NA catalyzes the hydrolysis of such ligands to allow the virus to escape from the cell during the budding process. M2 protein serves as a pH-activated ion channel that enables acidification of the interior of the virion. M2 is

important in the uncoating of viruses and in viral assembly. M1 is the most abundant structural protein which lies beneath the envelope and associates with the ribonucleoprotein (RNP) complex. The 8 single stranded RNA molecules are encapsidated with NP and associated with the 3 RNA polymerase proteins: PB1, PB2 and PA. They are responsible for the transcription and replication of viral RNA. NS is expressed in infected cells. [18;27;25].

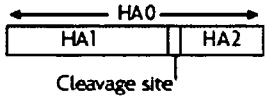


Figure 1.2: haemagglutinin (HA) (image taken from Subbarao, 2007; [25])

The HA glycoprotein is synthesized as an HA0 molecule. Post-translationally HA0 is cleaved into HA1 and HA2 subunits. This cleavage is essential for virus infectivity and membrane fusion (figure 1.2). Only HA0 of highly pathogenic viruses like H5N1 viruses displays multiple basic amino acids at the cleavage site. Thus, it is easily cleaved by ubiquitous intracellular proteases in extrapulmonary sites and as a consequence the virus can replicate in various organs including the brain [25].

1.2. H5N1 - Host Range, Transmission and Pathology

The major reservoir of influenza A viruses are aquatic birds. Normally, influenza viruses are highly species-specific due to the difference in cell receptor binding requirements of the HA molecule (see 1.4). Nevertheless, some subtypes of influenza viruses have crossed the species barrier and became established in certain mammals. Avian influenza viruses have been isolated from pigs, horses, sea mammals, wild waterfowl, cats, poultry and humans [18;29].
The H5N1 subtype of avian influenza virus, or commonly known as "bird flu", is the source of a contagious viral infection that normally affects the fowl but in 1997, in Hong Kong, the first human H5N1-infections have been confirmed [12;29]. All evidence to date indicates that close contact with dead or sick birds combined with a high virus concentration is the principal source of human infection with H5N1 viruses [12;18]. Exposure to an environment that may have been contaminated by faeces from infected birds is a second, though less common source. Although there has been evidence of occasional human-to-human-transmission, to date, neither genetic reassortment with human flu nor a significant change of receptor specifity of the H5N1 virus towards binding to human α2,6-receptor (see 1.4) was confirmed (WHO, May 2007, [21;29]).
In most of the patients the disease caused by H5N1 follows a very aggressive clinical course with rapid deterioration and high fatality. The incubation period for H5N1 avian influenza ranges from 2 to 17 days. The spectrum of clinical symptoms is broad: high fever, Diarrhoea, vomiting, abdominal pain, chest pain, respiratory distress, bleeding from the nose and gums, encephalitis, viral pneumonia not responding to antibiotics and multiorgan dysfunction [21].
Until today 306 laboratory-confirmed cases of H5N1 infections of humans including 185 deaths have been reported to the World Health Organization (WHO). Most of the incidents occurred in Vietnam (93 cases), Indonesia (96 cases), Egypt (34 cases), Thailand (25 cases) and China (24 cases) (WHO, May 2007, [21]).

1.3. Evolution of Influenza Virus

Influenza A viruses are prone to undergo antigenic variations in their HA and NA glycoproteins.
Evolution of influenza viruses occurs due to 2 principal mechanisms. The first is "antigenic shift": a mechanism of gene-reassortment in which genetic material is exchanged between influenza viruses (for example human and avian influenza viruses) during co-infection of an adequate host (for example humans or pigs). This results in the appearance of a novel combination of HA and NA for which human population has no immunity. The second mechanism is "antigenic drift", including point mutations, substitutions, deletions and insertions due to the lack of proofreading among RNA polymerases. Antigenic drift occurs all the time and makes yearly changes in influenza vaccine composition necessary [27;18].

In the past century at least three influenza pandemics have occurred due to antigenic shift and/or antigenic drift: the devastating Spanish influenza pandemic in 1918-1919, which was caused by a H1N1 subtype claimed about 50 million lives, and the Asian (H2N2 subtype, 1957) and Hong Kong (H3N2 subtype, 1968-1969) pandemics, which each resulted in 1-2 million deaths. It was confirmed that the viruses of both the Asian (H2N2) and Hong Kong (H3N2) pandemics were reassortants of genome units between avian and human influenza viruses. In contrast, the 1918 virus (H1N1) which caused the Spanish pandemic may have arisen wholly from an avian influenza virus by adaptive mutations, not by reassortment [29].

During the past 4 years most of the HA sequences of avian H5N1 viruses have separated into 2 distinct phylogenetic clades (genetic groups). Clade 1 viruses were responsible for human infections in Vietnam, Thailand and Cambodia. Clade 2 viruses circulated in Indonesia and China and spread westwards to Middle East, Europe and Africa during 2005. Multiple subclades of clade 2 have evolved, three of them differing in geographical distribution (evolution-tree of the H5N1 haemagglutinin gene see appendix 10.1) [24].

1.4. Receptor Binding Specifity

The first event in infection with influenza virus is the specific binding of the surface glycoprotein haemagglutinin (HA) to cell surface receptors containing glycans with terminal sialic acids (SAs) such as *N*-acetylneuraminic acid. These types of cell surface receptors are present on epithelial cells of either the respiratory tract in humans or the intestine in birds. Binding efficiency is dependent on the type of SA and its linkage to the vicinal galactose of carbohydrates [22].
Human influenza viruses prefer binding to receptors containing *N*-acetylneuraminic acid α2,6-galactose (SAα2,6Gal) linkages whereas avian and equine viruses preferentially bind to those containing *N*-acetylneuraminic acid α2,3-galactose (SAα2,3Gal) linkages [16;17].
Receptor specifity is an important determinant of the host range restriction of influenza viruses. A single amino acid substitution in the viral HA can change the

binding specifity from α2,3 to α2,6 [18]. This switch is believed to be a crucial step in the adaptation of avian viruses to human hosts [22].

1.5. Receptor Binding Specifity Affects the Sensitivity of Haemagglutination Inhibition Assay (HAI)

Haemagglutination inhibition assays (HAI) are used to detect antigen-specific antibodies in serum following vaccination or infection.
HAI tests rely on inhibition of binding between HA of the virus and sialic acid of the erythrocyte by specific anti-HA antibodies of the serum [16]. In fact, HA is the main target antigen of humoral immune response to influenza viruses [27]. Antibody-titres resulting from HAI assays are considered correlates of protection against infection with H5N1 viruses [16;17].

Increasing the number of SAα2,3Gal linkages improves the sensitivity of detection of antibodies to H5N1 avian influenza virus. The proportion of SAα2,3Gal linkages can be increased by change of erythrocyte-species. [16].
Turkey and chicken erythrocytes express a mixture of SAα2,3Gal and SAα2,6 Gal linkages, whereas horse blood contains a high proportion of SAα2,3Gal linkages. The low proportion of SAα2,3Gal linkages on chicken and turkey RBCs which are conventionally used in HAI assays, appear to be responsible for the insensitivity of detecting antibodies against avian influenza viruses. [16;17]

1.6. Inhibitors of Virus Receptor Binding

Beside specific antibodies, soluble macromolecules (for example glycoproteins or proteoglycans) present in animal sera can contain sialic acid residues.
As a consequence, they compete with cellular receptors for virus binding and thus they are able to inhibit virus-mediated haemagglutination (binding of HA to erythrocyte receptors) [23].

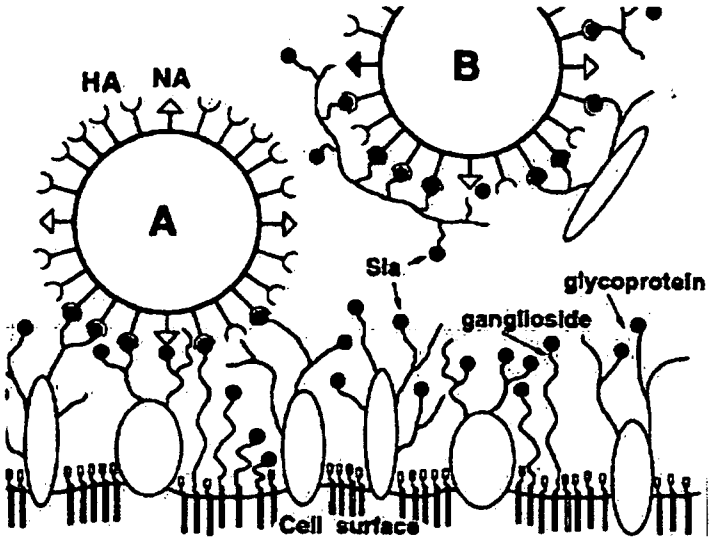


Figure 1.3 (A) and (B): influenza virus attachment to cell surface resp. to inhibitors (image taken from Matrosovich, 2003; [23])

Bindings between HA and the sialyloligosaccharides of the receptors are rather week. Thus, virus attachment requires binding of multiple HA spikes to cell-surface glycoproteins and gangliosides (figure 1.3A). Binding of inhibitors present in serum can create steric obstacles by blocking the HA receptor-binding sites and can lead to aggregation of virus particles (figure 1.3B) [23].

Inhibitors are classified into α, β and y types. The classification is based on their thermal stability, virus-neutralising activity, sensitivity to inactivation by periodate, trypsin, neuraminidase or bacterial sialidase. β-inhibitors play an important role in innate immunity. They are heat-labile mammalian collagenous lectins (collectins) and bind to N-linked glycans on HA and NA. In mouse serum among others inhibitors, mannose-binding lectins were found to inhibit haemagglutination. α-inhibitors are heat-stable and neuraminidase sensitive. У-inhibitors like for example α2-macroglobulin are also heat-stable but neuraminidase-resistant [23;26].

1.7. Treatment and Prevention of H5N1 infections

Humans do not have immunity to H5. If H5N1 acquires a mutation that allows human-to-human transmission, the virus will quickly spread. Vaccination is the most-effective preventive measure to control the influenza outbreak because of their potential to induce a high level of immunogenicity for immunologically naive persons and their ability to rapidly induce immunity [18;27;31]. The principle behind the generation of human influenza vaccines is to elicit protective antibodies directed primarily against HA, the major protective antigen of the virus that induces neutralizing antibodies [31].

Usually, influenza vaccine production is dependent on the availability of embryonated eggs for virus growth but due to that H5N1 is so deadly in chicken embryos, other techniques like plasmid-based reverse-genetics systems or virus production in cell lines were developed.
The Baxter Department of Virology has developed a cell-culture derived whole virus influenza vaccine from the H5N1 human isolates A/Vietnam/1203/04 (clade 1) and A/Indonesia/05/05 (clade 2), two H5N1 subtypes which have widely been accepted

as the potential origin for a pandemic outbreak. Baxter's H5N1 influenza candidate vaccines were grown in African green monkey kidney (VERO) cells.
Because the influenza virus strains always mutate, it is difficult to produce a vaccine in advance that exactly matches the circulating pandemic strain [27]. It was evidenced that the pandemic whole virus vaccine is more effective against antigenically different subtypes of H5N1 strains (cross-protection) than split or reverse-genetic-based vaccines [30]. In opposition to reverse-genetics and egg-based technologies, production of influenza vaccines in cell culture systems is less time-consuming, cost-effective and allows a quicker manufacturing process of large volumes of vaccine. Hence, a possible lack of supply of appropriate eggs is precluded and the availability of the vaccine during a pandemic is accelerated. Such egg-free vaccines might also be useful for those who have allergies to egg proteins [30]. In addition, a cell culture derived vaccine preserves the antigenic properties of clinical isolates better and elicits a stronger, long-lasting cell-mediated immunity than conventional egg-derived vaccines [27;30].

Nevertheless, the preparation of a vaccine takes about 4-6 months. Until a vaccine is available, antiviral drugs are essential [18]. There are 4 antiviral agents available to treat influenza. Amantadine and rimantadine which inhibit uncoating steps of the virus by blocking ion channel activity of the viral M2 protein [b]. Unfortunately, H5N1 viruses isolated from the Vietnamese outbreak in 2004 hardly responded to the M2 inhibitors [12]. In addition, there are the antiviral neuraminidase inhibitors: zanamivir and oseltamivir. Zanamivir is a sialic acid analogue with a three-dimensional structure of neuraminidase spikes. Zanamivir and oseltamivir block the step of release of the progeny virus from infected cells which is processed by the function of the neuraminidase spikes [18].

2. AIM

The aim of this work was to optimize the Haemagglutination Inhibition Assay for testing mouse serum and to develop a whole virus ELISA assay to quantify antibodies specific for different clades of influenza virus H5N1.

The Virology Research & Development Department at Baxter, Orth/Donau is currently developing inactivated whole virus vaccines to influenza strains having pandemic potential. As part of this work, the potency and immunogenicity of candidate vaccines based on different clades of influenza virus H5N1 is being assessed in small lab animals. The serology uses four standard assays: ELISA, Haemagglutination Inhibition (HAI) Assay, Micro-Neutralization (µ-NT) Assay, and Western Blotting.

The HAI Assay is considered the Gold Standard for the evaluation of the immunogenicity of influenza vaccines in humans. In addition, HAI tests are routinely applied to evaluate the serological response to pre-clinical influenza vaccines in small animal models. However, the HAI Assay has been found to work poorly in mice: H5N1 assays being particularly disappointing. The problem was associated with difficulties in reading, poor antibody-titres and high background interference from the mouse serum. This project was aimed to improve HAI Assay for mouse serum by attempting alternative pre-treatments of the sera to reduce interference and by evaluating erythrocytes from alternative species to see whether higher antibody-titres can be obtained.

Distinct antigenic groups or clades of H5N1 viruses are known to exist. Currently employed H5N1 ELISA assays use a recombinant H5 antigen as coating antigen. Presently this H5 antigen is only available for a single clade: H5N1 Vietnam/1203/04. Consequently, the ELISA assay was modified for use with whole virus antigen of Indonesia and Vietnam strain to allow the comparison of the immunogenicity of candidate vaccines to different H5N1 influenza clades. In addition, Western Blot analysis were performed to determine the antibody response in serum to non-HA antigens in the candidate vaccines.

3. MATERIAL AND METHODS

3.1. Antigens

Antigens derived of 2 different strains of H5N1 viruses were used: A/Vietnam/1203/04 (VN) and A/Indonesia/05/05 (ND).

name	H5N1 virus strain	type	propagation cell line	purification procedure	manufacturer
rHA5 [1]	A/Vietnam/1203/04	recomb. protein	Sf+ insect cells	downstream processing techniques	Protein Sciences
whole virus OR [2]	A/Vietnam/1203/04	whole virus antigen	VERO	ultracentrifugation through sucrose cushion	Baxter, Orth-Donau/AT
whole virus BH [3]	A/Vietnam/1203/04	whole virus antigen	VERO	ultracentrifugation through sucrose cushion and ultra/diafiltration	Baxter, Bohumil/CZ
VN (MDCK) [4]	A/Vietnam/1203/04	whole virus antigen	MDCK	ultracentrifugation through sucrose cushion	Baxter, Orth-Donau/AT
ND (MDCK) [5]	A/Indonesia/05/05	whole virus antigen	MDCK	ultracentrifugation through sucrose cushion	Baxter, Orth-Donau/AT

Table 3.1: antigens of H5N1 virus strains used for the development of HAI and whole virus ELISA assay for mouse serum

1) rH5-HA: recombinant, full-length, glycosylated H5 HA (rH5-HA), purified from Sf+ insect cells (a derivative of the Sf9 cell line), which were infected with the appropriate baculovirus vector; purchased from Protein Sciences (Meriden, CT, USA). Recombinant H5 HA of H5N1 influenza strain A/Vietnam/1203/2004 (Cat. No. 3006) was used. The protein is purified to >90% purity under conditions that preserve its biological activity and tertiary structure [14].
2) whole virus OR: A/Vietnam/1203/04 propagated in VERO cells; purified by ultracentrifugation through sucrose cushion; Department of Virology, Baxter AG, Orth/Donau/AT.
3) whole virus BH: A/Vietnam/1203/04 propagated in VERO cells; purified by ultracentrifugation through sucrose cushion and diafiltration; Manufacturing (GMP grade), Baxter AG, Bohumil/CZ.
4) VN (MDCK): A/Vietnam/1203/04 propagated in MDCK cells; purified by ultracentrifugation through sucrose cushion; Department of Virology, Baxter AG, Orth/Donau/AT.
5) ND (MDCK): A/Indonesia/05/05 propagated in MDCK cells; purified by ultracentrifugation through sucrose cushion; Department of Virology, Baxter AG, Orth/Donau/AT.

For the development of whole virus ELISA assay, microtiter plates of individual experiments were coated with an assortment of 8 different antigens (see table 3.2).

antigen number	name	H5N1 virus strain	protein concentration	ELISA-coating concentration
1	whole virus OR	VN	559 µg/ml	200 ng/100 µl
2	whole virus BH	VN	1091 µg/ml	200 ng/100 µl
3	VERO Mock [1)]	VN	355 µg/ml	200 ng/100 µl
4	rHA5	VN	1035 µg/ml	50 ng/100 µl
5	VN (MDCK)	VN	104 µg/ml	200 ng/100 µl
6	ND (MDCK)	ND	118 µg/ml	200 ng/100 µl
7	BSA [2)]	---	---	50 ng/100 µl
8	MDCK-lysate [3)]	---	363 µg/ml	200 ng/100 µl

Table 3.2: coating antigens used for the development of whole virus ELISA assay

1) VERO Mock: VERO cell lysate of a mock-infected cell culture provided by the Baxter Department of Microbial Fermentation.
2) BSA: bovine serum albumin (Sigma)
3) MDCK-lysate: Protein-lysate made from MDCK cells at passage 68. MDCK cells were provided by the Baxter Department of Cell Culture.

3.2. Serum

Animal housing and experimental work with animals was done by the Baxter Department of Animal Models.

name	immunization code	species	antigen	doses [µg]	adjuvant	application in immune assay
# 470 group A	# 470 [1)]	CD1 mice	rH5-HA (VN)	5	alum [8)]	ELISA
# 470 group B	# 470 [1)]	CD1 mice	H5N1 whole virus (VN)	5	alum [8)]	ELISA
# 470 group C (control group)	# 470 [1)]	CD1 mice	diluent	---	alum [8)]	ELISA
# 255 anti-VERO-cell mouse serum	# 255 [2)]	CD1 mice	VERO-protein	20	FCA [9)]	ELISA
PC	H5N1-22 [3)]	guinea pigs	H5N1 whole virus (VN)	3.75	---	HAI
# 28	H5N1-28 [4)]	CD1 mice	H5N1 whole virus (ND, VN)	3.75 - 0.000048	---	HAI
# 33 pool A	H5N1-33 [5)]	CD1 mice	H5N1 whole virus (VN)	0.75	---	HAI/ELISA
# 33 pool B	H5N1-33 [5)]	CD1 mice	rH5-HA (VN)	0.75	---	HAI/ELISA
# 37	H5N1-37 [6)]	CD1 mice	H5N1 whole virus (ND, VN)	3.75 - 0.000048	alum [8)]	HAI
NC	NC 17/10/06 [7)]	CD1 mice	---	---	---	HAI

Table 3.3: sera used for the development of HAI and whole virus ELISA assay for mouse serum

1) Immunization # 470: CD1 mice were immunized with purified Baculovirus-expressed recombinant HA-5 of VN clade (group A) or double-inactivated A/Vietnam/1203/04 virus (48 h at 0.025% formalin followed by 2 cycles of UV-irradiation at a flow rate of 260 l/h) that was purified by sucrose gradient ultracentrifugation (group B).
2) Immunization # 255: To generate anti-sera directed against VERO-cell-proteins, CD1 mice were immunized four times with 20 µg VERO-protein.
3) Immunization H5N1-22: Due to the fact that antisera of mice showed interactions in HAI assays, antisera of guinea pigs of immunization H5N1-22 were applied as positive reference serum for the development of HAI assay for mouse serum. The guinea pig antiserum was prepared by vaccination of the animals with two (one primary and one booster immunization) 3.75 µg doses without adjuvant of H5N1 A/Vietnam/1203/04 strain (HA content: 211.1 µg/ml; total protein content: 796 µg/ml; HA to protein ratio: 0.27).
4) Immunization H5N1-28: CD1 mice were immunized twice via the subcutaneous route with eight different doses (5-fold dilutions beginning at 3.75 µg HA per dose) of the A/Indonesia/05/2005 candidate vaccine preparation or with 2 different doses (3.75 and 0.03 µg) of A/Vietnam/1203/04 influenza strain. Only non-adjuvanted vaccine preparations were used.
5) Immunization H5N1-33: CD1 mice were immunized with either H5N1 A/Vietnam/1203/2004 isolate (group A) or Baculovirus-expressed rH5-HA of VN clade (group B). For the development of HAI assay for mouse serum, pools of these antisera were used. First, antibody-titres of H5N1-33 were determined by standard-ELISA assay (see 3.6) using rH5-HA of A/Vietnam/1203/2004 (Protein

Sciences [14]) as coating antigen. Then, sera from individual mice with highest ELISA antibody-titres within group A (H5N1 whole virus; VN clade) and group B (rH5-HA; VN clade) respectively, were pooled.

