Generation and characterisation of monoclonal antibodies against influenza virus A, subtype H5N1

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Abstract

The emergence of highly pathogenic avian influenza A virus (HPAIV) subtype H5N1 in 1997 has since resulted in large outbreaks in poultry and in transmission from poultry to humans, mostly in southeast Asia, but also in several European countries. Effective diagnosis and control measures are essential for the management of HPAIV infections. To develop a rapid diagnostic test, a panel of murine monoclonal antibodies (mAbs) against influenza virus A subtype H5 was generated. Eleven mAbs were produced and characterised according to their reactivity by indirect and sandwich ELISA and western blotting against different H5 subtypes representing past and viruses currently circulating. Ten out of 11 mAbs reacted strongly with the haemagglutinin (HA) protein of H5 viruses, whereas one mAb reacted with the M1 protein. Targeted HA protein epitopes seemed to be conformational. One hybridoma clone binds to a linear epitope of the M1 protein. One specific mAb reacts with HPAIV H5 in the immunofluorescence test, and two antibodies neutralised H5 viruses. On the basis of the results, the set of seven mAbs is appropriate for developing diagnostic tests. With the generated mAbs, a sandwich ELISA was developed recognising all H5N1 strains tested but no other influenza viruses. With this ELISA, as little as 0.005 HA units or 0.1 ng/ml H5N1 was detected, surpassing other ELISA tests. The novel reagents have the potential to improve significantly available rapid antigen detection systems.

1. Introduction

Influenza virus A, subtype H5N1 infects birds and has been thought to have limited zoonotic potential and public health significance. In 1997, with the emergence and spread of the new Asian lineage H5N1 virus, this assumption has changed dramatically, as this virus is able to infect a broad spectrum of non-avian species, including humans (Capua and Alexander, 2007). Viruses of the genus influenza A in the family of *Orthomyxoviridae* are grouped into 16 haemagglutinin (HA) subtypes (H1–16) according to their reactivity in serological tests (haemagglutination inhibition test) and into nine neuraminidase (NA) subtypes (N1–9). The eight single-stranded RNA segments of the influenza A virus genome encode 11 viral proteins. According to their virulence, avian influenza viruses are categorised into two groups: highly pathogenic avian influenza viruses (HPAIV), which cause high mortality approaching 100% in chickens, and low pathogenic avian influenza viruses (LPAIV), which cause mild respiratory diseases in poultry ([Deregt et al., 2006] and [Alexander, 2007]). Infection with HPAIV, generally caused by strains of subtypes H5 and H7, results in high economic losses in the poultry industry. Retrospective studies have shown that domestic poultry plays a substantial role in generating novel influenza A virus strains with the capacity to cross the species barrier ([Capua and Alexander, 2007] and [Yang et al., 2008]). The recent HPAIV H5N1 virus has been transmitted occasionally from fowl to human, and a few human-to-human transmissions (family clusters) have been reported. Until August 2010, 505 human cases were laboratory confirmed, 300 of which (59%) had a fatal outcome (WHO, 2010).

Laboratory diagnosis of influenza is essential for surveillance, treatment and vaccine development (Petric et al., 2006). The diagnosis of HPAIV H5 generally includes conventional virus culture followed by serological differentiation but also may include rapid and more cost-effective technologies that allow for the detection of subtype-specific viral antigens or nucleic acids. For the diagnosis of HPAIV H5

infections in humans, the WHO recommended RT-PCR, real-time RT-PCR or other molecular methods, such as rapid antigen detection systems or so-called point-of-care testing and virus culture (WHO, 2007). Commercially available rapid antigen detection systems can, in principle, be used at the point of care, even by untrained personnel without laboratory equipment, and provide results within 15–30 min. However, point-of-care tests (generally lateral flow tests) vary greatly in their sensitivity and specificity, and laboratory confirmation of reactive samples is required ([Beigel et al., 2005], [Petric et al., 2006], [Chan et al., 2007] and [Cui and Tong, 2008]). Direct comparison of the sensitivity and specificity of the available point-of-care tests is difficult due to variable conditions for test evaluations. Preliminary results showed a poor clinical sensitivity by commercial rapid antigen detection systems for the diagnosis of avian influenza (AI) in patients. Furthermore, some of the tests detect several subtypes of influenza A viruses and are therefore not H5-specific ([Chotpitayasunondh et al., 2005], [Chan et al., 2007] and [Ghebremedhin et al., 2009]). It has been suggested that these tests should be used only in situations with a high risk of avian influenza and when high viral loads are expected, and lateral flow ELISA tests are not recommended for surveillance programs (Charlton et al., 2009).

However, there is a need for specific and sensitive rapid antigen detection systems. Monoclonal antibodies (mAbs), as compared to polyclonal antibodies (pAbs), are able to increase the specificity and sensitivity of immunological assay systems (Yang et al., 2008) and are important for the selective detection of H5N1 viruses, antibody testing, and vaccine efficacy studies and might be of possible therapeutic use ([Du et al., 2009] and [Oh et al., 2009]). In this study, newly developed murine mAbs against influenza virus A, subtype H5, were analysed for their reactivity in immunochemical test systems, their ability to neutralise influenza virus A, subtype H5N1, and their suitability as tools for the development of improved point-of-care tests.