6) Immunization H5N1-37: CD1 mice were immunized twice via the subcutaneous route with eight different doses (5-fold dilutions beginning at 3.75 µg HA per dose) of the A/Indonesia/05/2005 candidate vaccine preparation or with 2 different doses (3.75 and 0.03 µg) of A/Vietnam/1203/04. Only alum-adjuvanted vaccine preparations were used.
7) NC 17/10/06: As negative reference (NC 17/10/06) for HAI assay, pooled naive sera of 20 CD1 mice were used.
8) alum: = Al(OH)3 = aluminium hydroxide
9) FCA: = Freund's adjuvant: antigen solution was emulsified in mineral oil

3.3. Red Blood Cells (RBCs)

Erythrocytes from chicken, turkey or horse were used for HA and HAI assays.
Horse and turkey blood was purchased from Charles River Labs (France).
Chicken blood diluted 1:2 in Alsever's solution was provided by the Department Animal Housing.

3.3.1. Standardization of RBCs

Blood was centrifuged at 1500 rpm at 2-8℃: chicken blood for 5 minutes and horse and turkey blood for 10 minutes. The supernatant was aspirated and the buffy layer of white cells was removed with a pipette. Erythrocytes were washed with HA-buffer and again centrifuged at 1500 rpm at 2-8℃: chicken blood for 5 minutes and horse and turkey blood for 10 minutes. Supernatant was discarded. Washing procedure was repeated two times for horse and chicken blood and three times for turkey blood. Washed and packed erythrocytes were resuspended in HA-buffer and diluted to a final concentration of 0.4%, 0.5%, 0.75% or 1% [3].

3.4. Haemagglutination Assay (HA)

The HA assay is used for the quantification of virus/antigen solutions and for the preparation of standardized antigen used in HAI assays [3].
The haemagglutinin (HA) protein of influenza viruses agglutinates erythrocytes.
The highest dilution of virus preparation that causes complete haemagglutination is considered the end point of HA-titration. The HA-titre is the reciprocal of the highest dilution of virus solution in the dilution series that still induces complete haemagglutination [1].

3.4.1. Procedure HA assay (= HA-titration of antigens)

HA assay and serial dilutions were performed in V-shaped 96-well microtiter plates. As a positive control a HA-standard with a defined HA-titre of 8 was used. The HA-standard was prepared from a virus suspension of the strain A/Sydney/#E42/02 and was provided by the Department of Experimental Virology. As a negative control 0.9% NaCl was used. Negative control must not show haemagglutination.

50 µl of 0.9% NaCl was added to an appropriate number of rows from well 2 to 12. 100 µl of antigen, negative or positive control respectively was applied to well 1. Serial twofold dilutions were made by transferring 50 µl from well 1 to successive wells. The final 50 µl from the last well were discarded. 50 µl of standardized RBC suspension (see 1.2.1) were added to each well. The plates were mixed gently and incubated at room temperature (18-28°C). Incubation time varied dependent on the erythrocyte species used. HA assays with chicken and turkey erythrocytes were incubated for 30 minutes whereas an assay with horse erythrocytes was incubated for 80 minutes.
After incubation the plates were tilted to determine the HA-titre. In the absence of haemagglutination the erythrocytes form a compact button on the bottom of the well and when plates are tilted they flow down in tear-shaped streams at the same rate as the negative control. Haemagglutination was recorded as positive when the RBCs were in suspension after the negative control has settled completely. The range of variation of HA assay is ± 1 HA-titre [1;3].

3.4.2. Preparation of standardized antigen for HAI assay

The standard virus-antigen routinely used for HAI assay (see 3.5) is 4 HA units/50 µl added to twofold dilutions of antisera. An HA unit is the amount of virus needed to agglutinate an equal volume of a standardized red blood cell suspension [1]. In case an antigen did not have 4 HA units it was adjusted by dilution (see table 3.4).

As a first step the HA-titre of the virus-antigen solution was determined by HA assay (see 3.4.1). The corresponding dilution to the HA-titre was divided by 4 to obtain the theoretical dilution factor (see table 3.4). Then the antigen was diluted in PBS pH 7.2 (Gibco) accordingly to the theoretical dilution factor.
To verify HA units a second HA assay (back titration) using the standardized antigen dilution was performed [1;3].

titre HA-assay	1	2	3	4	5	6
equal to dilution (1:X)	1 [1)]	2	4	8	16	32
theoretical dilution factor (1:X)	---	---	---	2	4	8
HAU/50 µl	1	2	4	8	16	32
titre HA-assay	7	8	9	10	11	12
equal to dilution (1:X)	64	128	256	512	1024	2048
theoretical dilution factor (1:X)	16	32	64	128	256	512
HAU/50 µl	64	128	256	512	1024	2048

Table 3.4: conversion table used for the determination of HA units and the preparation of standardized virus-antigen for HAI assay

1) undiluted

3.5. Haemagglutination Inhibition Assay (HAI)

HAI assay is used for the detection and quantification of specific antibodies directed against influenza virus haemagglutinin (HA) [2].
If there are specific antibodies present in serum samples, they attach to the antigenic sites on the HA molecule. As a consequence the binding between viral HA and erythrocyte receptors is inhibited. This effect is called haemagglutination inhibition and is the basis for the HAI test.
In general, a standardized quantity of HA antigen is mixed with serially diluted antisera. RBCs are added and specific binding of antibodies to the HA molecule is determined [1].
The HAI-titre is the reciprocal of the highest dilution in a dilution series of antiserum that still completely inhibits haemagglutination [1]. Is there already haemagglutination occurring at a dilution of 1:10 (titre < 10) the result is negative and was recorded as a titre of 5 [2].

3.5.1. Pre-treatment of antisera

Not only antibodies but also other serum components contain sialic acid residues that mimic RBCs receptors. Hence they are capable to bind to the HA molecule of the virus antigen being tested. This could lead to false interpretations of HAI assays. To ensure that sera do not contain such non-specific inhibitors, sera was pre-treated [1].
2 distinct pre-treatment methods were used for the development of HAI assay for mouse serum.

Pre-treatment with receptor destroying enzyme (RDE)

4 vol of RDE-solution were added to 1 vol of serum and incubated for 18 hours in a 37℃ incubator. After the incubation time samples were heated in a 56℃ waterbath for 45 minutes to inactivate remaining RDE. 5 vol of PBS pH 7.2 (Gibco) were added so that the final dilution of antisera was 1:10 [2].

Trypsin-heat-periodate pre-treatment

0.5 vol of trypsin-solution were added to 1 vol of serum and inactivated in a 56℃ waterbath for 30 minutes. Serum was cooled to room temperature and 3 vol of 0.011 M metapotassium periodate (KIO_4) was added. Solution was mixed and left at room temperature for 15 minutes. 3 vol of 1% glycerol saline were added and the samples were again mixed and left for 15 minutes at room temperature. 2.5 vol of 0.9% sodium chloride solution were added. Final serum dilution was 1:10 [2].

3.5.2. Procedure HAI assay

To control the quality of the erythrocytes (RBC control) used in HAI assay, 100 µl of 0.9% NaCl mixed with 50 µl RBCs was applied. As heamagglutination control, 0.9% NaCl mixed with the test-antigen and RBCs was used. Reference antisera (see table 3.3) from immunization H5N1-22 (positive control) and NC 17/10/06 (negative control) were tested in the same way like serum samples. RBC control must not show haemagglutination in contrast to the haemagglutination control where haemagglutination must occur completely. Positive reference antisera (H5N1-22; see table 3.3) must show a specific haemagglutination inhibition reaction.

HAI assay was performed in V-shaped 96-well microtiter plates (Greiner). Serial twofold dilutions of pre-treated antisera (already diluted 1:10) were made by diluting appropriate volumes in 0.9% NaCl. All the dilutions steps (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120) were carried out in micronics respectively deep wells. Then 50 µl of each dilution step were transferred to the HAI test plate. 100 µl 0.9% NaCl for the RBC control was applied to well 1 of each row. For the haemagglutination control 50 µl of 0.9% NaCl were added to column 12. 50 µl standardized antigen was applied to the HAI test plate to row A-H, column 2-12. Plates were mixed manually, covered and incubated at room temperature for 30 minutes. Then 50 µl of standardized RBCs (see 3.3.1) were added to all wells (row A-H and column 1-12). Plates were mixed as before and covered. The appropriate incubation time was dependent on the species of RBCs. For chicken and turkey erythrocytes incubation time was 60 minutes and for horse erythrocytes 130 minutes.

dilution HAI-titre		10	→	5120	
row	column				
	1	2	→	11	12
A	100 µl 0.9% NaCl	Serum 1	→	Serial twofold dilution	50 µl 0.9% NaCl
B	100 µl 0.9% NaCl	Serum 2	→	Serial twofold dilution	50 µl 0.9% NaCl
C	100 µl 0.9% NaCl	Serum 3	→	Serial twofold dilution	50 µl 0.9% NaCl
D	100 µl 0.9% NaCl	Serum 4	→	Serial twofold dilution	50 µl 0.9% NaCl
E	100 µl 0.9% NaCl	Serum 5	→	Serial twofold dilution	50 µl 0.9% NaCl
F	100 µl 0.9% NaCl	Serum 6	→	Serial twofold dilution	50 µl 0.9% NaCl
G	100 µl 0.9% NaCl	Serum 7	→	Serial twofold dilution	50 µl 0.9% NaCl
H	100 µl 0.9% NaCl	Serum 8	→	Serial twofold dilution	50 µl 0.9% NaCl
	RBC control	**sample**			**Positive control**
		50 µl standardized antigen			
	50 µl erythrocytes				

Table 3.5: application scheme of HAI assay

3.6. Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect antibody-ELISA (antigen-1stantibody-2ndantibody complex) was the method for the semi-quantitative measurement of antibody titres directed against H5-HA [4].
In general, specific antibodies in serum samples bind to the immobilized antigen on microtiter plates. Then horseradish peroxidase-linked secondary antibodies are added which bind to the specific antibodies. OPD-tablets (ortho-phenylenediamine) are applied as chromogen substrate. OPD is oxidized during the enzymatic degradation of H_2O_2 by horseradish peroxidase into an orange-coloured product. The rate of colour formation is proportional to the amount of specific antibodies [15].

3.6.1. Procedure ELISA

For each liquid removing respectively washing step of the ELISA procedure a plate washer (Skan Washer 300 Version B; Skatron Instruments) was used.

The assay was performed in microtiter plates (Nunc-Immuno Plate - MaxiSorp TM Surface; Nunc International). Serial dilutions were done in Micronics (Costar) and during incubation times plates were shaked on a Titramax shaker.

Antigen coating

Antigen solution (see table 3.2) was diluted to the appropriate concentration in 50 mM $NaHCO_3$ buffer pH 9.6. Each well of the microtiter plate except row A, column 1-12 was coated with 100 µl/well with the above mentioned antigen solution. Plates were covered and incubated overnight at +2-8℃ [4].

Blocking

The blocking-step is required to saturate the binding capacity of the microtiter plate and thus prevent problems with non-specific bindings [5]. After overnight incubation time the plates were brought to room temperature for at least 1 hour. Coating solution was removed and plates were washed with washing-buffer 4 times. Washed plates were carefully beaten on cotton tissue to remove remaining buffer. Then 100 µl of blocking-buffer was added to each well. Plates were covered and shaked while incubating at room temperature for 1 hour [4].

Primary antibody reaction

Serum samples were diluted in antibody-buffer. Serial fourfold dilutions were made starting with 1:100 (1:100, 1:400, 1:1600, 1:1600, 1:6400, 1:25600, 1:102400, 1:409600). 100 µl of the diluted serum was added to appropriate wells.
Microtiter plates were covered and placed on the shaker for 1 hour at room temperature. After incubation time coating-solution was removed and plates were washed four times with washing-buffer. Then they were carefully beaten on cotton tissue to remove remaining buffer [4].

Application of secondary antibody

As a secondary antibody a horseradish peroxidase-conjugated AffiniPure goat anti mouse IgG (H+L) purchased from Jackson Immuno Research was used.
The antibody was diluted 1:5000 in antibody-buffer. 100 µl of this solution were added to each well except to blank control. Plates were covered and shaked for 1 hour. Then washed 4 times with washing-buffer and beaten on cotton tissue [4].

Development

During the last incubation time and immediately before use the enzyme substrate-solution was prepared. As a substrate SIGMA FAST^{TM} OPD tablets were used.
200 µl of the freshly prepared substrate was added to each well. Orange colour developed in positive wells after 10 minutes. The reaction was stopped with 50 µl 5 M H_2SO_4 and absorbance was read using a microplate reader (Ultra Microplate Reader EL808) at 490 +/- 20 nm (reference wavelength: 620 +/- 20 nm) [4].

The cut-off value for the determination of the ELISA-titre was calculated by multiplying the average of the negative controls with factor 4.

3.7. Western Blot

A protein (antigen) mixture is electrophoresed through an SDS gel and thus separated based upon size and charge. Separated antigens are transferred from the gel onto a membrane which is then flooded with a solution of primary antibodies (samples). Antibodies directed against the separated antigens are bound and others are washed away at the end of incubation period. In order to detect the bound antibodies, a secondary antibody-enzyme conjugate (horseradish peroxidase conjugated secondary antibody) which recognizes the primary antibody is added. After an incubation time the secondary antibody is removed and DAB (3,3-diaminobenzidine tetrahydrochloride) is added as chromogen substrate to visualize peroxidase reactions: in the presence of H_2O_2, horseradish-peroxidase catalyzes the oxidation of DAB into an insoluble brown precipitate and therefore enables the detection of bound antibodies [6;7].

3.7.1. Concentration of samples that are low in protein concentration

Before Western Blot was performed, virus samples (antigens) with very low protein concentrations were concentrated by ultracentrifugation.
Samples were diluted appropriately and spinned down in an ultracentrifuge (Beckman OPTIMA TLX Ultracentrifuge; Beckman rotor TLA 120.2, 120 K rpm) at 100.000 rpm for 10 min. Supernatant was discarded and pellet was dissolved in an appropriate volume of RIPA-buffer. The solution was kept on ice for 30 min. $PAPU_{red}$ was added and samples were stored at 2-8℃ until application in Western Blot procedure [19].

3.7.2. Procedure

SDS Page

Antigens were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-gel consisted of 5% stacking gel (see table 3.6) and 12% separating gel (see table 3.7). Transfer-buffer was added and electrophoresis was performed at 200 V for 45 min. Then gels were transferred to a PVDF membrane [7].

reagents	stacking gel 5%
SDS-stacking gel buffer	937 µl
40% acrylamide	423 µl
aqua bidest.	1640 µl
Temed	3.75 µl
10% APS	30 µl

Table 3.6: stacking gel for Western Blot

reagents	separating gel 12.5%
SDS-separating gel buffer	2.5 ml
40% acrylamide	3.1 ml
aqua bidest.	4.4 ml
Temed	10 µl
10% APS	50 µl

Table 3.7: separating gel for Western Blot

Sample preparation

Primary antibodies (= sera) and the protein-standard (Precision Plus Protein™ Standards Dual Color; Bio-Rad) were appropriately diluted in PBS pH 7.2 and $PAPU_{red}$. Then all samples (except the protein standard) were heated at 95℃ for 5 min. After that they were sonicated in an ultrasonic bath (Bandelin Sonorex TK 52) for 10 min. Samples and standard were stored at 2-8℃ until use [7].

Blotting

Transfer of separated antigens from the SDS-gel onto a PVDF membrane was done by tank blotting at 200 mA for 90 min. The tank was filled with blotting-buffer and cooled at 4℃.
After the transfer the membrane was incubated in blocking-buffer for 30 min and washed 3 times with TTBS for 5 min [7].

Application of primary antibody

Primary antibodies (= sera after sample preparation) were incubated with the membrane at room temperature over night [7].

Application of secondary antibody

Anti-immunoglobulin antibodies coupled to the enzyme horseradish peroxidase (horseradish peroxidase-conjugated AffiniPure goat anti mouse IgG purchased from

Jackson Immuno Research) were added to the membrane at an appropriate dilution [7].

Detection

The blotting membrane was washed 3 times with TTBS and twice with TBS for 5 min. The bands were developed for 10 minutes by adding substrate solution containing hydrogen peroxide and 3.3' diaminobenzidine tetrahydrochloride (DAB; Sigma). The membrane was washed with aqua bidest. for 5 minutes and the positive reactions were determined by the visualization of clearly defined brown bands [7].

3.8. Microneutralisation Assay (µ-NT)

Due to the fact that µ-NTs are performed with infectious H5N1 virus material, they were done by specially trained persons of the Baxter Department of Virology. Data of µ-NT for the correlation with HAI assay (see 4.2.2) were provided.

In general, microneutralisation assays (µ-NTs) are a serological method used for the detection of H5N1 specific neutralising antibodies in sera. Serum samples are diluted serially and incubated with a defined amount of infectious virus. Subsequently, VERO cells are infected with the serum-virus-dilutions. Inoculated cells are observed for cythopathic effect (CPE) one week after infection. In case that specific antibodies are present in serum, no CPE appears due neutralization of infectious H5N1 virus before infection of the cells occurs. The dilution of the serum protecting more than 50 % of the cell sheet from CPE is the viral antibody-titre [20].

3.9. VERO and MDCK Cell-Lysates

VERO cell lysate was made from mock infected VERO-cells at passage 138. The VERO lysate was provided by the Baxter Department of Cell Culture Fermentation. MDCK cell lysate was made from cells at passage 68. MDCK cells were provided by the Baxter Department of Cell Culture.