2. Materials and methods

2.1. Cells and antigens

MDCK cells were propagated in Eagle's minimal essential medium (EMEM; Invitrogen, Paisly, UK) supplemented with 10% foetal bovine serum (FBS, PAN, Aidenbach, Germany), 1% glutamine (PAA, Pasching, Austria), and 1% non-essential amino acids (PAA).

Thirty influenza A viruses, representing all 16 HA subtypes, including 11 H5 isolates with representatives of clades 1 and 2.2, and two isolates of influenza B virus (Table 1) were grown either in 10-day-old embryonated chicken eggs (specific pathogen-free SPF eggs; Lohmann Tierzucht, Cuxhaven, Germany) or in MDCK cells. Stocks from H1N1, H3N2, H5N2, H5N6, and all H5N1 isolates, H7N1, and influenza B viruses were produced on MDCK monolayers, whereas subtypes H2, H5, and H7-H16 were propagated in embryonated eggs. The virus was harvested either from allantoic fluid or cell supernatant. For the latter, the cells were scratched and centrifuged with the cell supernatant at 4,500 × *g* (Multifuge 3 S-R, Thermo Fisher Scientific, Langenselbold, Germany) for 10 min at room temperature. The supernatant or the allantoic fluid was collected, inactivated with beta-propiolactone (Ferak Berlin, Berlin, Germany), and stored at −80 °C. Inactivation was performed with an ice-cold dilution of beta-propiolactone (1:10 in Tris-HCl, pH 8.0). The virus material was mixed thoroughly with beta-propiolactone. The pH of the mix was adjusted to a value between 7.4 and 8.4. The solution was stored at 4 °C overnight, followed by incubation at 37 °C for 2 h. Inactivation was verified by infection of cells or eggs. Neither cytopathic effects in cell culture nor HA activity in the allantoic fluid or supernatant were observed after four passages. Furthermore, an H5N1-specific TaqMan-PCR was negative in such samples, proving the inactivation of virus. Only inactivated viruses were used for ELISA, western blot, and for the immunisation of mice under bio-safety level (BSL)-2 conditions. Virus quantitation of infectious materials was performed by HA assay (egg-grown virus) or by plaque tests on MDCK cells. In the case of infectious HPAIV H5, virus propagation and experiments were performed in a BSL 3 laboratory. The specific reaction of the generated mAbs was further tested with 16 different respiratory viruses, 10 enteroviruses (Table 1), and 16 bacteria causing influenza-like symptoms in humans (Table 2).

2.2. Antigen preparation

Virus from allantoic fluid or from cell culture supernatant was inactivated and then concentrated by ultracentrifugation at 70,000 × *g* for 2 h at 4 °C through a 30% sucrose cushion. The virus pellet was resuspended in PBS, and aliquots were stored at −80 °C. One aliquot was used for determining the protein concentration by a BCA-protein assay (Thermo Fisher Scientific, Schwerte, Germany).

2.3. Control sera

Polyclonal rabbit anti-AIV H5N1 serum was produced by the immunisation of a rabbit with inactivated H5N1 (A/Whooper Swan/Germany; R65-2/06). Polyclonal mouse sera were collected from the mice immunised for hybridoma generation. Pre-immunisation sera served as negative controls.

2.4. Generation of monoclonal antibodies against avian influenza virus subtype H5

Female BALB/c and C57BL/6 mice, 8–9 weeks old, were immunised intraperitoneally with 17 µg of H5N1 A/Whooper Swan/Germany (R65-2/06) mixed with an equal volume of complete Freund's adjuvant (Sigma–Aldrich Chemie, Steinheim, Germany), followed by a booster immunisation four weeks later of the same dose mixed with incomplete Freund's adjuvant (Sigma–Aldrich). The mice received three boosts of the same amount of antigen in PBS on days −3, −2, and −1 prior to fusion. The spleens were excised and the splenocytes were fused with myeloma cells (P3X63Ag8.653) according to the previously described protocol ([Dorner et al., 2003] and [Pauly et al., 2009]). Beginning on day 10 after fusion, hybridoma supernatants were screened for specific antibodies.

2.5. Screening of supernatants for the production of antibodies

A three-step approach was used: initial screening of hybridoma supernatants was performed by indirect ELISA, followed by selection of antibody-producing hybridoma cells by cell-ELISA and sandwich assay. MAbs were purified using a HiTrap Protein G HP column (GE Healthcare, Munich, Germany) and subsequently dialysed in PBS overnight. After dialysis, the protein concentration was determined using a NanoPhotometer™ UV/Vis spectrophotometer (Implen, Munich, Germany). If values were lower than 1 mg/ml, antibodies were concentrated by means of the centrifugal filter device Amicon® Ultra-4 (Millipore Corp., Bedford, USA).

Isotyping of purified mAbs was performed with the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche-Applied Sciences, Mannheim, Germany).

For specificity and sensitivity testing of purified mAbs and for the definition of optimal antibody pairs, indirect ELISA and sandwich ELISA were used.

2.6. Indirect ELISA

Nunc Maxisorb® microtitre plates (VWR International, Darmstadt, Germany) were coated overnight at 4 °C with viral antigens (1 µg/ml in PBS) or, as negative controls, with BSA, ovalbumin, or allantoic fluid (50 µl each per well). After blocking (blocking buffer: 2% skimmed milk in PBS containing 0.1% Tween 20 [PBS-T]), 50 µl of hybridoma culture supernatants or purified mAbs (1 µg/ml in blocking buffer) were added, and the plates were incubated for 1 h at room temperature (RT). Each well was then incubated for 30 min at RT with 50 µl peroxidase-conjugated, Fcγ-specific goat anti-mouse IgG (Dianova, Hamburg, Germany) diluted 1:2000 in blocking buffer. PBS-T washes were performed three times in between incubations and six times before adding substrate, using an automated plate washer (Tecan, Crailsheim, Germany). The binding of antibodies was determined by 15 min of incubation with 100 µl/well 3,3′,5,5′-tetramethylbenzidine substrate (TMB dihydrochloride tablets; Sigma-Aldrich) according to the manufacturer's instructions. The reaction was stopped by adding 100 µl of 1 M H_2SO_4. The absorbance (A) was measured at 450/620 nm using an automated plate reader (Tecan).

Unspecific cross-reactivity to bacterial antigens were only tested by indirect ELISA using 1×10^6 cfu/ml in 0.1 M $NaHCO_3$, 50 µl per well.

2.7. Cell-ELISA

The cell-ELISA was used as a confirmation assay for the detection of influenza virus-specific hybridoma cell supernatants, as this assay permits the differentiation of reactions of antibodies with influenza A virus and cellular antigens. The test was performed essentially as described previously (Pauli et al., 1984). Briefly, MDCK cells were cultivated on 96-well plates until they reached 100% confluence, infected with 100 µl of H5N1 virus (1×10^3 PFU/ml, A/Whooper Swan/Germany [R65-2/06]), and incubated for 24 h at 37 °C with 5% CO_2. After fixing with 3% formaldehyde in PBS, 50 µl of hybridoma cell supernatant was added, and the plates were incubated for 30 min at 37 °C in a humid chamber. After washing five times with PBS, the plates were incubated with 50 µl of HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific) as described. Finally, 50 µl of the freshly prepared substrate solution (10 mg 3-amino-9-ethyl-carbazole [Sigma-Aldrich Chemie] dissolved in 3 ml dimethylsulphoxide added to 50 ml of 20 mM sodium acetate buffer [pH 5.0–5.5] and 400 µl 3% H_2O_2) was added to each well of the plates. The reaction was stopped after 30 min by washing with tap water and was then analysed by light microscopy.

2.8. Sandwich ELISA

The sandwich assay was used to (a) evaluate the ability of the mAbs to function as a detector or to capture antibodies in a rapid antigen detection system (antigen capture assay) and to (b) determine the detection limit of selected mAb pairs. For (a), 96-well plates were coated with 50 µl of appropriately diluted anti-H5 polyclonal rabbit antibody (1:1000) and incubated overnight at 4 °C. Coated wells were incubated with the antigen solution (1 µg/ml in PBS, 50 µl/well) for 2 h at RT. MAbs were biotinylated, diluted in blocking buffer according to ELISA pre-testings, and incubated with the immobilised virus for 1 h at RT. Finally, HRP-conjugated streptavidin 1:2000 in blocking buffer (Dianova) was added to each well and was further processed as described for the indirect ELISA. To examine the ability of mAbs to capture H5 antigen, the wells were coated with mAbs (1 µg/ml; capture antibody), and anti-H5 polyclonal rabbit antibodies (1:1000) were used as detector antibodies. Bound rabbit antibodies were detected by HRP-conjugated goat anti-rabbit IgG, 1:2500 (Dianova). For (b), capture mAbs were diluted to 0.5, 1.0, and 5.0 µg/ml. Antigen (H5N1 A/Whooper Swan/Germany [R65-2/06]) was applied in serial dilutions from 0.1 ng/ml to 10 µg/ml. Appropriately diluted biotinylated mAbs were used as detector antibodies. Binding of biotinylated antibodies was determined either via peroxidase-conjugated streptavidin (SA-POD) or, for increased sensitivity, with PolyHRP40-conjugated streptavidin (Senova Immunoassay Systems, Jena, Germany) and processed further as described above.

2.9. Western blot

Virus preparations of inactivated subtype H5 viruses (see Table 1) or H1N1 virus (A/New Caledonia/20/99) in Laemmli buffer (Laemmli, 1970) with or without 2-mercaptoethanol were boiled for 5 min before loading. Samples were separated by a 10% or 12.5% SDS-polyacrylamide gel under reducing or non-reducing conditions and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Munich, Germany). The membrane was submerged in blocking buffer for at least 30 min, incubated with the purified mAbs (1:2000 dilution in blocking buffer) for 1 h at RT, washed extensively with PBS-T, and bound antibodies were detected by incubation (30 min, RT) with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) (Thermo Fisher Scientific; diluted 1:20,000 in blocking buffer). Finally, the antibody-binding was visualised using the Immun-Star™ HRP substrate Kit (Bio-Rad Laboratories), followed by exposure to Amersham Hyperfilm ECL (GE Healthcare).