3.9.1. Preparation of MDCK cell lysate

Medium from 3 roux flasks completely covered with adherent MDCK cells was decanted. The confluent cell layer was washed twice with 15 ml 1xPBS/EDTA solution. To remove the cells from the bottom of the flask 2 ml 1x trypsin/EDTA solution were added. Flasks were incubated at 37℃ for 15 minutes. Cells were scraped off with a plastic cell scraper and a total vol of 6 ml cell suspension was transferred into a centrifuge tube. 0.6 ml heat-inactivated fetal calf serum (FCS) was added and the suspension was centrifuged (Beckman Coulter™; Avanti™ J-20 XPI centrifuge) at 3000 rpm and 4℃ for 10 minutes. The supernatant was discarded and

the pellet was resuspended in 1.5 ml PBS pH 7.2 and centrifuged like before. The supernatant was decanted and the remaining pellet resuspended in 2 ml PBS. The suspension was kept on ice and sonicated twice with 15 impulses (20 % amplitude) of 2 sec bursts and at 2 sec intervals using a sonificator (Branson Ultrasonics Corporation).
To hydrolyze nucleic acids and to reduce cell lysate viscosity 5 µl 1 M magnesium chloride ($MgCl_2$) and 2 µl benzonase (250 U/µl; Merck) were added. The suspension was incubated at 37℃ over night on a shaker. Lysed cells were sonicated like before and centrifuged for 10 min at 3000 rpm at 4℃. The protein content of the supernatant was determined by BCA™ protein assay kit or Micro BCA™ protein assay kit respectively (PIERCE; see 3.11). Remaining solution was frozen at ≤ 60℃ [8,9].

3.10. Virus Purification by Ultracentrifugation through Sucrose Cushion

Virus was thawed at 37℃ in a waterbath and was then kept on ice. 6 ml of sucrose buffer were added into clear ultracentrifuge tubes (Beckman). The virus suspension was added carefully up to 28 ml in each tube without disturbing the sucrose cushion. To one tube MEM-EARLE-medium was added up to 28 ml to assure all tubes were balanced. The samples were spinned down (Beckman ultracentrifuge L8-60M; Beckman rotor SW 28) at 23000 rpm at 4℃ for 3 hours. The supernatant was removed and the tubes were left inverted on cotton tissue for a couple of minutes. The walls of the tubes were cleaned with a cotton paper wrapped pipette. Into each tube 1 ml Tris/HCl buffer pH 7.3 was added and tubes were incubated on ice for 30 minutes. The fractions were pooled and filled up to a final volume of 7 ml with Tris/HCl buffer pH 7.3. The protein content of the purified virus suspension was quantified by BCA™ protein assay kit or Micro BCA™ protein assay kit respectively (PIERCE; see 3.11). Then samples were stored at ≤ 60℃ [8;10]

3.11. Protein Determination by BCA Protein Assay Kit

All quantifications of virus protein were done by BCA™ protein assay kit or Micro BCA™ protein assay kit respectively purchased from PIERCE.
PIERCE protein assay kits uses bicinchoninic acid (BCA) for the detection of protein. In general, the assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present. Cysteine, cystine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+}. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins at absorbance maximum 562 nm.
The working range is 20–2.000 µg/ml for BCA™ protein assay kit and 0.5-20 µg/ml for Micro BCA™ protein assay kit [10].

3.12. Buffers and Reagents

40% acrylamide (Bio-Rad)
10% ammonium persulfate (APS): 1 g ammonium persulfate (Bio-Rad) dissolved in 10 ml aqua bidest.; stored at 2-8℃ stable for 1 mo nth
Antibody-buffer (ELISA): 3% Skim Milk Powder (Fluka) dissolved in washing-buffer (ELISA); prepared freshly;
Antibody-buffer (Western Blot): 1% gelatine (Bio-Rad) dissolved in 700 ml TTBS; autoclaved (121℃, 20 min) and filled up to 1l with TTBS; stored at 2-8℃ stable for 3 months
Benzonase (Merck # 1.01695.0001; 100.000 U/vial; 341 U/µl)
Blocking-buffer (ELISA): 1% Skim Milk Powder (Fluka) dissolved in washing-buffer (ELISA); prepared freshly;
Blocking-buffer (Western Blot): 1% gelatine (Bio-Rad) dissolved in 700 ml TBS; autoclaved (121℃, 20 min) and filled up to 1l with TTBS; stored at 2-8℃ stable for 3 months
Blotting-buffer (Western Blot): 200 ml 10x Tris-glycine stock buffer (Bio-Rad) mixed with 400 ml methanol (Baker) and filled up to 2 l aqua bidest.; prepared freshly
Bromophenolblue (Merck)
FCS: fetal calf serum (PAA Laboratories); heat-inactivated in a watherbath at 56℃ for 30 min
HA-buffer (0.5% bovine serum albumin in 0.9% NaCl): 18 g NaCl (Merck) and 10 g bovine serum albumin (Sigma) dissolved in 2l aqua bidest.
1 M $MgCl_2$: 20.33 g magnesium chloride hexahydrate (Merck) dissolved in 60 ml aqua dest. and filled up to 100 ml aqua dest.; stored at 2-8℃ stable for 6 months
1x MEM-EARLE-medium (Biochrom AG): w 2.2 g/l $NaHCO_3$, w stable glutamine LE, Cat # FG 0320
1x 0.011 M metapotassium periodate (KIO_4)-solution: 230 mg metapotassium periodate (KIO_4) dissolved in PBS pH 7.2 and filled up to 100 ml; sterile filtered; prepared freshly and used within one week
2x 0.011 M metapotassium periodate (KIO_4)-solution: 460 mg metapotassium periodate (KIO_4) dissolved in PBS pH 7.2 and filled up to 100 ml; sterile filtered; prepared freshly and used within one week
0.9% NaCl: 9 g NaCl (Merck) dissolved in 1l aqua bidest; stored at 2-8℃ stable for 6 months
1.8% NaCl: 1.8 g NaCl (Merck) dissolved in 1l aqua bidest; stored at 2-8℃ stable for 6 months
50 mM $NaHCO_3$ buffer pH 9.6: 4.20 ± 0.04 g $NaHCO_3$ dissolved in 900 ml aqua bidest.; pH adjusted with 1 N NaOH to 9.6 ± 0.1; filled up to 1l with aqua dest.; stable at 2-35℃ for 1 year
1 N NaOH: 40.0 ± 0.4 g NaOH (Merck) diluted in 1l aqua bidest.; stable at 2-35℃ for 1 year
PBS pH 7.2 (Gibco)
1x PBS/EDTA solution: 100x PBS/EDTA stock solution 1:100 diluted in PBS pH 7.2 (Gibco)
100x PBS/EDTA stock solution: 2 g EDTA (Titriplex III; Merck KB 273818) diluted in 100 ml PBS pH 7.2 (Gibco); sterile filtrated; stored at 2-8℃

0.1 M phosphate buffer, pH 7.2: 17.8 g Na_2HPO_4 (dibasic sodium phosphate dihydrate) and 13.8 g $NaH_2PO_4OH_2$ (monobasic sodium phosphate monohydrate) dissolved in 500 ml aqua bidest.; stored at 2-8℃ s table for 1 year
0.1 M phosphate buffer, pH 8.2: 13.8 g $NaH_2PO_4OH_2$ dissolved in 1000 ml sterile distilled water (0.1 M monobasic sodium phosphate monohydrate); 14.2 g Na_2HPO_4 dissolved in 1000 ml sterile distilled water (0.1 M dibasic sodium phosphate); 1 vol of monobasic sodium phosphate monohydrate mixed with 31 vol of dibasic sodium phosphate (0.1 M phosphate buffer, pH 8.2); pH adjusted by adding extra volumes of 0.1 M monobasic sodium phosphate monohydrate to lower pH or 0.1 M dibasic sodium phosphate to raise pH
Protein-standard: Precision Plus Protein™ Standards Dual Color; Bio-Rad
PVDF membrane (Millipore): Immobilon™ transfer membranes, Cat # IPVH09120, pore size: 0.45 µm, cut size: 9 cm x 12 cm
RDE-solution: neuraminidase from Vibrio cholerae (1.24 U/ml, sol. in 50 mM sodium acetate, content: 25 µg/ml HAS; Serva Electrophoresis GmbH) diluted 1:20 in PBS pH 7.2 (Gibco); prepared freshly before use
RIPA-buffer: 0.33 g (= 40 mM) NaF (sodium fluoride; Sigma), 0.20 g (= 0.1%) SDS (sodium dodecyl sulfate; Bio-Rad), 2 ml (= 1%) Triton X-100 (Sigma) and 0.149 g (= 2 mM) EDTA (Titriplex III, Merck) dissolved in 20 ml 0.1 M phosphate-buffer pH 7.2; filled up to 200 ml with aqua dest.; stored at 2-8°C stable for 1 year
Sample-application-buffer ($PAPU_{red}$): 18 ml 2x Tris/glycine/SDS sample buffer (Novex) mixed with 1 ml ß-mercaptoethanol and 1 ml aqua bidest.; stored at 2-8℃ stable for 3 months
Secondary antibody (enzyme-linked): horseradish peroxidase-conjugated AffiniPure goat anti mouse IgG (H+L); Jackson Immuno Research
Standardized erythrocytes (chicken, turkey, horse): 0.4%, 0.5%, 0.75% or 1% solutions in HA-buffer; stored at 2-8℃; stable for 10 days
Substrate-solution (ELISA): o-phenylenediamine dihydrochloride (OPD); 1 OPD tablet (10 mg tablets, SIGMA/P-8287) was dissolved in 1 ml methanol (J.T.Baker/8045) + 99 ml aqua bidest. (Fresenius); immediately before use 20 µl H_2O_2 (30%; Merck/822287) were added; solution must be prepared freshly
Substrate-solution (Western Blot): 3 DAB tablets added to 100 ml TBS and dissolved in an ultrasonic bath for 10 min; 30 µl H_2O_2 were added; prepared freshly
PBS pH 7.2 (Gibco)
20% sucrose in 10 mM Tris/HCl pH 7.3: 40 g sucrose (Merck) dissolved in 200 ml 10 mM Tris/HCl pH 7.3; sterile filtered through 0.22 µm GP Express Plus membrane (Millipore); stored at 2-8℃ stable for 6 months
5 M sulphuric acid (H_2SO_4): 278 ml concentrated sulfuric acid (95-97%; Merck) added to 722 ml aqua bidest.; stable at room temperature for 6 months
1x TBS-buffer: 100 mL 10x TBS mixed with 900 mL Aqua bidest.; stored at 2-8℃ stable for 5 months
10x TBS-buffer: 400 ml 1M Tris/HCl pH 7.5 and 584.4 g NaCl filled up to 2l aqua bisdest.; stored at 2-8℃ stable for 3 months
Transfer-buffer for SDS page: 10x Tris/glycine/SDS stock buffer (Bio-Rad) diluted in aqua bidest. 1:10; prepared freshly
12% trichloric acid (TCA): 12 g trichloric acid (Fluka) dissolved in 100 ml aqua bidest; stored at 2-8℃ stable for 5 years
1 M Tris/HCl pH 7.5: 121.14 g Tris (Merck) diluted in 800 ml aqua bidest; pH adjusted to 7.5 with 37% hydrochloric acid and filled up to 1l with aqua bidest.; stored at 2-8℃ stable for 6 months

1 M Tris/HCl pH 7.3: 121.14 g Tris (hydroxymethyl) aminomethane (Merck) dissolved in 800 ml aqua bidest.; pH adjusted to 7.3 by adding extra volumes of hydrochloric acid (37% HCl; Merck) and filled up to 1l with aqua bidest.; stored at 2-8℃ stable for 6 months
10 mM Tris/HCl pH 7.3: 2 ml 1 M Tris/HCl pH 7.3 diluted with 198 ml aqua bidest.; stored at 2-8℃ stable for 6 months
1x trypsin/EDTA solution: 10x trypsin/EDTA solution (Gibco) diluted 1:10 in 1x PBS/EDTA solution
10x trypsin/EDTA solution (Gibco)
1x trypsin-solution*:* 200 mg of trypsin (Gibco 27250-018) dissolved in 25 ml of 0.1 M phosphate buffer pH 8.2; sterile filtered; stored at -70℃; stable for 6 months
2x trypsin-solution: 400 mg of trypsin dissolved in 25 ml of 0.1 M phosphate buffer pH 8.2; sterile filtered; stored at -70℃; stable f or 6 months
1x TTBS-buffer: 100 ml 10x TBS mixed with 900 ml Aqua bidest. and 500µl Tween20 added; stored at 2-8℃ stable for 5 months
Washing-buffer (ELISA): 20 PBS tablets (Gibco; Cat # 18912-014) and 10 ml Tween 20 (Sigma) dissolved in 10l aqua dest.; stable at 2-8℃ for 1 month

3.13. Equipment

Beckman Coulter™ Avanti ™ J-20 XPI centrifuge
Beckman ultracentrifuge L8-60M
Beckman OPTIMA TLX Ultracentrifuge
Beckman rotor TLA 120.2, 120 K rpm
Beckman rotor SW 28
Electrophoresis chamber Mini PII cell; Bio-Rad
ELISA-plate-washer: Skan Washer 300 Version B; Skatron Instruments
Mighty Small Blotting Tank; Hoefer
Photometer: Ultra Microplate Reader EL808; BioTek
Power Supply; Hoefer, Bio-Rad
SemiDry Blotting Apparatur; Pharmacia Multiphor II
Sonificator: Branson Ultrasonics Corporation digital sonifier
Ultrasonic bath: Omega Instruments, Vienna; Bandelin Sonorex TK 52

4. RESULTS AND DISCUSSION

4.1. Development of HAI Assay for Mouse Serum

The virus-antigens and appropriate antisera used in following HAI assays are described in detail in chapter 3.1 and 3.2.
Unless otherwise mentioned, antisera were pre-treated with neuraminidase (RDE) before application in HAI assay and the incubation time of HAI assays performed with horse erythrocytes was 130 min. HAI-titres below the lower limit of detection were given a value of 5 respectively a titre of 1:5.

4.1.1. Difficulties in reading of HAI assays performed with mouse serum

Currently employed HAI assays are performed with standardized chicken red blood cells (RBCs) and work poorly when mouse antisera are tested: reading of these HAI assays appears to be very difficult and determination of an endpoint is impossible. The problem is associated with low levels of specific antibodies and with high background interference possibly caused by non-specific inhibitors (see 1.6) present in the mouse serum. In contrast, reading of HAI assays performed with chicken RBCs and guinea-pig antisera displays no problem: the difference between viral haemagglutination (figure 4.1) and antibody-induced inhibition of haemagglutination (figure 4.2) is obvious. Viral haemagglutination shows a homologous turbid suspension of RBCs (figure 4.1) and haemagglutination inhibition is indicated by sliding-down erythrocytes (figure 4.2). In mouse HAI assays, a pellet of sedimented erythrocytes (RBCs) at the bottom of the wells is clearly visibly (figure 4.3). But there is no tear-shaped smear of sliding down RBCs like in guinea-pig HAI assays (figure 4.2) when plates are inverted. This effect is referred to as interaction and as a consequence the endpoint of HAI assay respectively the HAI-titre of mouse serum samples can not be defined.

Figure 4.1: viral haemagglutination of erythrocytes (RBCs), performed with guinea-pig antisera and chicken RBCs

Figure 4.2: antibody-induced inhibition of viral haemagglutination of RBCs in HAI assay, performed with guinea-pig antisera and chicken RBCs

Figure 4.3: interaction in HAI assay, performed with mouse antisera and chicken RBCs

To improve reading of HAI assays with mouse serum and hence to reduce interactions, different pre-treatment methods of serum were tested (see 4.1.4) and erythrocytes from different species (turkey, horse) were evaluated (see 4.1.2).

4.1.2. Evaluation of erythrocytes from different animal species

Conventionally, HA and HAI assays are performed with 0.4% chicken red blood cells (RBCs). They work fine with antisera of guinea-pigs but they are relatively insensitive for the detection of antibody response in mouse serum.
To obtain higher antibody titres and therefore to optimize reading of HAI assay for mice sera, standardized erythrocytes from horse and turkey were evaluated.

RBCs from chicken, horse and turkey were standardized in HA-buffer to a concentration of 0.4%. HA assays with the different RBC species were performed to adjust VN antigen concentration for HAI assay. HA-titres showed no difference depending on the used RBCs species (data not shown). Thus, all HA assays in following experiments were performed with 0.4% chicken RBCs. One reason for the choice of using chicken RBCs for HA assays was that the incubation time is only 30 min in relation to horse HA assays where incubation time takes 80 min. Another reason was that the provided HA-standard (see 3.4.1) did not react with horse RBCs at all (data not shown). Mouse antiserum (see 3.2) used in HAI assay was taken from immunization H5N1-33 pool A (immunized with rHA-5) and pool B (immunized with whole virus VN).

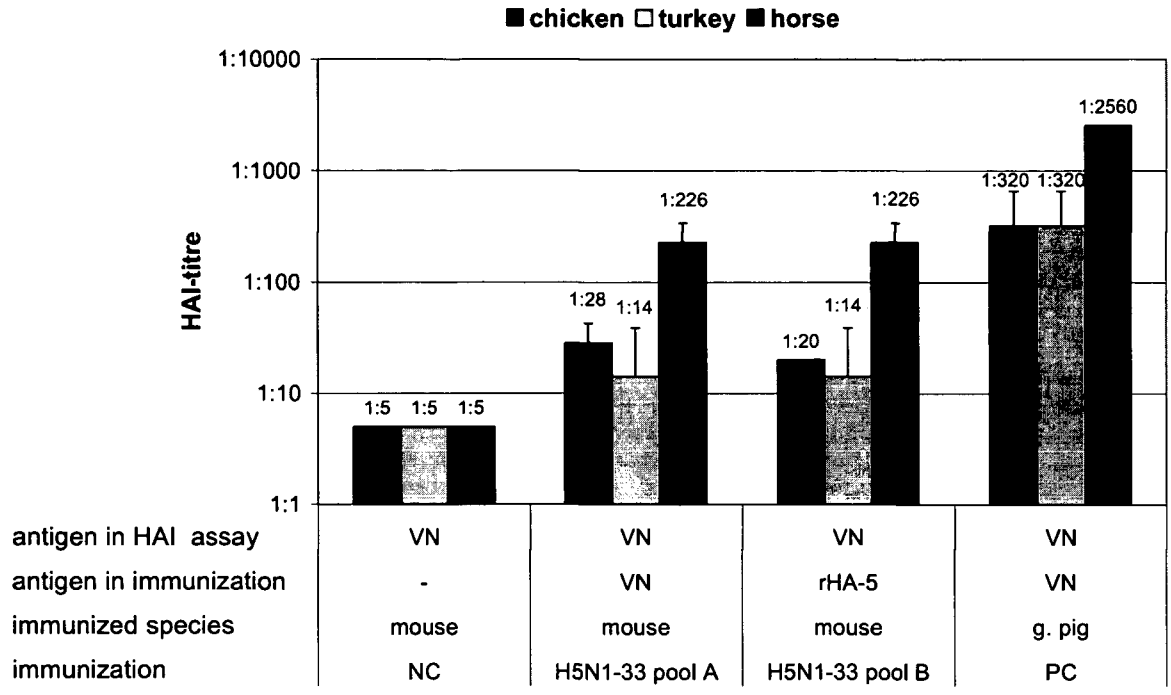


Figure 4.4: HAI-titres using chicken, turkey or horse erythrocytes of immunization H5N1-33 pool A (mice immunized with whole virus VN), H5N1-33 pool B (mice immunized with rHA-5 of VN clade), NC (NC 17/10/06, negative control) and PC (H5N1-22, positive control, guinea-pigs immunized with whole virus VN). Each bar represents the GMTs including standard variation of HAI-titres of two separate experiments. HAI-titres < 1:10 are below the detection limit and were given a value of 5 respectively a titre of 1:5.