2.10. Deglycosylation

To confirm the binding reaction of mAbs to the influenza virus HA protein, viral H5 glycoproteins were N-deglycosylated with PNGase F (New England Biolabs Inc., Frankfurt/Main, Germany), based on the

manufacturer's instructions and the protocol of Wagner et al. (2000). Briefly, a 10 µl sample, containing 680 ng of inactivated H5N1 virus and 1 µl of denaturing buffer, was combined with 500 U of PNGase F in G7 buffer and 1% Nonidet P-40 to a final reaction volume of 20 µl and was incubated for 6 h at 37 °C, without a denaturation step at 100 °C prior to enzyme treatment. Controls were treated similarly in the absence of PNGase F. The deglycosylation reaction of the HA proteins was analysed by western blotting under non-reducing conditions as described above, with mAb 492/3/E6 as the primary antibody and the polyclonal serum of the immunised mouse as a positive control.

2.11. Immunofluorescence staining of cells infected with H5N1 virus

H5N1 (A/Whooper Swan/Germany [R65-2/06])-infected MDCK cells and mock-infected cells were mixed and incubated on microscope slides with reaction wells for 6 h. The incubation time of 6 h was selected after prior experiments with different incubation times. Then, the slides were fixed in 3% formaldehyde in PBS and stored in 1.5% formaldehyde at 4 °C until use. For immunofluorescence staining, slides were incubated in 0.1% TritonX-100. The purified mAbs were titrated in serial dilutions from 100 to 0.1 µg/ml in sample buffer (PBS with 0.2% sodium azide and 2% BSA). After a washing step, slides were incubated with FITC-conjugated goat anti-mouse secondary antibody (Dianova) in a final dilution of 1:160. After washing, the slides were covered with mounting medium and were investigated under a fluorescence microscope (EURO Star II, Euroimmun, Lübeck, Germany). As controls, immunofluorescence assays were similarly performed with H1N1- and H3N2-infected cells.

2.12. Microtitre neutralisation assay

A microtitre neutralisation (MN) assay was used to test the neutralising capacity of the mAbs. First, mAbs were serially diluted from 100 µg/ml to 0.1 ng/ml in a volume of 50 µl of MDCK cell culture medium and incubated with 50 µl (100 $TCID_{50}$) of the infectious virus (A/Whooper Swan/Germany [R65-2/06]) for 1 h at 37 °C. The solution was added to MDCK cell monolayers in 96-well plates (Renner, Dannstadt, Germany) and incubated for three days at 37 °C in the presence of 5% CO_2. After incubation, the cells were fixed with 3% formaldehyde and stained with naphthalene black for analysis under a light microscope. The neutralising titre (ND_{50}) was determined as the mAb dilution resulting in 50% neutralisation of infectious virus. If a neutralising effect was determined, mAbs were serially diluted 2-fold, starting from the mAb concentration that resulted in 100% neutralisation, and each dilution was tested as described above. Eight parallel cultures were infected with the virus-mAb mix. The ND_{50} was calculated according to Reed and Muench (1938). To compare the neutralisation effect of mAbs for different H5N1 strains, a concentration of 100 ng/ml was applied. This test was done in triplicate.

3. Results

3.1. Selection of monoclonal antibodies

To select mAbs highly specific for the H5 antigen, a 3-step screening approach was performed: starting from more than 5000 hybridomas, clones that were reactive to the H5 antigen immobilised on a plastic surface were selected using an indirect ELISA. From the first screening round, 531 clones were identified that reacted with the antigen preparation. With one exception, no unspecific reactions to BSA, allantoic fluid, or ovalbumin were detected. In an independent second step of the screening process, cell-ELISA testing of hybridoma cell supernatants was used as a tool to select hybridomas reacting only with H5N1 virus-infected cells and to eliminate hybridomas reacting with cellular antigens. Non-specific reactions to cell proteins did not occur. Forty-six selected hybridoma cell culture supernatants were further evaluated in a third screening step performed as sandwich ELISA. There, 21 antibodies were selected that were capable of reacting with the H5 antigen in solution. After the three screening procedures and after subcloning and further culturing of hybridoma cells, 11 clones were obtained that produced the desired antibodies for further characterisation.

3.2. Reactivity of mAbs

The reactivity and specificity of the selected mAbs against AIVH5 isolates and other viruses and bacteria (Table 1 and Table 2) were investigated by indirect ELISAs. No cross-reactions with influenza B (*n* = 2), other respiratory viruses (*n* = 26), or bacteria (*n* = 16) were observed (data not shown). The reaction of the mAbs with influenza A viruses was tested with representative strains of subtype H5 (*n* = 11) and the other 15 influenza A subtypes (*n* = 19) in three different test systems: indirect ELISA, sandwich assays, and western blot. Although all 11 mAbs showed strong reactivity with AIV H5 isolates either immobilised or in solution, test result differences were observed depending on mAb, isolate, and method used (Table 3). In indirect ELISA, three mAbs (20/1/E6, 107/1/E4, and 140/1/D9) showed strong reactivity with almost all of the influenza A HA subtypes, whereas mAb 10/1/D6 demonstrated a weak reaction against HA subtypes 1, 3, and 12. The other seven mAbs reacted exclusively with subtype H5 viruses. MAbs 1/03/F2 and 23/3/G5 detected all of the H5 isolates tested, except for the LPAIV H5N2 isolate. In the sandwich assay, where the biotinylated mAbs were applied as detector antibodies, fewer cross-reactivities with other influenza A subtypes were observed. Eight antibodies detected the subtype H5 viruses. With the exception of the broadly reacting mAb 20/1/E6, mAbs did not bind to any seasonal human influenza virus strain (H1N1, H3N2). Similar to the indirect ELISA, none of the antibodies recognised the LPAIV H5N2 strain, but several mAbs detected the HPAIV H5N6 isolate.

3.3. Detection of isolates from different clades

The H5N1 virus used for immunisation (A/Whooper Swan/Germany [R65-2/06]) is a German isolate from 2006 belonging to clade 2.2.2 (Starick et al., 2008). The results revealed that, besides clade 2.2 (subclades 2.2.1, 2.2.2, and 2.2.3), the established mAbs – with the exception of clone 23/3/G5 – also recognised H5N1 clade 1 viruses (A/Hong Kong/213/03/28, A/Thailand [KAN-1]/2004). Six mAbs reacted with H5N1 strains isolated a year later than the immunisation virus. Remarkably, five antibodies detected not only the H5N1 strains circulating recently, but also the earlier AIV H5 isolate from 1959 (A/Chicken/Scotland/59). Nevertheless, none of the mAbs detected all nine of the HPAIV H5N1 isolates examined.

3.4. Optimal mAb combinations

To find mAb combinations able to detect all H5N1 isolates, extended sandwich ELISA studies were conducted. Selected mAbs alone or mixes of two mAbs together were used as capture and biotinylated detector antibodies, respectively. Finally, several pairs of mAb were identified that detected all nine H5N1 isolates analysed without any cross-reactions with other influenza A subtypes or H5N2/H5N6 viruses. One example of such a superior mAb combination is shown in Table 4.

3.5. Sensitivity testing

To evaluate the potential of selected mAb pairs to function in rapid antigen detection tests, their detection limit was examined in a sandwich ELISA format, which is in general the underlying principle of lateral flow immunochromatographic assays. An example of results are shown in Fig. 1. Using peroxidase-conjugated streptavidin (SA-POD) as the detection reagent, the limit of detection was 1–10 ng/ml, depending on the concentration of the capture antibody. The use of Poly-HRP40 further improved the detection limit 10- to 100-fold, to 0.1–1 ng/ml, with 1 ng corresponding to 0.04–0.05 HA units.

3.6. Western blot

Western blot analysis was used to verify ELISA results obtained for the reactivity of mAbs and to identify the antigen. Ten mAbs recognised a band with a molecular weight of ~75 kDa, corresponding to the HA_0 protein of purified influenza virus. All of these antibodies reacted exclusively with the respective antigen under non-reducing conditions, suggesting that these mAbs target conformational epitopes. Interestingly, antibody 10/1/D6 identified a band of ~28 kDa, corresponding to the M1

protein of the virus. This mAb reacted with the antigen separated on gels under both reducing and non-reducing conditions, implying that mAb 10/1/D6 targets a linear epitope within the M1 protein (Fig. 2a). To investigate the reactivity of the antibodies to other influenza A virus antigens, all 11 AIVH5 viruses and one isolate of the H1 subtype causing most of the cross-reactions were separated under reducing and non-reducing conditions and probed with the different mAbs. Six mAbs reacted exclusively with H5 antigens, whereas five mAbs identified influenza virus H1N1 A/New Caledonia/20/99 as well (Table 3), indicating good correspondence with the findings obtained by indirect ELISA. However, in contrast to indirect ELISA results, six mAbs demonstrated a weak reaction with the LPAIV H5N2 isolate.

To confirm the binding reaction of mAbs with the HA protein, the haemagglutinin was deglycosylated with PNGase F. Antibody 492/3/E6 and polyclonal mouse serum were used as positive controls for detecting the HA protein in western blot. The amount of glycosylation varies between HA proteins from different influenza A virus isolates. Deducing from a calculation based on the protein sequence alone, using a web-based application for the calculation of molecular weights and glycosylation sites from ExPASy (http://us.expasy.org/), it was expected that a shift of the HA_0 band by ~20 kDA for the respective H5 isolate would be seen. After PNGase F treatment, the molecular weight of HA_0 protein was reduced from ~75 kDa to ~55 kDA, as hypothesised. This observation confirmed that mAbs reacted with conformational epitopes on the HA protein separated under non-reducing conditions, and that the binding reactions of the antibody and antigen were not affected by deglycosylation (Fig. 2b).

3.7. Immunofluorescence assay for virus antigen detection

All purified mAbs were tested for their ability to detect H5N1-infected cells by immunofluorescence (Table 5). Two mAbs reacted with virus-infected cells fixed in formaldehyde (A/Whooper Swan/Germany [R65-2/06] and A/Cygnus olor/Germany [R1349/07]), but not with H1N1- and H3N2-infected cells. The antibody 492/3/E6 reacted poorly with infected cells, whereas mAb 22/03/D9 showed the same specific fluorescence staining as the positive control mAb (influenza type A [pooled] of the WHO Influenza Reagent Kit [data not shown]). Neither antibody reacted with the uninfected cells.

3.8. Microtitre neutralisation assay

Two mAbs were identified that neutralised H5N1 (A/Whooper Swan/Germany [R65-2/06] mAb 1/03/F2 and 23/3/G5) (Table 5). A concentration of 74.1 ng/ml for mAb 23/3/G5 and 33.2 ng/ml for mAb 1/03/F2 neutralised H5N1 (100 $TCID_{50}$/ml) by 50%. Further neutralisation assays with another seven H5N1 strains were conducted using 100 ng/ml, demonstrating that both antibodies had a broad neutralisation capacity against H5N1 viruses. The antibody 1/03/F2 neutralised all eight H5 strains tested, whereas mAb 23/3/G5 neutralised five out of eight strains (Table 6).