HAI assay performed with turkey or chicken erythrocytes clearly underestimated antibody response compared to HAI assay done with horse erythrocytes where geometric mean titres (GMTs) are obviously increased. The GMTs of horse HAI-titres of pool A and pool B are about 8-fold higher than chicken and even 16-fold higher than turkey HAI-titres. The positive control (guinea-pig serum) also displayed an 8-fold increase in HAI-titre when horse RBCs were used. NC sera did not harbour haemagglutination inhibiting antibodies (figure 4.4).
HAI assays performed with horse RBCs were found to be more sensitive than those performed with the conventional used chicken RBCs. The reason for this finding is that horse erythrocytes have more receptors with N-acetylneuraminic acid α2,3-galactose (SAα2,3Gal) linkages which are important for the effective binding of avian viral haemagglutinin to RBCs (see 1.4 and 1.5). Results suggest that the predominant binding between erythrocytes and avian viral haemagglutinin is SAα2,3Gal and horse blood is an outstanding source of availability of those receptors [17].

4.1.3. Optimization of HAI incubation time and RBCs concentration

Horse erythrocytes do not sediment as well as avian erythrocytes (turkey, chicken) due to the fact that horse RBCs are non-nucleated and smaller [16]. This results in a

long incubation time of HAI assays performed with horse RBCs (130 min) and makes the determination of the endpoint time-consuming.
To optimize HAI incubation time in compliance with optimal RBCs concentration, HAI assays were performed with 1%, 0.75%, 0.5% and 0.4% suspensions of horse RBCs in HA-buffer. Mice antisera were taken from immunization H5N1-33, pool A (mice immunized with whole virus VN) and pool B (mice immunized with rHA-5 of VN clade). HAI assays were read after 60, 100 and 130 min.

Incubation times of 60 and 100 min were unable to produce consistent endpoints due to not settled horse RBCs. Different erythrocyte concentrations had no obvious effect on HAI-titre (data not shown). Although all RBCs suspensions showed consistent endpoints when HAI was read after 130 min, 0.4% horse RBCs were found to visualize the endpoint best (data not shown).
Based on these results, optimal conditions for HAI assays performed with horse RBCs and mouse serum were defined with 130 min incubation time and 0.4% erythrocyte suspension.

4.1.4. Pre-treatment of antisera to destroy interfering substances

Standard pre-treatment procedure of sera for HAI assay uses neuraminidase from Vibrio cholerae (Serva) to lower levels of non-specific inhibitors (see 1.6) of hemagglutination.
It was assumed that reading difficulties namely interactions (see 4.1.1) in HAI assays performed with mouse serum derive from such inhibitors. To improve reading of HAI assays with mouse serum and to eliminate interference, different pre-treatment methods of sera were evaluated.

Mouse antisera were pre-treated with:
RDE-solution (neuraminidase) with or without (±) heat-inactivation
trypsin-periodate ± heat-inactivation
RDE-solution combined with trypsin-periodate ± heat-inactivation

Mouse sera of pool A (immunized with whole virus VN) and pool B (immunized with rHA-5 of VN clade) were taken from immunization H5N1-33 (see 3.2). Negative control (NC 17/10/06) was naive mouse serum and positive control (PC) was guinea-pig serum of immunization H5N1-22 (immunized with whole virus VN, see 3.2). Heat-inactivation was carried out at 56°C for 30 min in a waterbath.

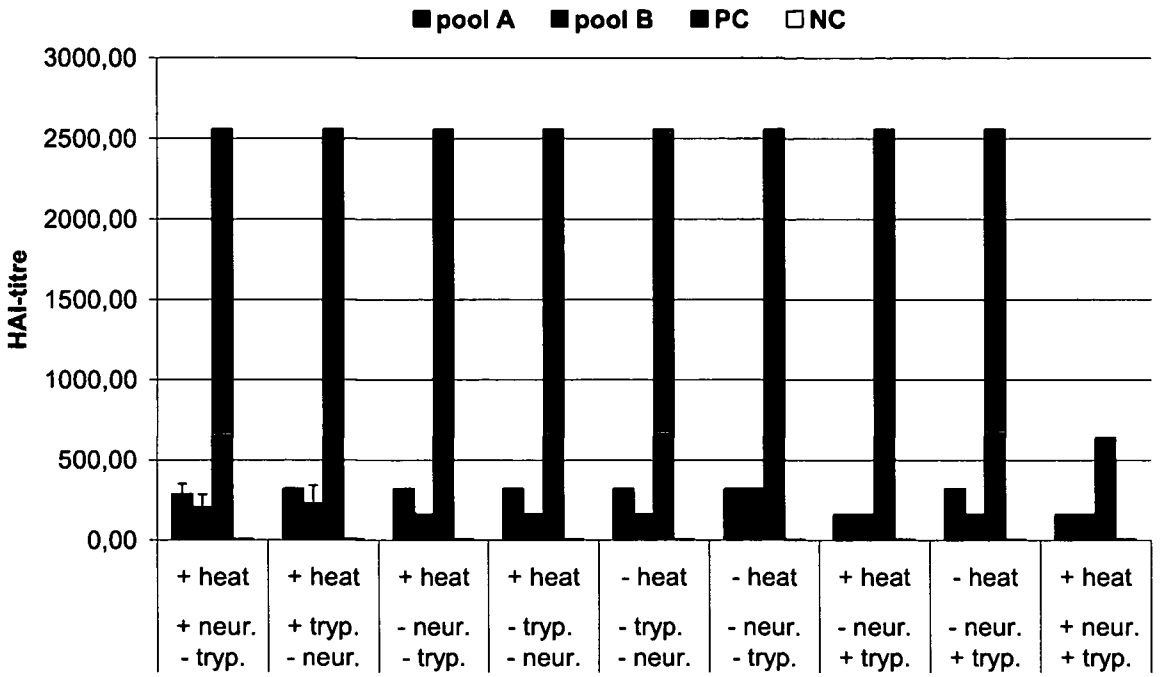


Figure 4.5: GMTs of HAI-titres including standard variation after different pre-treatment methods of mouse serum: ± RDE-solution (+neur.; -neur.), ± trypsin-periodate (+tryp.; -tryp.) and ± heat-inactivation (+heat; -heat). + heat, + neuraminidase experiments were performed 6 times and +heat, +trypsin tests were performed as duplicates. All the other pre-treatment experiments were performed once. Horse RBCs were added as 0.4% solutions and VN antigen was used as test antigen in HAI assay. Incubation time of HAI assay was 130 min. Mouse sera of pool A (immunized with whole virus VN) and pool B (immunized with rHA-5 of VN clade) were taken from immunization H5N1-33. Negative control (NC 17/10/06) was naive mouse serum and positive control (PC) was guinea-pig serum of immunization H5N1-22 (immunized with whole virus VN).

Almost none of the pre-treated serum samples displayed either improvement in reading of HAI assays or impairment in HAI-titres (fig. 4.5). Resulting HAI-titres were nearly the same (with a variation of ± 1 titre level) just like without any treatment at all (- heat, - neur., - tryp.), independent of the pre-treatment method. Only the combination of RDE-solution (+neur.), + trypsin-periodate (+tryp.) and + heat-inactivation (+heat) eliminated reading difficulties (= interactions) at all but also demonstrated a detrimental effect to the HAI-titre of the positive control (PC, guinea-pig serum of immunization H5N1-22: whole virus VN immunized). With this treatment, the titre of the guinea-pig positive control was found to be markedly lower in opposition to pool A and pool B where HAI-titres were in the range of variation (figure 4.5).

Due to unaltered HAI-titres, even when mouse serum was not pre-treated at all (- heat, - trypsin, - neuraminidase), it is likely that mouse antiserum has either low respectively undetectable levels of non-specific inhibitors of hemagglutination or that there are other inhibitors present which are thermal stable and not sensitive to inactivation by periodate, trypsin or neuraminidase (see 1.6) [23].

Although all tested alternative pre-treatment methods were ineffective in reducing interactions and hence improving the reading of HAI assay with mouse serum was not achieved, the standard pre-treatment procedure with neuraminidase (RDE) was maintained in following experiments. Retaining the RDE treatment was a precaution to play safe and avoid possible serum-interference which has been observed to emerge and to disappear again from time to time in previous experiments performed

at the Baxter Department of Virology. In the case of experiments performed in this work no effect due to neuraminidase pre-treatment was observed.

4.1.5. Influence of species as source of antibodies tested in HAI

To evaluate the impact of animal species in HAI assay, rabbit, hamster, rat, mouse and guinea-pig antisera raised against H5N1 influenza strain A/Vietnam/1203/2004 were tested against VN antigen using 0.4% horse erythrocytes. Animal models were immunized with different doses of virus-antigen with (+) or without (-) adjuvant (alum).

Due to different body weight and varying doses of antigen applied, only a qualitative comparison of HAI-titres was possible (see table 4.1).

species	age at immunization	average body-weight [g]	antigen dose [µg]	ratio: body-weight/antigen dose
Wistar rat	8 - 9 weeks	180 - 240	45	4.7
hamster	6 - 8 weeks	150	5	30
rabbit	12 weeks	2800	15	186.7
guinea-pig	6 - 7 weeks	350 - 400	0.3	1250
CD1 mouse	7 - 9 weeks	28 - 35	0.3 or 0.03	105 or 1050

Table 4.1: age, body-weight and vaccinated antigen-dose of different animal species tested in HAI

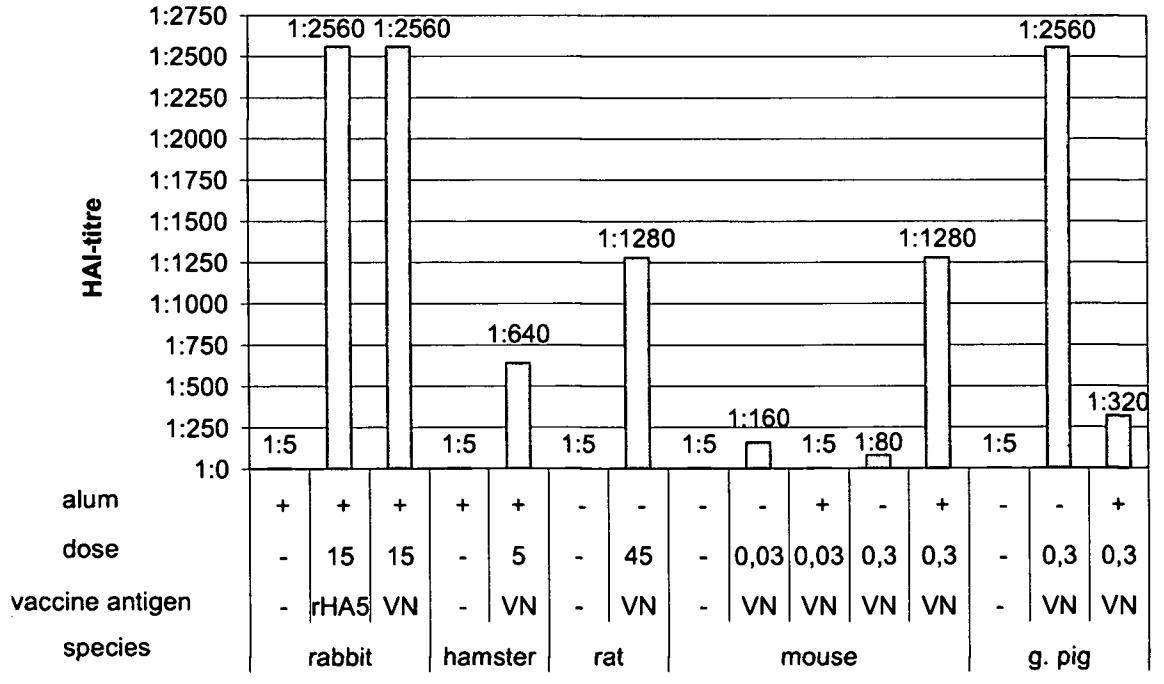


Figure 4.6: HAI-titres of different animal species raised to H5N1 VN clade. HAI assay was performed against VN antigen using 0.4% horse erythrocytes.

Guinea-pigs and rabbits demonstrated highest antibody-titres and thus best response in the performed HAI assay. Surprisingly, the serum of a guinea-pig receiving non-adjuvanted vaccine (- alum) showed a lower antibody-titre than a guinea-pig receiving adjuvanted vaccine. However, this finding is based only on a single sample and would have to be repeated to allow a conclusion concerning the influence of alum. Rats and hamsters showed with respect to the high immunization dose and their body weight, very poor antibody titres. Regarding to the different body weight and the immunization dose, the comparison of HAI-titres was most convenient between guinea-pigs with an antigen dose of 0.3 µg and CD1 mice with an antigen dose of 0.03 µg (see table 4.1). Mice HAI-titres were much lower than those of guinea-pigs even when immunized with the same doses regarding to their difference in body weight. To summarize, related to body weight and immunization doses, rabbits and guinea-pigs are presumably the most sensitive animal models (figure 4.6).

4.1.6. Specifity of HAI assay to H5N1 virus

The H5 specifity of HAI assay was evaluated by using an anti-H7-chicken-serum (VLDIA043 HAR-INFH7 avian influenza, H7 hemagglutinin antiserum). 2 different lots (H5-lot1 resp. H5-lot2) of anti-H5-chicken-serum (VLDIA042 HAR-INFH5 avian influenza H5 hemagglutinin antiserum) were applied as positive control and chicken naive serum (VLDIA030 SPF-CH chicken negative control serum) as negative control (NC). All samples were purchased from Animal Health Service/Netherlands.

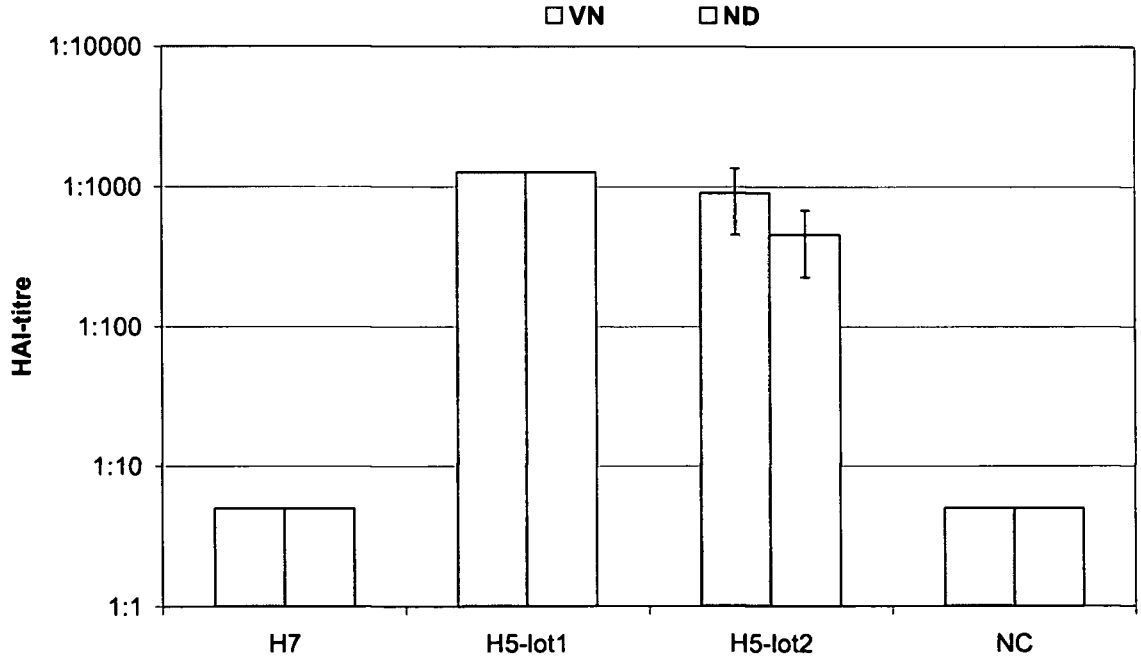


Figure 4.7: HAI assay performed with H5 or H7 specific chicken sera. Each bar represents the GMTs including standard variation of HAI-titres of 2 independent experiments performed as duplicates. HAI assay was performed with 0.4% horse erythrocytes and 130 min incubation time. Both antigens, VN and ND were tested.

There was no antibody-titre detected using anti-H7-chicken-serum (H7) in comparison to markedly higher titres demonstrated with the two different lots of anti-H5-chicken-serum (H5-lot1, H5-lot2). Results imply a specific detection method to antibodies directed against H5 type of haemagglutinin (figure 4.7).

4.1.7. Impact of virus antigen tested in HAI assay with mouse serum

To evaluate the impact of the VN and the ND antigen on the HAI antibody titre when tested in HAI assay, mouse antisera from immunization H5N1-33, pool A (whole virus VN immunized) and pool B (rHA-5 immunized) were applied in HAI assay. The experiment was performed with 0.4% horse RBCs and 130 min incubation time. Serum samples were tested 7-times against VN antigen and twice against ND antigen.

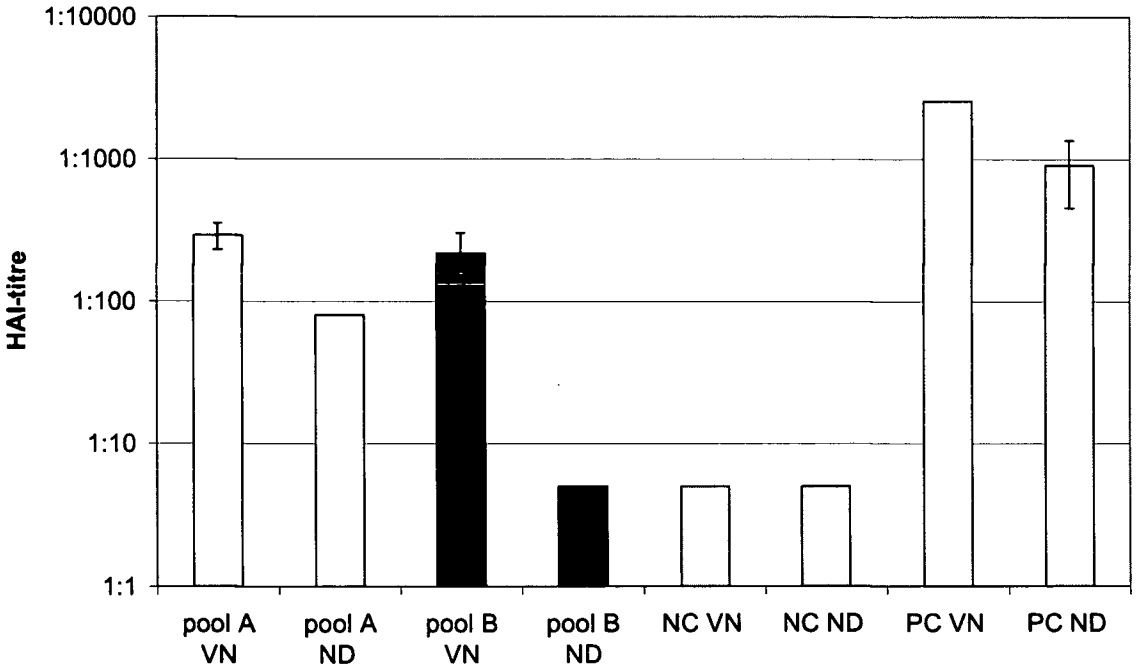


Figure 4.8: mouse antisera from pool A (whole virus VN immunized) and pool B (rHA-5 immunized) tested in HAI assay against ND and VN antigens. Bars show the GMTs of HAI-titres including standard variation. Negative control (NC) is naive mouse serum and positive control (PC) is guinea-pig serum (whole virus VN immunized).