4. Discussion

The usefulness of rapid antigen detection tests for the diagnosis of influenza is associated with their predictive values and is thus highly dependent on the sensitivity and specificity of the test systems and the prevalence of disease in the population (Petric et al., 2006). This project was initiated because there was a growing demand for the improvement of commercially available test kits for diagnostics of H5N1 in human samples. Available rapid antigen detection systems either did not discriminate between influenza A virus subtypes or showed a low sensitivity, leading to such tests having questionable suitability for routine application in the clinical setting ([Chan et al., 2007], [Rouleau et al., 2009] and [Ghebremedhin et al., 2009]). Ideally, rapid antigen detection tests would have a high sensitivity for detection of H5N1 virus and would also be able to differentiate between subtypes (Chan et al., 2007). Compared to polyclonal antibodies, mAbs offer the unique advantages of increased specificity, unlimited availability, and consistent properties that may be exploited for diagnostic applications ([Yang et al., 2008] and [Oh et al., 2009]).

The immunisation strategy is one of the most important steps for the generation of antibodies to detect native antigens. Different approaches for the immunisation of mice with influenza viruses were

reported: immunisation with Bromelain-purified BHA (Russ et al., 1987), recombinant antigens ([He et al., 2007] and [Yang et al., 2008]), formaldehyde-treated whole virus (Huang et al., 2007), or γ-irradiated immunogen (Oh et al., 2009). It is known that epitopes of virus fixed in formaldehyde can be altered and therefore can induce mAbs, which do not react with native antigenic determinants (Duque et al., 1989). Beta-propiolactone (BPL)-inactivated virus was chosen for the immunisation used here on the basis of the following rationale: BPL preserves viruses close to their native structure and interacts preferentially with the viral genome (Wainberg et al., 1971) Surface proteins remain intact, which induces the production of mAbs against both linear and conformational epitopes. Mice that were immunised twice with this antigen preparation produced a long-lasting, stable titre. Furthermore, it was predicted that immunisation with whole virions would result in the generation of mAbs against the main surface protein haemagglutinin. After the fusion and application of a staged selection system, 10 of 11 mAbs were achieved that target the HA protein, which represents about 35% of the total viral protein of human influenza virus (Oxford et al., 1981). Only one mAb reacted against a linear epitope of the viral matrix protein (M1 protein), which is associated with the inner membrane of the virion.

Investigation of the generated mAbs revealed that the antibodies recognise distinct epitopes ranging from those “specific for the immunisation strain” (data not shown) to more conserved epitopes of influenza A viruses. No viruses or bacteria other than influenza A were recognised by the mAbs by indirect ELISA. The majority of the antibodies were specific for H5 strains, whereas four mAbs (107/1/E4, 140/1/D9, 10/1/D6, and 20/1/E6) also reacted with other influenza virus A subtypes, suggesting that there was binding to conserved epitopes. In a previous report using ELISA, none of four mAbs generated against a distinct H5N1 isolate detected H5 viruses that were isolated before the isolate was used for immunisation (Oh et al., 2009). It is suggested that the HA proteins of H5 viruses have changed significantly since the first isolation of this virus more than 30 years ago and that, therefore, the mAbs directed against recent H5N1 viruses do not bind to earlier H5 virus isolates. However, the present study revealed that three of the antibodies with a broader reaction pattern (20/1/E6, 107/1/E4, and 140/1/D9), as well as five H5-specific mAbs (1/03/F2, 23/3/G5, 132/03/D8, 157/1/F4, and 492/3/E6), were able to detect recent as well as older H5 isolates (e.g., A/Chicken/Scotland/59). Thus, it is assumed that stable epitopes are present on the HA protein of H5 strains, confirming observations published earlier by Varečková et al. (2008). With one exception, all of the antibodies detected both clade 1 and clade 2.2 H5N1 isolates, indicating that these mAbs react broadly with circulating H5 viruses. Western blot analysis suggested that 10 mAbs react with conformational HA epitopes, as they detect HA under non-reducing conditions only, whereas the one antibody that reacts with the M1 protein, 10/1/D6, detects a linear epitope, as it worked under reducing and non-reducing conditions. To verify the binding of the mAbs with the HA protein, PNGase F was used for deglycosylation of the haemagglutinin. The level of glycosylation of the HA protein can vary significantly between virus strains and can thus have a considerable impact on the molecular weight of the glycoprotein (Harvey et al., 2008). Previous reports have shown the altered migration characteristics for deglycosylated HA1/HA2 proteins after denaturation and under reducing conditions ([Harvey et al., 2008] and [Li et al., 2010]). As expected, enzyme treatment resulted in a smaller-sized, faster-migrating HA protein, and a band shift from ~75 kDa to ~55 kDa was demonstrated, confirming that the generated mAbs detected exclusively the protein backbone of the HA molecule.

Furthermore, it has been determined that the ectodomain of the HA protein undergoes conformational changes when the virus preparation is treated with temperatures ≥63 °C (Epand and Epand, 2002). This could explain the finding that the mAbs did not react with the HA protein after an incubation at 100 °C under reducing conditions.

The HA protein of influenza virus is the target of neutralising antibodies. Former studies described five distinct neutralisation epitopes for H5N9 on the HA protein (Philpott et al., 1989). Recombinant human antibodies against influenza A H5N1 viruses described recently showed a 50% neutralisation effect against 100 $TICD_{50}$ H5 viruses at antibody concentrations between 0.2 µg/ml and >500 µg/ml, depending on the clade of H5N1 (Sun et al., 2009). However, two antibodies (1/03/F2 and 23/3/G5) were able to protect MDCK cells against H5N1 infection. Treatment of H5N1 viruses with antibody concentrations of 33 ng/ml and 75 ng/ml, respectively, resulted in a 50% neutralisation of the immunisation strain of H5N1. Neutralisation was not only observed for the virus used for immunisation, but also for other H5N1 isolates tested. The antibody 1/03/F2 neutralised viruses from H5N1 clades 1 and 2, whereas the antibody 23/3/G5 reacted only with viruses from H5N1 clade 2. Whether or not such antibodies could be used as source for the production of therapeutic antibodies has yet to be seen.

The purpose of the present investigation was to develop mAbs which could be used for the development of antigen detection systems. Therefore, the mAbs were used in a sandwich ELISA, either as capture or detector antibodies. Eight mAbs were shown to react specifically with the non-immobilised H5 antigen, which is important for their application in the sandwich-based lateral flow assays. In general, there was a good correlation regarding antibody specificity between the three immunological assay systems used. Differences in epitope recognition must be expected because availability of the antigenic sites is probably dependent on the test system used. It has long been known that antigen conformation on a solid phase appears to be different from one in solution (Vaidya et al., 1985). On the one hand, a portion of injected material may have changed its conformation, which exposed immunogenic sites that are hidden on the native protein. On the other hand, there are sites which are exposed on the native protein but are masked when the antigen is adsorbed onto plastic surfaces and vice versa (Mierendorf and Dimond, 1983). While these are only conformational changes, in western blotting, the denaturation of proteins will lead to changes in antigen presentation and epitope accessibility, thereby affecting antigen–antibody interaction.

Several H5-specific antibodies were generated that are able to recognise recently circulating and older H5 viruses, both immobilised and in solution. However, one mAb alone was not enough to detect all nine H5N1 isolates examined. To achieve this aim, different combinations of mAbs were evaluated using sandwich ELISA. Several mAb pairs were identified that detected all H5N1 viruses analysed, without any cross-reactions with other influenza viruses. This was of great significance for the intended application of generated mAbs in an H5N1-specific rapid antigen detection system. Moreover, established combinations of mAbs resulted in higher absorbance readings for some H5 viruses, suggesting that combined mAbs were directed to different epitopes on the virus particles. These findings are in agreement with previous studies and have confirmed that mAb combinations can be used to increase ELISA sensitivity (He et al., 2007).

The present study demonstrated the ability of mAbs to detect low antigen concentrations, which makes them appropriate for developing enhanced rapid antigen detection systems. According to their instruction leaflets, available tests generally have a sensitivity of ≥1 HA (WHO, 2005). Importantly, the results of the established sandwich ELISA showed that the detection limit for H5N1 virus can be improved to 0.04–0.05 HA (1 ng/ml), indicating the potential for increasing the sensitivity of rapid antigen detection tests. Furthermore, when using Poly-HRP40 conjugate instead of HRP-conjugated streptavidin, the detection limit was significantly improved, to 0.004–0.005 HA (0.1 ng/ml). Based on these results, it is expected that the application of the newly generated mAbs in rapid antigen detection tests using Poly-HRP40 conjugate would result in a 25- to 250-fold higher sensitivity of such an assay in comparison with the ones currently available. Prospective studies should be performed to test the generated antibodies with clinical samples to verify whether the sensitivity of the test systems might be influenced by sample matrices or by poor quality of the sample. Samples that are not collected appropriately may contain smaller amounts of virus and, thus, are associated with a lower sensitivity. The type of transport media is also important, as some of its components may influence the sensitivity of the test. In addition, a dilution of swabs for additional laboratory tests may also lead to negative rapid test results. Therefore, the authors suggest using at least one nasal swab, undiluted, directly for screening and a second swab (nasal or/and throat swab) in transport medium for further investigations, such as PCR or virus culture.

Regarding the H1N1 pandemic starting in 2009, the need for rapid antigen detection tests with reliable results remains essential. Such systems are useful in clinical settings where fast assays guide physicians to make the best possible clinical decisions, for example, when a quarantine of infected patients is needed, followed by clinical and therapeutic monitoring. Next to the clinical application, the use of rapid antigen detection tests is desirable for community surveillance, in facility outbreaks and other epidemiological situations, in order to detect epidemiological trends (Rouleau et al., 2009).The discussion of rapid antigen detection systems mainly involves sensitivity and specificity. A recent evaluation of several test methods for the detection of the novel 2009 influenza A (H1N1) confirm that both criteria remain questionable ([Ginocchio et al., 2009], [Vasoo et al., 2009] and [Sambol et al., 2010]). The problems of sensitivity and specificity in rapid antigen detection systems remain, whether H5N1 or pandemic H1N1 viruses are present, and therefore, the need for better assays is essential. So far, the World Health Organization does not recommend the routine use of rapid tests alone for surveillance purposes or patient management without confirmatory testing ([WHO, 2005], [WHO, 2007] and [WHO, 2009]).