Although mice and guinea-pigs were immunized with H5N1 influenza strain of VN clade, determination of HAI-titre against ND antigen was possible in pool A and PC. HAI-titre of pool A against ND antigen was lower than HAI-titre against VN antigen due to immunization of animals with VN strain. That implies a better protection of mice against the homologues virus strain. The HAI-titre of pool B (mice immunized with purified rHA-5 of VN clade) against ND antigen was negative in comparison to a markedly higher titre of pool A when tested against the same antigen (ND). These results suggest a presumably better cross-protection of the whole virus Vietnam candidate vaccine against Vietnam and Indonesia subtype than highly purified rHA-5 vaccine (figure 4.8).

4.2. Application of optimization of HAI assay for mouse serum

Following experiments were performed with 2 similar immunizations of CD1 Mice: H5N1-28 and H5N1-37 (see 3.2). Immunizations were only differing in presence (H5N1-37) or absence (H5N1-28) of 0.2% adjuvant Al(OH)3 (alum).
In brief, 8 groups of CD1 mice were immunized twice via the subcutaneous route with eight different doses (5-fold dilutions beginning at 3.75 µg HA per dose) of the A/Indonesia/05/2005 candidate vaccine preparation (ND Vaccine).
2 groups received 3.75 µg or 0.03 µg of A/Vietnam/1203/04 (VN vaccine).

4.2.1. Monitoring of humoral immune response of immunized mice by different immune assays (HAI, ELISA, µ-NT)

The presence of specific antibodies in sera (= seroconversion) against H5N1 vaccine was monitored by different immune assays: HAI, ELISA and µ-NT.
For a more detailed description regarding the comparability of above mentioned immune assays see chapter 4.2.2.
For the monitoring of seroconversion, mouse serum was always examined 6 weeks post vaccination of the animals.

HAI assay

HAI assays of immunizations H5N1-28 (- alum) and H5N1-37 (+ alum) were performed with 0.4 % horse RBCs against both antigens: ND and VN.

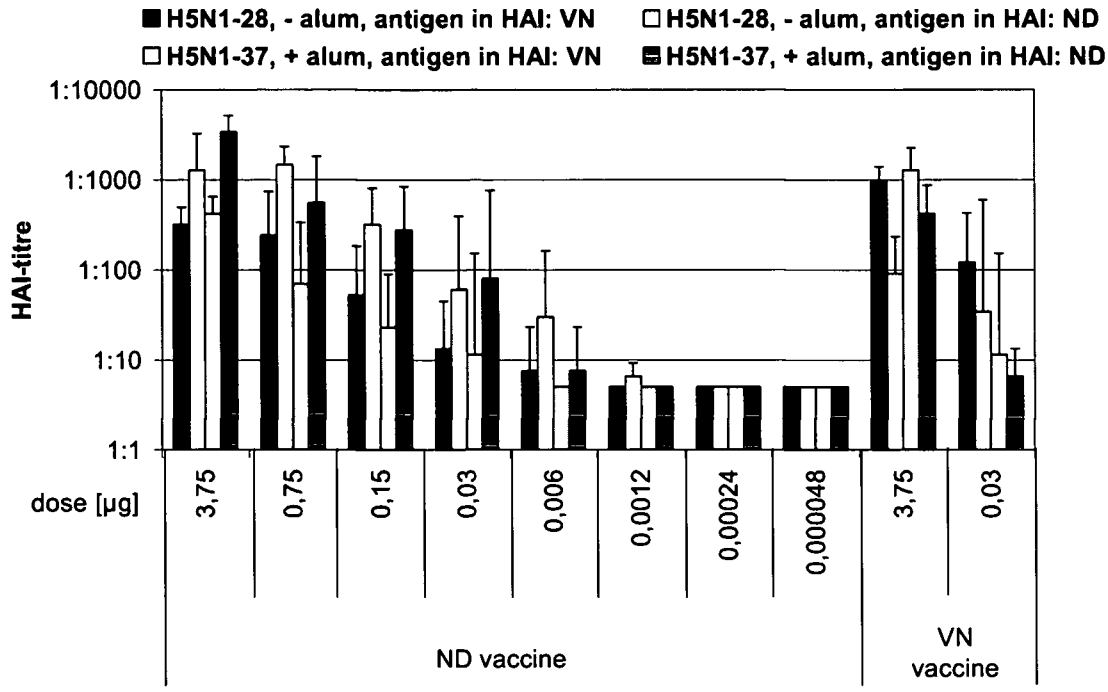


Figure 4.9: dose-response curve of HAI-assay with horse RBCs of immunization H5N1-28 (- alum) and H5N1-37 (+ alum). The GMT HAI-titres including standard variation from 5 mice per group respectively doses are shown.

In both immunizations, HAI-titres against the homologues target (ND vaccine against ND antigen) were distinctly higher in contrast to HAI-titres against the heterologues target (ND vaccine against VN antigen). That means for example, the heamagglutination inhibition reaction against ND antigen in HAI was better (higher HAI-titre) if vaccination of mice was performed with ND virus preparation (ND vaccine). Mice infected with lower vaccine doses than 0.0012 µg had no detectable antibody-titre in HAI assay with horse RBCs. At high immunization doses (3.75 µg) with ND and VN vaccine, adding of the adjuvant alum showed a beneficial effect resulting in higher HAI-titres to both antigens (ND and VN) tested in HAI assay. The lower the immunization doses the less pronounced was this positive alum-effect. At very low doses there was either no alum-effect or even an adverse impact of alum detected (figure 4.9).

ELISA assay

Conventionally employed ELISA assays were performed with rHA-5 (VN clade) as coating antigen. Data was provided by the Baxter Department of Virology.

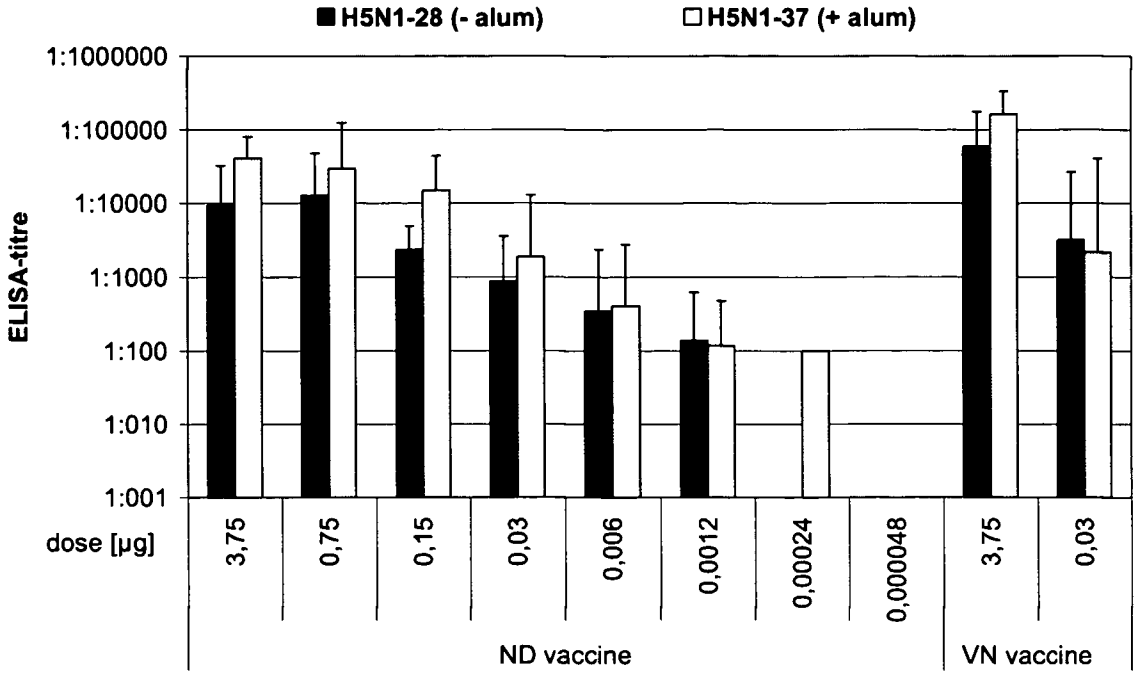


Figure 4.10: dose-response curve of ELISA assay (coated with rHA-5 of the VN clade) of immunization H5N1-28 (- alum) and H5N1-37 (+ alum). Each bar displays the GMTs of ELISA-titres of 18 mice including standard variation. Limit of detection is an ELISA-titre of 1:100.

ELISA-titres of both immunizations showed a clear dose-response curve even up to low doses in case of the ND vaccine. In comparison to the detection limits of the horse-HAI and the µ-NT (horse-HAI: 0.006 µg and µ-NT: 0.03 µg), ELISA detected an antibody-response until a ND-vaccine dose of 0.00024 µg (figure 4.10).

µ-NT assay

In µ-NT assays, serum samples were not tested individually (like in ELISA or HAI assays) but groupwise in pools of 18 mice per dose. Data was provided by the Baxter Department of Virology.

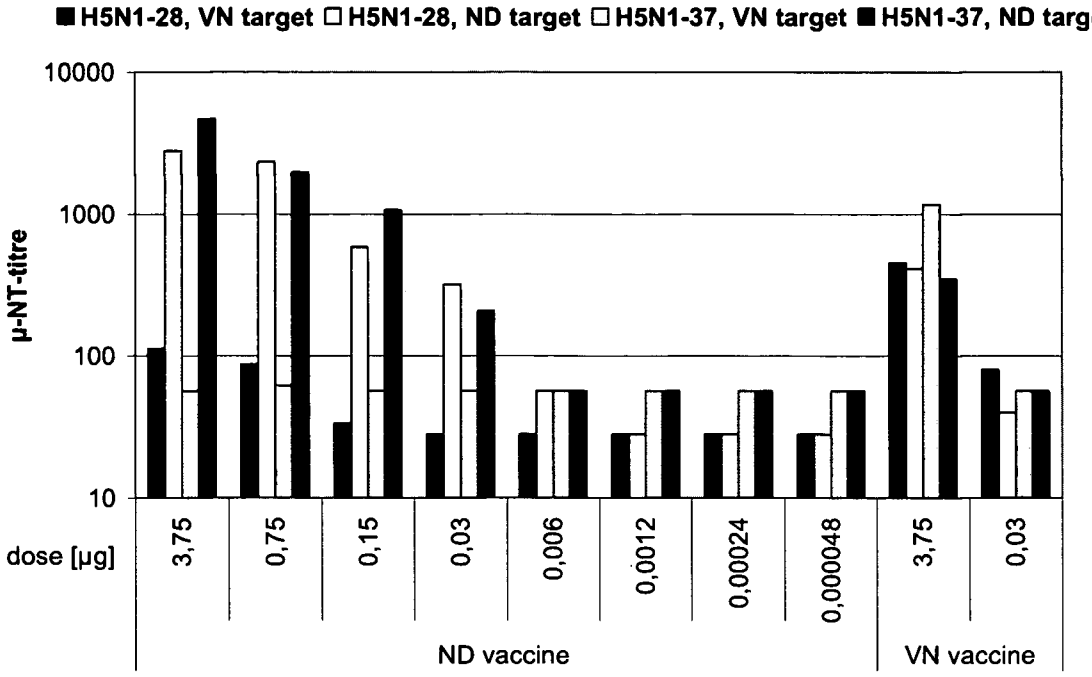


Figure 4.11: dose-response curve of µ-NT assay of immunization H5N1-28 (- alum) and H5N1-37 (+ alum) against VN and ND target. Each bar represents the neutralising antibody-titres of pools of 18 mice per dose. For immunization # 28 the detection limit was a µ-NT-titre ≤ 28.3 and for immunization # 37 it was ≤ 56.6.

The dose-response curve of ND vaccine shows that in both immunizations (# 28 and #37) only the homologues ND-specific-target detected an antibody response. The VN-specific target is in case of the ND vaccine for the most part below the detection limit. On the other hand looking at the VN vaccine, beside the fact that highest antibody titres were also detected with the homologues VN-target, the heterologues ND-target was able to detect a cross-reactivity of antibodies. Detected cross-reactivity indicates that VN vaccine is presumably better at protecting mice against a heterologues infection with the ND subtype (figure 4.11).

4.2.2. Correlation of immune assays

The comparability of antibody-titres of different immune assays was investigated. On this account, HAI-titres achieved with horse erythrocytes were correlated against titres of the conventionally employed H5-specific indirect ELISA (rHA-5 of VN clade used as coating antigen) and the Micro-Neutralisation (µ-NT) assay (see also 4.2.1).
For the correlation, sera from mice vaccinated with ND vaccine from immunization H5N1-28 (- alum) and H5N1-37 (+ alum) were used. Correlation coefficients were determined by linear regression.
HAI-titres were correlated with ELISA-titres using results from individually tested mice: 5 mice per dose or group, respectively. In µ-NT assays, sera are always tested in pools and therefore correlation with HAI was performed groupwise: GMTs of HAI-titres of 5 mice per group/dose were correlated against GMTs of µ-NT-titres of 18 mice per group/dose. Data of µ-NT assays of recent experiments were provided by the Baxter Department of Virology.

H5N1-28 (- alum)	coefficient of correlation [R^2]		
	ELISA (rHA-5)	HAI (VN target)	HAI (ND target)
HAI (VN target)	0.66	---	---
HAI (ND target)	0.7702	---	---
µ-NT (VN target)	---	0.5551*	0.434*
µ-NT (ND target)	---	0.9904*	0.9597*
H5N1-37 (+ alum)	**coefficient of correlation [R^2]**		
	ELISA (rHA-5)	**HAI (VN target)**	**HAI (ND target)**
HAI (VN target)	0.5491	---	---
HAI (ND target)	0.472	---	---
µ-NT (VN target)	---	-917.9*	-924.2*
µ-NT (ND target)	---	0.8909*	0.9006*

Table 4.2: correlation of antibody titres in mouse serum detected by different immune assays. Antisera from mice, vaccinated with ND virus-antigen, were taken from immunization H5N1-28 (- alum) and H5N1-37 (+ alum).

* groupwise correlation

Best correlation coefficients at around 0.9 were achieved between the ND target of µ-NTs and both targets (VN and ND) of HAI assays. Most of the compared titres correlated poorly. For example, there was rather weak correlation evinced between ELISA and HAI assay. One possible reason for that could be that in contrast to the ELISA, which detects all HA-5-specific antibodies in serum, the µ-NT and the HAI, have the advantage to identify strain-specific (target VN or ND) antibodies generated against viral HA [28]. This results in higher but therefore more unspecific antibody titres detected by ELISA and presumably lead to the bad correlation coefficients between HAI and ELISA assays (table 4.2). Furthermore, the H5-specific ELISA is coated with highly purified rHA-5 from VN clade and the tested mice-sera derived from an immunization with ND vaccine, which depicts the heterologues virus target. Hence, a ND-specific ELISA would represent a tool for a better comparability.
In general, it is believed that µ-NT assays are the most sensitive tests to detect antibodies against avian influenza [28]. But when VN-target specific µ-NT-titres from immunization # 37 were compared with horse-HAI-titres against both targets (VN and ND), the µ-NT reached the detection limit earlier than the HAI assay resulting in even negative correlation coefficients: -917.9 and -924.2. The same observation did not apply to immunization # 28, where correlation coefficients (0.5551 resp. 0.434) between µ-NT (VN target) and HAI (VN and ND) target were a little better because in this case µ-NT assay was also able to detect lower antibody titres (table 4.2). Based on these results the µ-NT assay appeared less sensitive than the HAI assay performed with horse erythrocytes due to the inability of the µ-NT assay to detect the lower levels of antibodies in serum. On the other hand the best correlation coefficients about 0.9 were achieved between ND-target specific µ-NT and HAI-titres against both targets (ND and VN). Presumably that's due to the fact that mice were immunized with ND vaccine resulting in the generation of high ND-specific antibody-titres and thus ND-target specific µ-NT didn't reach the detection limit so early. Another reason that applies for the better sensitivity of horse-HAI, in comparison to the µ-NT, was the good correlation (0.9904 and 0.8909) between VN-specific target of HAI and ND-specific target of µ-NT (table 4.2). In that case the correlation is

presumably good because even though HAI assay was performed with the heterologues vaccine-target, the VN target, high HAI-antibody-titres were detected which in turn correlate to the high antibody-titres of the homologues ND-target specific µ-NT. To sum up, these results suggest that HAI assay performed with horse RBCs might be more sensitive than the µ-NT assay. But it should be kept in mind not to over-assess these findings because of the groupwise correlation and the small number of analysed values respectively titres.

4.2.3. Correlation of HAI-titre with protection of mice against H5N1 infection

To demonstrate the efficacy of the vaccination and to determine the protective dose 50% (PD50) value against infection with H5N1, challenge experiments were performed.
For this purpose, mice from immunizations with ND and VN vaccine, H5N1-28 (- alum) and H5N1-37 (+ alum) were inoculated intranasally with the different H5N1 strains (i.e. VN or ND) and were observed for disease signs respectively death as result of infection with H5N1. The protective doses of vaccination were then calculated by the logit-algorithm with Baxter in-house software. Data was provided by the Baxter Department of Virology.
For immunization H5N1-28 (- alum) the PD50 value was 6 ng when mice were challenged with H5N1 Vietnam (VN) strain and 1 ng when challenged with H5N1 Indonesia (ND) strain. For immunization H5N1-37 (+ alum) the PD50 value was 24 ng when mice were challenged with H5N1 Vietnam (VN) strain and 3 ng when challenged with H5N1 Indonesia (ND) strain (see also figure 4.12).

To evaluate whether there is a correlation between protection of mice and high HAI-titres, the antibody-titres of horse HAI assays against both antigens (ND and VN) were classified according to survival or dead of the animals when challenged with H5N1 VN strain (challenge of mice with ND strain was not considered because there was insufficient mouse-serum available for testing it in HAI assay). This was done for ND vaccine for both immunizations: H5N1-28 (- alum) and H5N1-37 (+ alum) (see table 4.3). Another table with additional information about the immunization doses can be found in the appendix (see appendix 10.2). Differences between the two groups (alive and dead) were investigated using a paired t-test.

H5N1-28 antigen in HAI: ND		H5N1-28 antigen in HAI: VN		H5N1-37 antigen in HAI: ND		H5N1-37 antigen in HAI: VN	
alive	dead	alive	dead	alive	dead	alive	dead
5120	1280	640	80	1280	80	160	5
640	5	160	5	5120	5	640	5
2560	40	320	5	2560	5	640	5
640	10	320	5	5120	80	320	5
640	40	320	5	5120	5	640	5
2560	5	320	5	2560	5	640	5
2560	5	1280	5	640	5	5	5
1280	5	320	5	160	5	320	5
640	5	80	5	2560	5	320	5
640	5	20	5	1280	40	5	5
5	5	5	5	1280	5	5	5
640	5	80	5	5	5	5	5
80	5	10	5		5		5
320	5	40	5		5		5
	5		5		5		5
	5		5		5		5
	5		5		5		5
	5		5		5		5
					5		5
					5		5
					5		5
					5		5
					5		5
					5		5
P-value: 0.0011		P-value: 0.0020		P-value: < 0.001		P-value: < 0.001	

Table 4.3: HAI-titres (tested against VN and ND antigen) of mice from immunization H5N1-28 (- alum) and H5N1-37 (+ alum) classified into 2 groups: 'alive' and 'dead', after challenge with H5N1 Vietnam (VN) strain

In all cases, HAI results in group 'alive' gave a significant larger estimate of the antibody titre than HAI results in group 'dead'. P-values were: $p = 0.0011$, $p = 0.0020$ and twice $p = < 0.001$. P-values below 0.05 indicate that there is a significant correlation between high HAI-titres and survival rate of the animals (table 4.3).

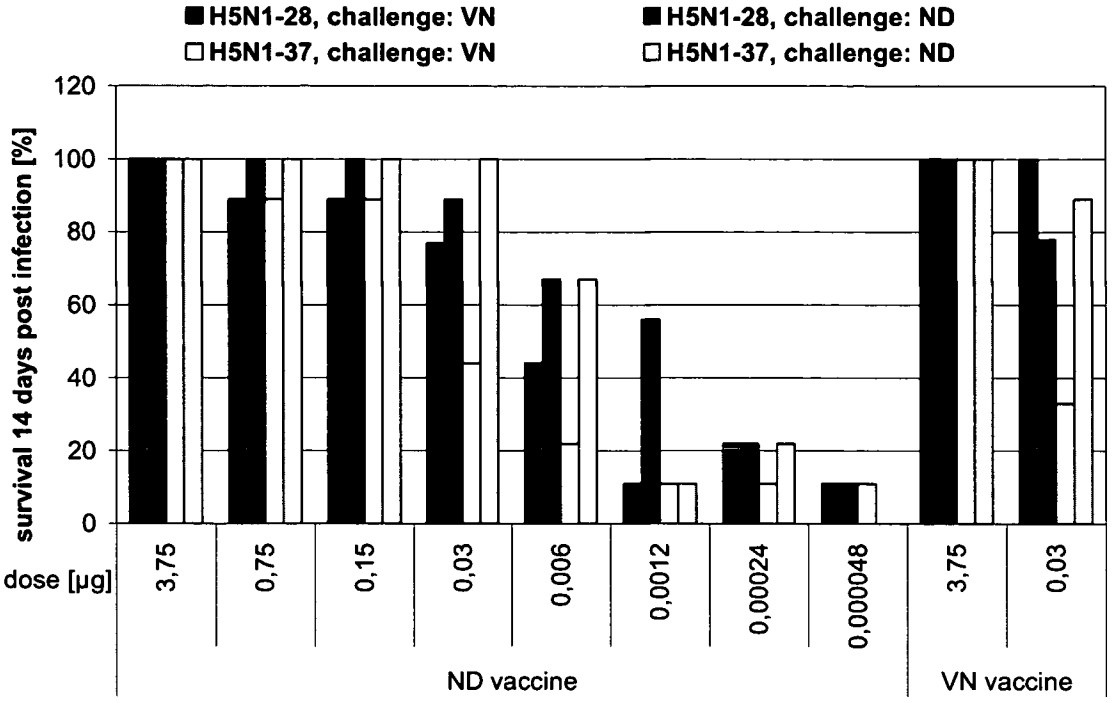


Figure 4.12: survival curve of mice 14 days post intranasal infection (challenge) with ND or VN H5N1 virus strain. Mice were taken from immunization H5N1-28 (- alum) and H5N1-37 (+ alum) and were vaccinated with different doses of either ND or VN vaccine. Each bar represents the percentage of mice (10 mice per challenge virus) that survived 14 days after infection with either VN or ND virus strain.

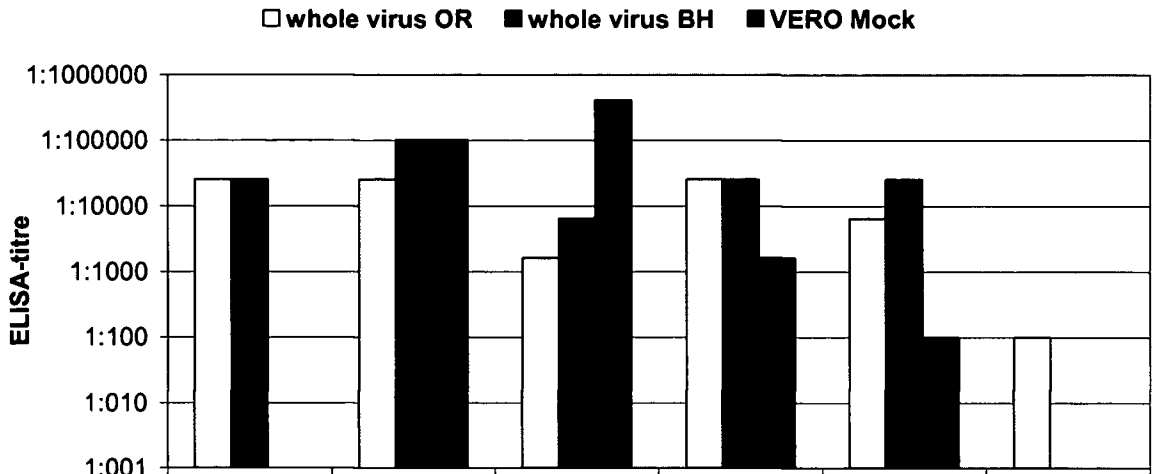
With an immunization dose of 3.75 µg the survival rate of the mice is 100 percent for both vaccines, ND and VN. With declining vaccine doses, the survival rate also decreased gradually which showed clearly that the survival rate is dose dependent. In general, the protection of mice was better against the homologues virus challenge. That means for example mice immunized with ND vaccine were better protected against ND challenge than mice immunized with VN vaccine. Surprisingly more than 80 % of mice vaccinated with 0.03 µg adjuvanted VN vaccine (# 37) survived when challenged with H5N1 Indonesia (ND) strain, although the challenge strain (ND) is heterologue to the vaccine strain (VN). Considering all the other experiments, this would be a solitary exception where a heterologues vaccine protected better than a homologues vaccine. Thus, this survival rate of more than 80 % for VN vaccine and ND challenge is not likely to be valid (figure 4.12).

4.3. Development of ELISA assay for mouse serum coated with whole virus antigen

Standard-ELISA assays to monitor immunogenicity of Baxter's H5N1 vaccine use rH5-HA, a baculo virus derived gene product (Protein Sciences [14]) as a coating antigen.
Currently, rH5-HA only exists for a single clade, the H5N1 clade 1 A/Vietnam/1203/2004 strain. rH5-HA from H5N1-Indonesia strain A/Indonesia/05/05 is not available for purchase yet.
The aim of this work was to design an indirect ELISA assay coated with whole virus antigen purified from H5N1 VN and ND virus representing clade 1 and clade 2 subclade 1 viruses, respectively (see appendix 10.1). This would create a tool to determine antibody titres directed against both clades (VN and ND) and would allow comparisons between the results.

4.3.1. ELISA-coating with whole virus antigen

In a first experiment, it was investigated whether accurate ELISA-titres can be obtained by the use of whole virus antigen as coating material.
ELISA microtiter plates were coated at a concentration of 200 ng/well with 3 different antigens: H5N1 whole virus OR (OR = Orth/Donau), H5N1 whole virus BH (BH = Bohumil) and VERO Mock cell-lysate (see table 3.2). Both whole virus antigens (whole virus OR and whole virus BH) derived from H5N1 virus of Vietnam (VN) strain. Whole virus BH differs from whole virus OR in purification status: Both antigens were ultracentrifuged through a sucrose cushion but whole virus BH was subjected to an additional ultra- and diafiltration step and is therefore more purified than whole virus OR.
Sera tested in ELISA assay derived from CD1 mice, immunized with either rH5-HA (VN) or H5N1 whole virus preparations: # 470 group A (immunized with rH5-HA of VN virus) and # 470 group B (immunized with H5N1 whole virus VN).



	#470 groupA rH5-HA (VN)	#470 groupB H5N1 whole virus (VN)	# 255 anti-VERO-cell-mouse-serum	# 33 pool A H5N1 whole virus OR (VN)	# 33 pool B rH5-HA (VN)	#470 groupC (control group)
□whole virus OR	1:25600	1:25600	1:1600	1:25600	1:6400	1:100
■whole virus BH	1:25600	1:102400	1:6400	1:25600	1:25600	1:000
■VERO Mock	1:000	1:102400	1:409600	1:1600	1:100	1:000

Figure 4.13: titres of an ELISA experiment coated with differentially purified whole virus antigens of VN virus (whole virus OR, whole virus BH) and VERO Mock protein-lysate. The following immunizations of CD1 mice were used: # 470 group A (immunized with rH5-HA of VN clade), # 470 group B (mice immunized with H5N1 whole virus VN), # 33 pool A (mice immunized with H5N1 whole virus OR of VN clade) and # 33 pool B (mice immunized with rH5-HA of VN clade). Limit of detection is a titre of 1:100.

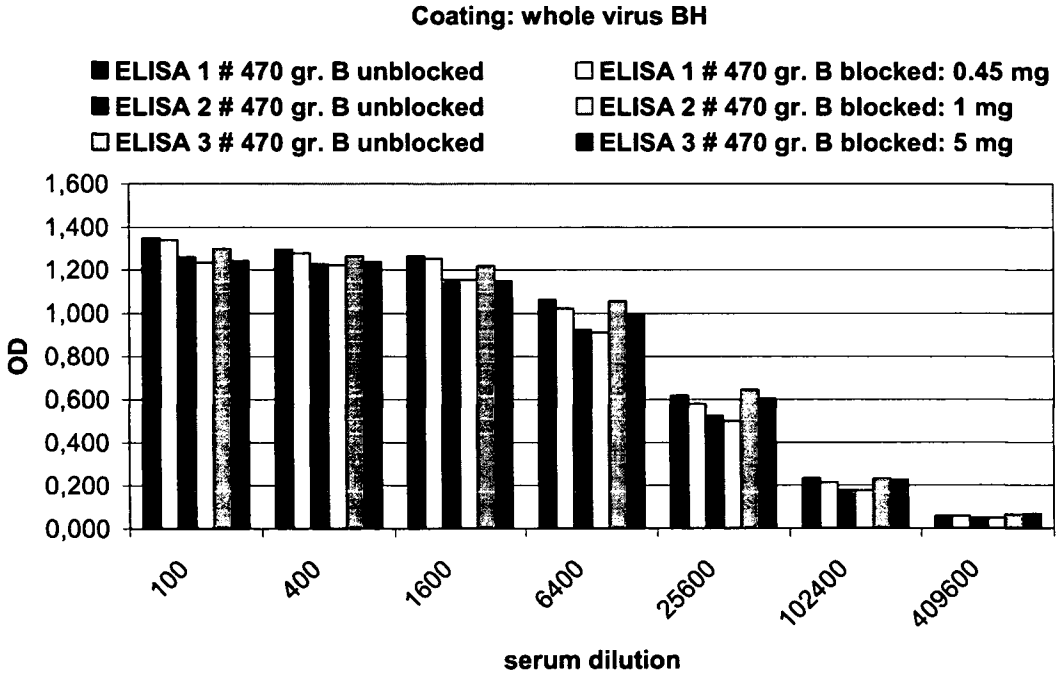
In all cases of tested sera (except the negative control: # 470 group C), ELISA plates coated with whole virus antigen showed high ELISA-antibody-titres. In addition, moderate reactivity between VERO Mock protein and anti-VERO-antibodies in serum (immunization # 470 group B, # 255 anti-VERO-cell-mouse-serum, # 33 pool B) was detected. Especially serum from groups immunized with whole virus vaccine (# 470 group B, # 33 group A) showed rather high antibody-titres against VERO-protein, whereas groups immunized with rH5-HA gave no respectively almost no (titre of 1:100) response. In fact, because the baculovirus-expressed rH5-HA is highly purified, the detection of antibodies in sera that cross-react with VERO protein was unlikely anyway. Anti-VERO-cell-mouse-serum (# 255) showed highest ELISA-titre when exposed to VERO Mock cell-lysate. However, antiserum of # 255 also reacted with the whole virus antigens (figure 4.13).
Anti-VERO-antibodies in serum were likely detected because the H5N1 virus used for immunizations was propagated in VERO cells. Normally, Baxter's H5N1 candidate vaccine virus preparations experience various purification steps before they are vaccinated but sera of the immunization # 470 was only purified by sucrose gradient ultracentrifugation. As a consequence VERO protein was applied together with vaccine-antigen and therefore anti-VERO-antibodies were generated as well. However, antisera are derived from immunization # 470 which used an only partially purified H5N1 vaccine preparation which does not represent Baxter standard H5N1 candidate vaccine preparations.
Based on these results 2 different possibilities to deal with anti-VERO-antibodies in mouse serum were tested. It was evaluated whether it makes sense to block anti-VERO-antibodies in serum by adding VERO-cell-lysate (see 4.3.2) and in addition H5N1 whole virus strains of VN and ND clade were grown in MDCK cells instead as usual in VEROs (see 4.3.3).

4.3.2. Blocking of anti-VERO-antibodies

To intercept anti-VERO-antibodies in serum, VERO-cell-lysate was added to the antibody-buffer used for the dilution of sera. This should enable the capture of anti-VERO-antibodies in sera before application onto the microtiter test plate.

In 3 separate experiments VERO-cell-lysate was added to antibody-buffer in different concentrations: serum in ELISA 1 was blocked with 0.45 mg/10 ml antibody-buffer, in ELISA 2 with 1 mg/10 ml and in ELISA 3 with 5 mg/10 ml.
ELISA assays were performed with the same 3 coating antigens as in 4.2.1: whole virus OR, whole virus BH, VERO Mock cell-lysate. Whole virus BH antigen preparation was more purified than whole virus OR (see table 3.2).
To illustrate the blocking effect, serum-samples were taken from that group which showed highest antibody-titre against VERO-protein: # 470 group B.
As pointed out in 3.3.1, serum from this group (# 470 group B) derived from an immunization of CD 1 mice, which received an only partially purified H5N1 vaccine preparation which does not represent Baxter's standard H5N1 candidate vaccine. In Baxter's standard H5N1 candidate vaccine preparations the amount of VERO protein is controlled strictly and must not exceed 2 %. However, serum of immunization # 470 group B was chosen to optimize the whole virus ELISA assay.
Sera from # 470 group C (data not shown) and from # 255 (anti-VERO-cell-mouse-serum) were used as controls.

A)



B)

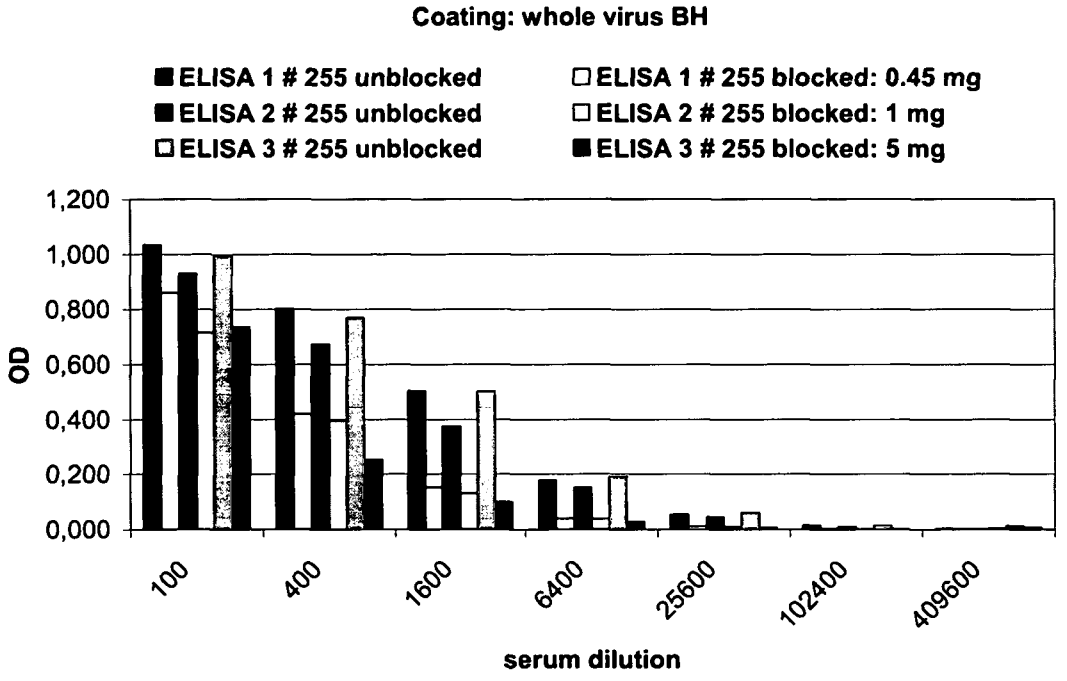
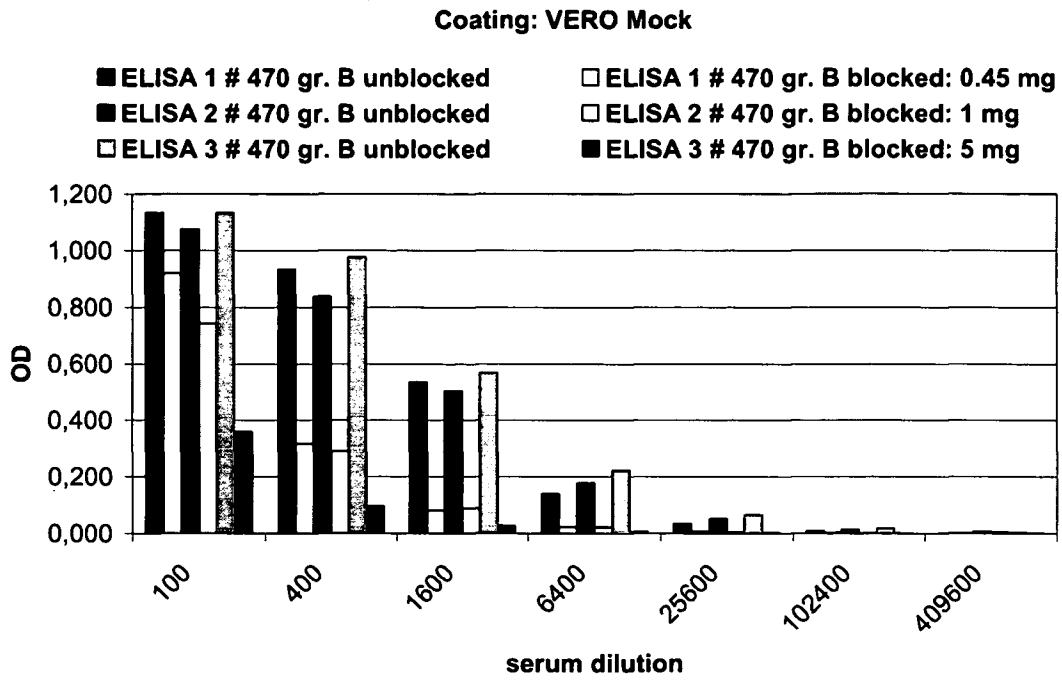


Figure 4.14: A): mouse serum from immunization # 470 group B (immunized with whole virus VN) tested in ELISA assay coated with whole virus antigen. Serum was blocked with VERO-cell-lysate of 3 different concentrations to intercept anti-VERO-antibodies. B): anti-VEROcell-mouse-serum from immunization # 255 tested in ELISA assay coated with whole virus antigen BH. Serum was blocked with VERO-cell-lysate of 3 different concentrations to intercept anti-VERO-antibodies.

When ELISA plates were coated with whole virus OR (data not shown) or whole virus BH preparations (fig. 4.14AB), serum from # 470 group B (CD1 mice immunized with H5N1 whole virus of VN clade) showed nearly no difference in OD with blocked or unblocked application. Results were independent of the applied blocking concentration and of the serum dilution (figure 4.14A). These results indicate that blocking of serum from # 470 group B (CD1 mice immunized with H5N1 whole virus of VN clade) by VERO-cell-lysate has only a minor effect. This is presumably due to a low amount of anti-VERO-antibodies present in the tested serum as a consequence of a small proportion of VERO protein in the antigen preparation used for vaccination (figure 4.14A).

On the contrary to above mentioned findings, serum from immunization # 255 (anti-VEROcell-mouse-serum) showed that blocking affected ODs when ELISA plates were coated with whole virus BH preparation (figure 4.14B). In this case the blocking-effect is plainly visible presumably due to a higher amount of anti-VERO-antibodies in serum: mice of immunization # 255 were vaccinated with pure VERO-cell-protein. In addition, the blocking-effect seemed to be depended on the serum dilution and furthermore on the amount of added VERO-cell-lysate. For example, at a dilution of 1:400, anti-VEROcell-antibodies in serum of immunization # 255 were blocked better with 5 mg VERO-cell-lysate than with 1 mg (figure 4.14B).

A)



B)

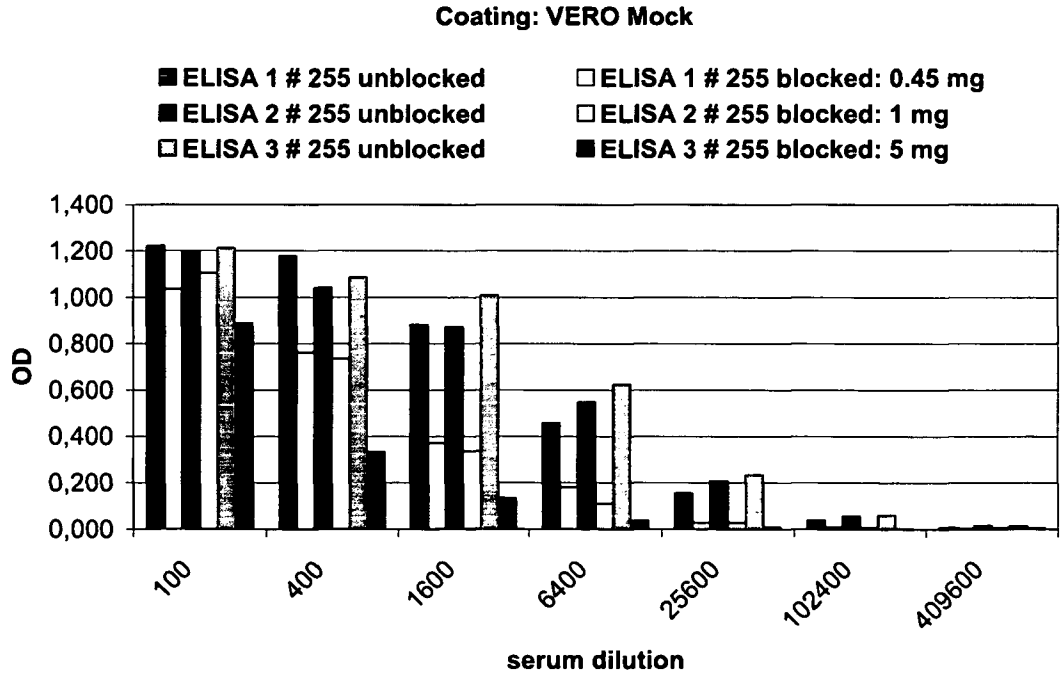


Figure 4.15 A): mouse serum from immunization # 470 group B (immunized with whole virus VN) tested in ELISA assay coated with VERO Mock antigen. Serum was blocked with VERO-cell-lysate of 3 different concentrations to intercept anti-VERO-antibodies. B): anti-VEROcell-mouse-serum from immunization # 255 tested in ELISA assay coated with VERO Mock antigen. Serum was blocked with VERO-cell-lysate of 3 different concentrations to intercept anti-VERO-antibodies.

When ELISA plates were coated with VERO Mock, in contrast to coating with whole virus antigen BH (see fig. 4.14A), serum from # 470 group B revealed an obvious blocking effect. Concomitant with an increasing amount of VERO-cell-lysate

decreased OD signals were obtained (fig. 4.15A). In contrast, serum from # 255 displayed less remarkable results attributed to blocking at lower dilutions (fig. 4.15B).

To summarize, although a positive blocking effect was conspicuous when either serum from immunization # 255 (anti-VEROcell-mouse-serum) was tested on plates coated with whole virus antigen (fig. 4.14B) or serum from # 470 group B (H5N1 whole virus VN clade) on plates coated with VERO Mock (fig. 4.15A), adding of VERO-cell-lysate showed no consistent positive effect throughout the experiments in relation to added amount and varying serum dilution. Further, blocking of anti-VERO-antibodies of # 470 group B (mice immunized with H5N1 whole virus of VN clade) showed hardly any effect on ELISA plates coated with whole virus antigen. In conclusion, blocking-experiments were considered to be of use but because of incomplete respectively inconsistent blocking-results, this method is not applicable to prevent the detection of anti-VERO-antibodies in the ELISA assay.

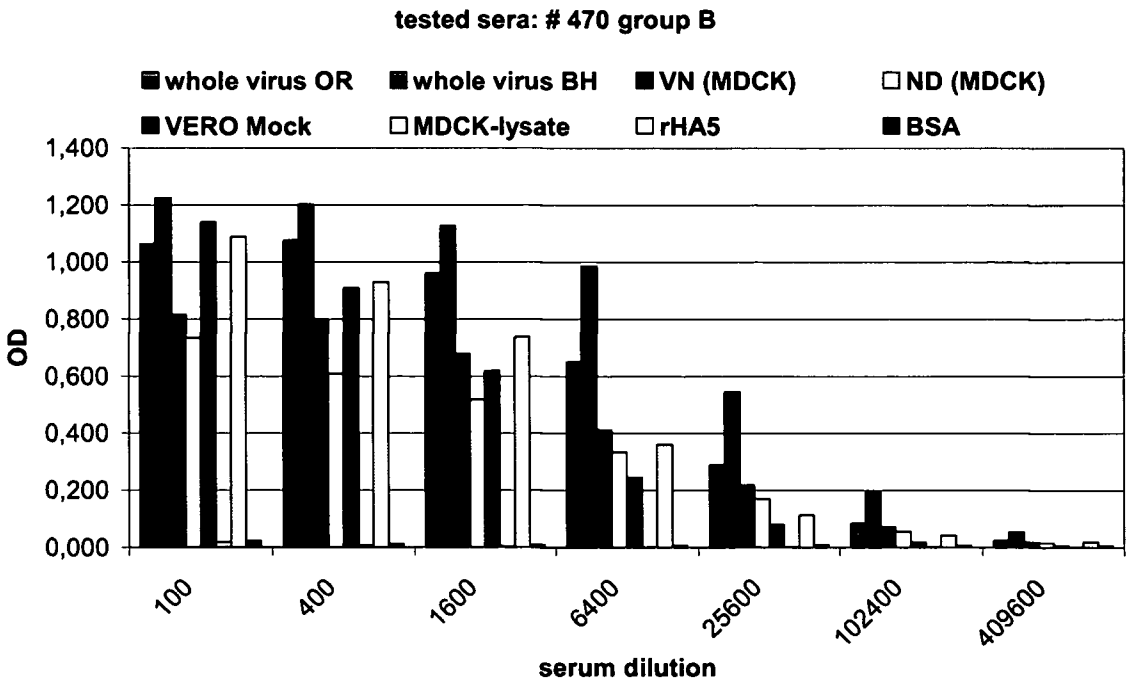
4.3.3. Propagation of H5N1 virus in MDCK cells

To produce VERO-cell-free coating antigens for the development of whole virus ELISA assay, H5N1 virus strains of A/Vietnam/1203/04 and A/Indonesia/05/05 subtype were propagated in MDCK cells of passage 67 for 2 days. Virus strains were inactivated by formalin for 24 h at 33℃ in a water bath. Inactivation of virus was confirmed by a safety assay. Then strains were purified by ultracentrifugation through a sucrose cushion and finally the protein content was determined by BCA™ protein assay kit or Micro BCA™ protein assay kit respectively.

ELISA assay was performed with 8 different coating antigens: whole virus OR, whole virus BH, VERO Mock cell-lysate, rHA-5 (recombinant H5 haemagglutinin of VN clade), VN (MDCK), ND (MDCK), BSA (bovine serum albumin) and MDCK-cell-lysate (see table 3.2).
VN (MDCK): H5N1 whole virus antigen of Vietnam (VN) clade propagated in MDCK cells and purified by ultracentrifugation through sucrose cushion.
ND (MDCK): H5N1 whole virus antigen of Indonesia (ND) clade propagated in MDCK cells and purified by ultracentrifugation through sucrose cushion.
Whole virus OR: H5N1 whole virus antigen of Vietnam (VN) clade propagated in VERO cells and purified by ultracentrifugation through sucrose cushion.
Whole virus BH: H5N1 whole virus of Vietnam (VN) clade propagated in VERO cells and purified by ultracentrifugation through sucrose cushion and ultra/diafiltration.

Coating concentration of the different antigens was 200ng/well except for rHA-5 and BSA it was 50 ng/well.

A)



B)

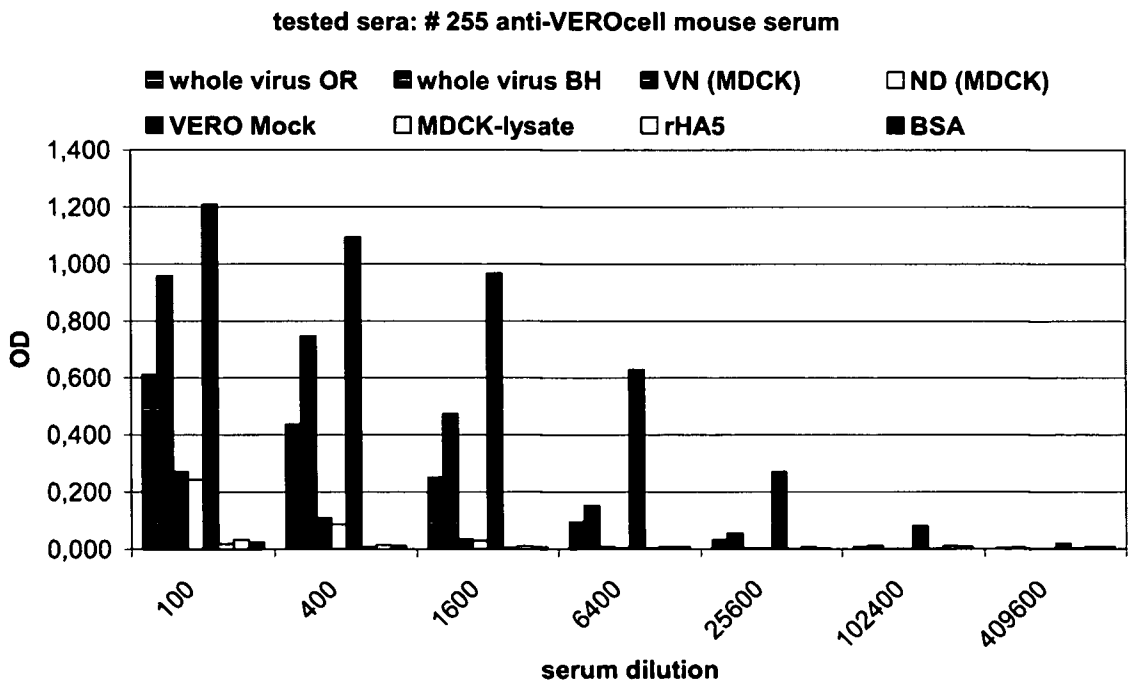


Figure 4.16 A): mouse serum from immunization # 470 group B (immunized with whole virus VN) tested in ELISA assay coated with 8 different antigens B): anti-VEROcell-mouse-serum from immunization # 255 tested in ELISA assay coated with 8 different antigens

Whole virus antigen OR and notably whole virus antigen BH, denoted higher ODs than the conventionally used rHA5 antigen when sera from immunization # 470 group B (immunized with H5N1 whole virus of VN clade) or # 255 (anti-VEROcell-mouse-serum) were tested (fig. 4.16AB). In general, antigens derived from the virus clades VN and ND propagated in MDKC cells, demonstrated lower ODs than whole virus

antigen propagated in VERO cells (OR and BH). Although, VN (MDCK) and ND (MDCK) also revealed a reactivity with anti-VEROcell-mouse-serum (# 255), indicating a presumably cross-reactivity between anti-VERO-antibodies in serum and MDCK protein, ODs were much lower than those denounced for whole virus OR and BH (fig. 4.16B). Interestingly, VN (MDCK) antigen displayed slightly higher ODs than ND (MDCK) antigen though sera from mice immunized with VN clade were tested (figure 4.16A). As expected, VERO Mock antigen showed highest ODs when sera of immunization # 255 were tested (fig. 4.16B).

Baxter's H5N1 candidate vaccine derived from viruses propagated in VERO cell cultures. Vaccine preparations are highly purified and strictly controlled regarding the amount of VERO protein which must not exceed 2%. Due to the fact that antigen preparations of H5N1 viruses propagated in MDCK cells showed very low reactivity with VERO protein, they were optimal antigen-material for the establishment of the whole virus ELISA.

4.3.4. Western Blot

For the further investigation of the reactivity between VERO-cell-protein and anti-VERO-antibodies in serum, Western Blot analysis was performed.
6 different antigens used in ELISA as well (see 4.3.3), were subjected to a SDS-page: purified MDCK (VN), purified MDCK (ND), MDCK-lysate, whole virus OR, whole virus BH and VERO Mock (see table 4.4).

lane	sample	definition
1	Precision Plus Dual Color Standard (Bio-Rad)	protein standard
2	MDCK (VN) after sucrose cushion purification	H5N1 whole virus antigen of Vietnam (VN) clade propagated in MDCK cells and purified by ultracentrifugation through sucrose cushion
3	MDCK (ND) after sucrose cushion purification	H5N1 whole virus antigen of Indonesia (ND) clade propagated in MDCK cells and purified by ultracentrifugation through sucrose cushion
4	MDCK-lysate	lysate of MDCK cells
5	whole virus OR	H5N1 whole virus antigen of Vietnam (VN) clade propagated in VERO cells and purified by ultracentrifugation through sucrose cushion
6	whole virus BH	H5N1 whole virus of Vietnam (VN) clade propagated in VERO cells and purified by ultracentrifugation through sucrose cushion and ultra/diafiltration
7	VERO Mock	VERO cell-lysate of a mock-infected cell culture
8	Precision Plus Dual Color Standard (Bio-Rad)	protein standard

Table 4.4: antigens applied in Western Blot assay

Serum (1st antibody) was used from immunization # 470 group B (H5N1 whole virus of VN clade) or from immunization # 255 (anti-VERO-cell mouse serum). As a control served a Western Blot assay with serum from immunization # 470 group C (naive serum) applied as 1st antibody. In this assay no bands were detected (data not shown).

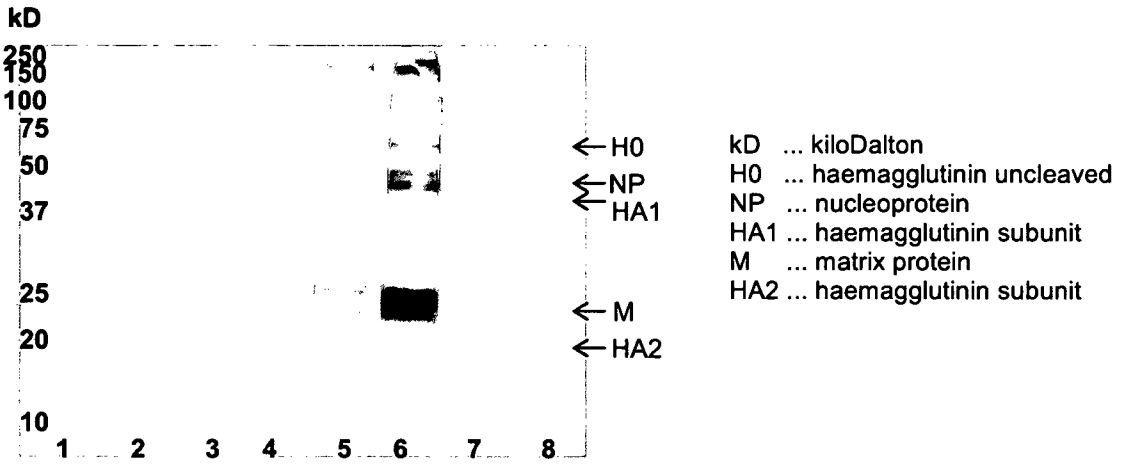


Figure 4.17: antigens used for ELISA assay separated in Western Blot: lane 1 + 8: Precision Plus Dual Color Standard, lane 2: MDCK (VN clade) after sucrose cushion purification, lane 3: MDCK (ND clade), lane 4: MDCK cell-lysate, lane 5: whole virus OR, lane 6: whole virus BH, lane 7: VERO Mock cell-lysate. The amount of the separated antigens was 1 µg, only for VERO Mock cell-lysate it was 3 µg. 1st antibody was applied (dilution 1:100) from serum of immunization # 470 group B (H5N1 whole virus VN clade). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson Immuno) at a dilution of 1:5000 was used as secondary antibody.

In lane 2, 3, 5 and 6 the anti-serum of # 470 group B (mice immunized with H5N1 whole virus of VN clade) detected a band at an apparent molecular weight of 75 kD, which presumably corresponds to uncleaved haemagglutinin (H0). In addition, the double bands at around 50 kD could be assigned to the nucleoprotein (NP) and the HA1 fragment of the HA0. The strong bands between 20-25 kD probably correspond to the matrix protein (M; 25 kD) and the HA2 fragment (22 kD) that is also generated by cleavage of HA0 (fig. 4.17). There was no band detected in lane 4 (MDCK cell-lysate) and surprisingly in lane 7 (VERO Mock cell-lysate) neither. Hence, anti-VERO-antibodies in serum were not able to detect VERO Mock protein in Western Blot. Reasons for that could be low sensitivity of the Western Blot assay or insufficient binding affinity of the antibodies. It might be that anti-VERO-antibodies are only able to recognize conformational epitopes but in Western Blot assays the protein is denatured and therefore presumably inaccessible to the antibody (fig. 4.17).

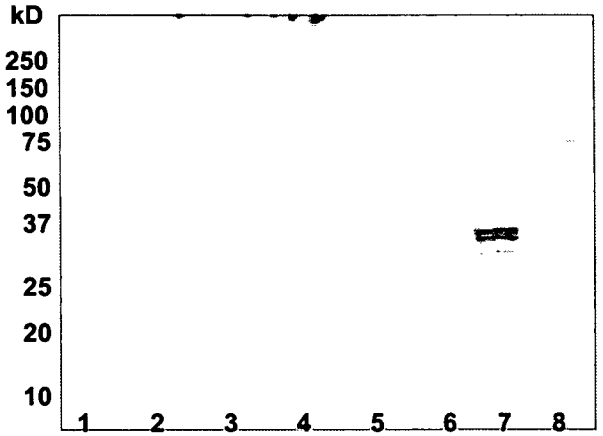


Figure 4.18: antigens used for ELISA assay separated in Western Blot: lane 1 + 8: Precision Plus Dual Color Standard, lane 2: MDCK (VN clade) after sucrose cushion purification, lane 3: MDCK (ND clade), lane 4: MDCK cell-lysate, lane 5: whole virus OR, lane 6: whole virus BH, lane 7: VERO Mock

cell-lysate. The amount of the separated antigens was 1 µg from lane 2-4 and 3 µg from lane 5-7. 1st antibody (dilution 1:100) was applied from serum of immunization # 255 (anti-VERO-cell mouse serum). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson Immuno) at a dilution of 1:5000 was used as secondary antibody.

Anti-VERO-cell mouse serum from immunization # 255 only reacted strong with VERO Mock cell-lysate in lane 7 (VERO Mock cell-lysate). No reactivity was observed against whole virus antigen derived from MDCK cells. In lane 5 (whole virus OR) and 6 (whole virus BH), a band at an apparent molecular weight of 15 kD was visible that presumably correspond to the band in lane 7. This band at about 15 kD could probably imply the existence of VERO-cell-protein in the virus preparation, which remained despite the purification process and therefore was recognized by anti-VERO-antibodies. On the other hand, in lane 5 (whole virus OR) and 6 (whole virus BH) a smear of several minor bands was also visible, possibly unspecific and due to overloading of the gel. Hence, an indeed correlation between the bands in lane 5 resp. 6 and 7 may be clarified by mass spectrometry (fig. 4.18).

5. CONCLUSION

Two different immune assays were set up to analyze the immune response of H5N1 candidate vaccine-immunized lab animals, i.e. an indirect ELISA assay coated with whole virus antigen and a Haemagglutination Inhibition assay using horse erythrocytes.

It was demonstrated that the modified HAI assay using horse erythrocytes is a highly specific and sensitive method for the measurement of antibody-titres to avian influenza virus HA in mouse serum following vaccination.
In fact, for the assessment of antibody response to H5N1 candidate vaccines, horse erythrocytes may be more appropriate for the application in HAI assays than the conventionally used chicken erythrocytes.

In addition, an indirect ELISA assay coated with whole virus antigen derived from H5N1 viruses of VN and ND clade propagated in MDCK cells was established.
In contrast to the conventionally employed H5N1 ELISA assay that uses a Baculovirus-expressed recombinant H5 antigen of the VN-clade as coating antigen, the whole virus ELISA was established with antigens of the H5N1 Indonesia and Vietnam subtype.
Hence, with the whole virus ELISA assay, it is now possible to compare the immunogenicity of candidate vaccines to different H5N1 influenza clades.

6. ZUSAMMENFASSSUNG

Im Jahre 1997 wurden in Hong Kong zum ersten Mal Infektionen von Menschen mit dem H5N1 Influenza Virus nachgewiesen. Seitdem wurden insgesamt 306 weitere Fälle gemeldet (Stand Mai 2007). Das H5N1 Influenza Virus hat das Potential, eine Pandemie auszulösen; seine Ausbreitung löste daher die Nachfrage zur Herstellung von spezifisch wirkenden Impfstoffen aus.
Die Abteilung Virologie der Firma Baxter hat einen inaktivierten, auf einer VERO-Zellkultur basierenden H5N1 Ganzvirus Impfstoff entwickelt, dessen Immunogenität und protektive Effizienz im Tierversuch gezeigt wurde. Die Immunogenität wurde durch den Nachweis von spezifischen Antikörpern mit Hilfe von verschiedenen Immuntests evaluiert.

Im Rahmen dieser Arbeit wurden zwei modifizierte, immunologische Verfahren zum Nachweis von spezifischen Antikörpern gegen das H5N1 Virus in Serum von Mäusen entwickelt: ein Hämagglutinations-Inhibierungstest (HAI), welcher mit Pferde-Erythrozyten durchgeführt wird und ein indirekter ELISA (enzyme-linked immunosorbent assay), der H5N1 Ganzvirus als Antigen benutzt.

Der HAI Test beruht auf der Antikörper vermittelten Inhibition der Hämagglutination von Erythrozyten durch Influenza Viren und stellt eine einfache Methode zur Beurteilung der Immunantwort gegen das Hämagglutinin der Influenza Viren dar. Herkömmliche HAI Tests zeigen aber geringe Sensitivität bei der Testung von H5N1 positiven Maus-Seren. Zudem ist das Ablesen schwierig und eine Auswertung der Ergebnisse unmöglich. Die Probleme sind generell mit geringen Antikörper-Titern assoziiert, aber auch mit dem Vorhandensein von interferierenden Serum-Bestandteilen, bei denen es sich nicht um Antikörper handelt, die aber einen hohen Hintergrund verursachen und somit das Testergebnis verfälschen können. Um das Serum von interferierenden Serum-Bestandteilen zu befreien und die Ablesbarkeit zu verbessern, wurden verschiedene Methoden - Behandlung mit Neuraminidase (RDE), Hitze oder Trypsin-Periodat und deren Kombinationen - evaluiert. Es konnte jedoch keine Verbesserung der HAI-Ablesbarkeit erzielt werden. Um die Sensitivität des HAI Tests für Maus-Serum zu verbessern, wurden Erythrozyten von verschiedenen Tierspezies getestet. Durch die Anwendung von Pferde-Erythrozyten konnten deutlich höhere Antikörper-Titer im Maus-Serum nachgewiesen werden, da Pferde-Erythrozyten vermutlich einen höheren Anteil von Rezeptoren besitzen, die von avinen H5N1 Influenza Viren erkannt werden. Die besten Ergebnisse wurden mit 0.4%igen Pferde-Erythrozyten bei 130 Minuten Inkubationszeit erzielt. Die Spezifität des HAI Tests wurde mit einem Hühner anti-H5 Antiserum nachgewiesen, welches im Gegensatz zu einem Hühner anti-H7 Antiserum, die H5 spezifische Hämagglutination von Erythrozyten inhibieren konnte. Die tatsächliche Anwendbarkeit des modifizierten HAI Tests wurde verdeutlicht, indem spezifische HAI-Antikörper-Titer mit Titern von anderen Immuntests verglichen wurden. Zusätzlich wurde demonstriert, dass ein hoher HAI Titer auch in Relation mit dem Schutz gegen eine Infektion mit H5N1 steht.

Eine andere Methode, um die Immunogenität eines pandemischen H5N1 Impfstoffs zu demonstrieren, ist der indirekte H5-Hämagglutinin-spezifische ELISA. Der indirekte ELISA benötigt hoch aufgereinigtes H5-Hämagglutinin, aber dieses ist kommerziell momentan nur für den H5N1 A/Vietnam/1203/2004 Subtyp erhältlich. Demzufolge wurde ein indirekter ELISA mit Ganzvirus-Antigen vom A/Vietnam/1203/2004 und A/Indonesia/05/05 Subtyp entwickelt. Während der Testentwicklung wurde moderate Reaktivität zwischen VERO-Zell-Protein in der Ganzvirus-Antigen Präparation und anti-VERO-Antikörpern im Maus-Serum festgestellt, wenn Serum von Mäusen benutzt wurde, die mit unvollständig aufgereinigtem Baxter H5N1 A/Vietnam/1203/2004 Ganzvirus Impfstoff immunisiert worden waren. Um den Ganzvirus-ELISA bestmöglich zu optimieren, wurde zum einen versucht, die anti-VERO-Antikörper mit VERO-Zell-Lysat abzufangen und zum anderen wurden H5N1 Viren beider Subtypen (Vietnam und Indonesia) in MDCK Zellen vermehrt, um die Viren dann als Antigen Material im ELISA einsetzen zu können. Zur genaueren Untersuchung der Reaktivität zwischen VERO-Zell-Protein und anti-VERO-Antikörpern wurden Western Blots durchgeführt, mit denen die beschriebene Reaktivität jedoch nicht bestätigt werden konnte. Das Blockieren der anti-VERO-Antikörper im Maus-Serum durch VERO-Zell-Lysat verminderte die Reaktivität gegenüber VERO-Zell-Protein in der ELISA Antigen Präparation mäßig. Eine ELISA Antigen Präparation von in MDCK vermehrten H5N1 Viren reagierte jedoch nur wenig mit anti-VERO-Antikörpern, die im Serum von mit H5N1 Ganzvirus Impfstoff immunisierten Mäusen enthalten waren, d.h. diese Virus Präparation stellte das optimale Antigen-Material für einen H5N1-Ganzvirus-ELISA dar.

7. ABSTRACT

The first human infections with avian H5N1 virus emerged in Hong Kong in the year 1997. Since that time a total of 306 cases were reported (WHO, May 2007).
H5N1 viruses have the potential to cause a human influenza pandemic and therefore the demand to produce effective vaccines has considerably increased.
The Baxter Department of Virology has developed a VERO-cell-derived H5N1 whole virus vaccine. Immunogenicity and efficacy of the candidate vaccine was assessed in animal experiments. The measurement of specific antibodies in serum by different immunoassays indicated that the H5N1 candidate vaccine induces functional antibodies capable of blocking virus infection.

In this work a modified Haemagglutination Inhibition (HAI) assay using horse erythrocytes and an indirect whole virus ELISA (enzyme-linked immunosorbent assay) to measure the amount of specific antibodies in mouse serum were developed.

HAI assays rely on the inhibition of haemagglutination of erythrocytes caused by influenza viruses and are a simple method to assess immune response to influenza haemagglutinin (HA) in sera. However, conventionally employed HAI assays are not sensitive enough for the detection of antibody responses to H5N1 avian haemagglutinin in mouse serum. Reading of HAI assays is difficult and consequential determination of the endpoint is impossible. The problem is associated with poor antibody titres and unspecific background interference caused by non-specific serum components. To diminish interferences in serum, alternative serum pre-treatment methods such as heat, neuraminidase, trypsin-periodate and combinations of those were tested but yet they were found to be ineffective in improving the reading. To establish an HAI assay that was more sensitive, erythrocytes of different animal species were evaluated. In addition, erythrocytes were applied at different concentrations and assays were read at varying points of time. By using horse erythrocytes and thereby probably increasing the proportion of specific linkages needed for the binding of viral HA to red blood cells, an improved detection of avian H5 antibodies in mouse serum was demonstrated. Best results were achieved by using horse erythrocytes at a concentration of 0.4% and after 130 minutes incubation time. The H5-specificity of the assay was confirmed by testing chicken anti-H5 and anti-H7 antisera against two different subtypes of H5N1 influenza viruses: H5-dependent hemagglutination was only inhibited by the chicken anti-H5 antiserum. The applicability of the modified HAI assay for mouse serum was demonstrated by the comparison of specific-HAI-antibody-titres with antibody-titres of other immune assays. Furthermore the detection of high HAI titres was related to the protection of mice against an infection with H5N1 virus.

Another method to illustrate the immunogenicity of the pandemic H5N1 vaccine is an indirect H5-haemagglutinin-specific ELISA. This indirect ELISA assay uses highly purified H5-haemagglutinin but this is currently only available for a single clade, the H5N1 A/Vietnam/1203/2004 subtype. Thus, an indirect ELISA assay coated with whole virus antigen purified from Vietnam and Indonesia clades was developed.

During the assay development, moderate reactivity between VERO-cell-protein in the whole virus-antigen preparation and anti-VERO-antibodies in mouse serum was detected, if antisera of mice vaccinated with less purified whole virus vaccine was used. To optimize the whole virus ELISA assay it was attempted to intercept anti-VERO-antibodies in serum by adding VERO-cell-lysate. In addition H5N1 virus strains of Vietnam and Indonesia subtype were propagated in MDCK cells to obtain virus antigens as coating material. For the more precise investigation of the reactivity between VERO-cell-protein and anti-VERO-antibodies, Western Blot analysis was performed but indicated reactivity could not be confirmed. The capture of anti-VERO-antibodies by VERO-cell-lysate sparsely attenuated reactivity to VERO-cell-protein but antigen preparations from H5N1 viruses grown in MDCK cells reacted little with anti-VERO-antibodies and thus provided an appropriate tool for the successful establishment of the whole virus ELISA assay.

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9. ABBREVIATIONS

APS	ammonium persulfate
BCA	bicinchoninic acid
bidest.	bidestillated
BSA	bovine serum albumin
conc.	concentration
DAB	3.3' diaminobenzidine tetrahydrochloride
FCS	fetal calf serum
fig.	figure
g.pig(s)	guinea pig(s)
GMTs	geometric mean titres
HA	Haemagglutination Assay
HAI	Haemagglutination Inhibition Assay
HAU	hemagglutination units
H+L	heavy and light chain of the antibody
l	litre
IgG	immunoglobuline G
LE	low endotoxine
MDCK	Madin-Darby canine kidney (cells)
min	minute(s)
ml	millilitre
NC	negative control
OPD	o-phenylenediamine dihydrochloride
$PAPU_{red}$	sample application buffer; reducing (Probenauftragspuffer; reduzierend)
PC	positive control
PVDF	polyvinylidene difluoride
RBCs	Red Blood Cells
resp.	respectively
rH5-HA	recombinant hemagglutinin5-hemagglutinin
rpm	rotations per minute
SA(s)	sialic acid(s)
sec	second(s)
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane buffer
TTBS	Tris buffered saline/Tween
V	Volt
VERO	kidney epithelial cells from African green monkey
VN	H5N1 influenza virus: A/Vietnam/1203/2004 isolate
vol	volume
WHO	World Health Organization
µ-NT	Micro-Neutralisation Assay

10. APPENDIX

10.1. Evolution-Tree of the H5N1 Haemagglutinin Gene

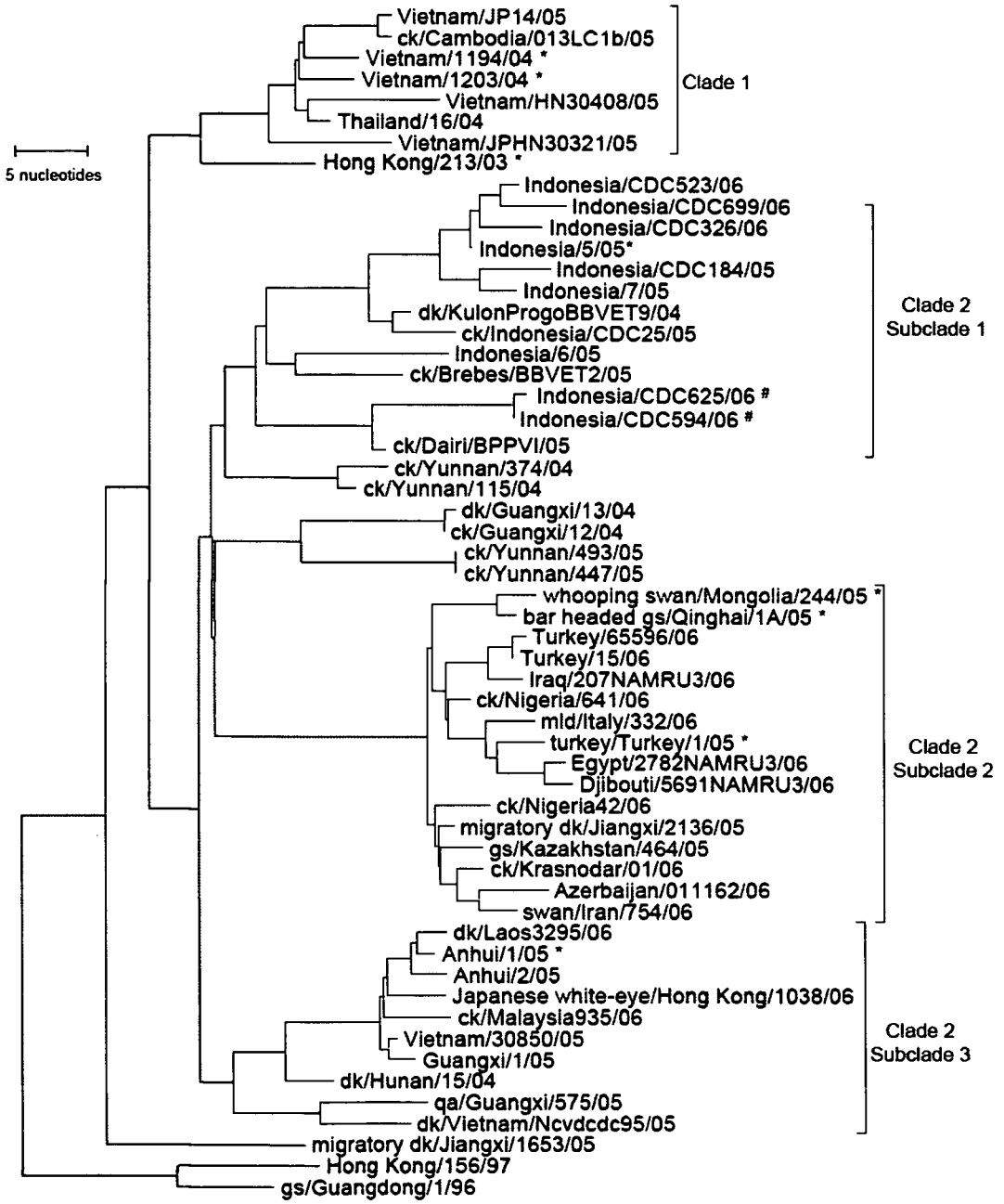

Figure 10.1: evolution-tree of the H5N1-haemagglutinin gene

10.2. HAI-titres of Mice from Immunization H5N1-28 and H5N1-37 'Alive' and 'Dead', after Challenge with H5N1 Vietnam (VN) Strain

dose [µg]	vaccine virus	challenge virus	H5N1-28 antigen in HAI: ND		H5N1-28 antigen in HAI: VN		H5N1-37 antigen in HAI: ND		H5N1-37 antigen in HAI: VN	
			alive	dead	alive	dead	alive	dead	alive	dead
3.75	ND	VN	5120	---	640	---	1280	---	160	---
3.75	ND	VN	640	---	160	---	5120	---	640	---
3.75	ND	VN	2560	---	320	---	2560	---	640	---
3.75	ND	VN	640	---	320	---	5120	---	320	---
3.75	ND	VN	640	---	320	---	5120	---	640	---
0.75	ND	VN	2560	---	320	---	2560	---	640	---
0.75	ND	VN	---	1280	---	80	640	---	5	---
0.75	ND	VN	2560	---	1280	---	160	---	320	---
0.75	ND	VN	1280	---	320	---	2560	---	320	---
0.75	ND	VN	640	---	80	---	---	80	---	5
0.15	ND	VN	---	---	---	---	---	5	---	5
0.15	ND	VN	---	---	---	---	1280	---	5	---
0.03	ND	VN	---	5	---	5	---	5	---	5
0.03	ND	VN	640	---	20	---	---	80	---	5
0.03	ND	VN	5	---	5	---	---	5	---	5
0.03	ND	VN	640	---	80	---	1280	---	5	---
0.03	ND	VN	80	---	10	---	---	---	---	---
0.006	ND	VN	---	40	---	5	---	5	---	5
0.006	ND	VN	---	10	---	5	---	5	---	5
0.006	ND	VN	---	40	---	5	---	5	---	5
0.006	ND	VN	---	5	---	5	---	5	---	5
0.006	ND	VN	320		40	---	---	40	---	5
0.0012	ND	VN	---	5	---	5	---	5	---	5
0.0012	ND	VN	---	5	---	5	---	5	---	5
0.0012	ND	VN	---	---	---	---	---	5	---	5
0.0012	ND	VN	---	---	---	---	---	5	---	5
0.0012	ND	VN	---	---	---	---	---	5	---	5
0.00024	ND	VN	---	5	---	5	---	5	---	5
0.00024	ND	VN	---	5	---	5	---	5	---	5
0.00024	ND	VN	---	5	---	5	---	5	---	5
0.00024	ND	VN	---	5	---	5	---	5	---	5
0.00024	ND	VN	---	5	---	5	---	5	---	5
0.000048	ND	VN	---	5	---	5	---	5	---	5
0.000048	ND	VN	---	5	---	5	---	5	---	5
0.000048	ND	VN	---	5	---	5	5	---	5	---
0.000048	ND	VN	---	5	---	5	---	5	---	5
0.000048	ND	VN	---	5	---	5	---	5	---	5
p-value			**0.0011**		**0.0020**		**< 0.001**		**< 0.001**	

Table 10.1: HAI-titres (tested against VN and ND antigen) of mice from immunization H5N1-28 (- alum) and H5N1-37 (+ alum) classified into 2 groups: 'alive' and 'dead', after challenge with H5N1 Vietnam (VN) strain