This paper describes the generation of highly specific mAbs against H5 viruses, which might be a useful tool for the development of rapid diagnostic systems. The mAbs produced can now be applied in a variety of immunoassays for point-of-care tests of AI infections. Based on the ELISA results, some of the newly produced mAbs and mAb combinations established are appropriate to improve available rapid antigen detection systems for the diagnosis of H5N1 infections in the areas of both specificity and sensitivity. Studies are under way to implement the novel antibodies in different test platforms.

Conflicts of interest

There are no competing interests.

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Tables and Figures

Table 1. Viruses used for the study.

Influenza viruses	Other viruses causing respiratory symptoms
Influenza A viruses	
H1N1 A/New Caledonia/20/99	Parainfluenza virus 1
H1N1 A/Solomon Island/3/2006	Parainfluenza virus 2 (Stuttgart) St. Geer
H1N1 A/Fukushima/141/06	Parainfluenza virus 3 (243)
H2N3 A/Mallard/Germany/WVv726/04	Parainfluenza virus 4A
H3N2 A/Wisconsin/67/2005	Adenovirus 973/90
H3N2 A/California/7/04	Adenovirus 5
H3N2 A/Brisbane/10/07	Adenovirus 7
H4N6 A/Mallard/Germany/WVv1806-09/03	Human metapneumovirus 625/02
H5N1 A/Whooper Swan/Germany (R65-2/06), clade 2.2	Human coronavirus NL63, 88-652
H5N1 A/Hong Kong/213/03/28, clade 1	Human coronavirus 229 E
H5N1 A/Common Buzzard/T206-91823, clade 2.2	Rhinovirus Netherlands 9501010
H5N1 A/Thailand (KAN-1)/2004, clade 1	Rhinovirus Netherlands 9300423
H5N1 A/Chicken/Scotland/59	Rhinovirus Netherlands 9501841
H5N1 A/Fulica atra/G/655/06, clade 2.2	Respiratory Syncytial virus A long
H5N1 A/Bubo bubo/G/R1166/06, clade 2.2	Respiratory Syncytial virus B 591/07
H5N1 A/Cygnus olor/G/R1349/07, clade 2.2	Coxsackievirus A6, patient 06-1105
H5N2 A/Chicken/Pennsylvania/1/83	Coxsackievirus A2, patient 07-500
H5N6 A/Duck/Potsdam/2216/84	Coxsackievirus B1, patient 08-134
H6N2 A/Turkey/Germany/R04/99	Enterovirus 71, patient 07-498
H7N1 A/Mallard/NVP/41/05	Echovirus 9, patient 08-130
H8N4 A/Turkey/Ontario/6118/68	Echovirus 11, patient 08-131
H9N2 A/Turkey/Germany/2/96	Echovirus 30, patient 07-572
H10N7 A/Mallard/NVP/WVv9417/04	Poliovirus 1, Sabin
H11N6 A/Duck/England/56	Poliovirus 2, Sabin
H12N5 A/Duck/Alberta/60/76	Poliovirus 3, Sabin
H13N6 A/Gull/Maryland/704/77	
H14N5 A/Mallard/Gurev/263/82	
H15N9 A/Shearwater/West Australia/2576/79	
H16N2 A/Herring gull/Germany/R27888/06	
Influenza B viruses	
Influenza B/Malaysia/2506/04	
Influenza B/Florida/4/2006	

Table 2. Bacteria used for the study.

Streptococcus strains	Staphylococcus strains
Streptococcus pneumoniae Spain 23F-1[Sp264]	Staphylococcus aureus PS80 (CC30)
Streptococcus pneumoniae Spain 6B-2 [6M17]	Staphylococcus aureus PS71 (CC121)
Streptococcus pneumoniae France 9V-3 [TL7/1993]	Staphylococcus aureus PS3A (ST9)
Streptococcus pneumoniae S. Africa 19A-7 [17619]	Staphylococcus aureus 8325 (CC8)
Streptococcus pneumoniae England 14-9 [PN96/872/B]	Staphylococcus aureus N315 (CC5)
Streptococcus pneumoniae TW 31 [Taiwan 19F-14]	Staphylococcus aureus PS94 (CC25)
Streptococcus pneumoniae 97-1177 [North Carolina 6A-23]	Staphylococcus aureus PS95 (CC45)
	Staphylococcus epidermidis (CCM2124)
	Staphylococcus haemolyticus (CCM1798)

Table 3. Evaluation of the specificity of mAbs against Influenza A viruses by indirect ELISA (immobilised antigen), sandwich ELISA (antigen in solution) and western blot.

mAb no.	Indirect ELISA		Sandwich ELISA[a]		Western blot	
	H5 viruses[b]	Other viruses[b]	H5 viruses[b]	Other viruses[b]	H5 viruses[b]	Subtype H1 virus
20/1/E6	10/11	16/19	7/11	10/19	11/11	1/1
107/1/E4	7/11	15/19	8/11	6/19	11/11	1/1
140/1/D9	7/11	13/19	9/11	4/19	11/11	1/1
10/1/D6	9/11	4/19	8/11	None	8/11	1/1
1/03/F2	10/11	None	7/11	None[c]	10/11	0/1
23/3/G5	10/11	None	4/11	None[c]	9/11	0/1
492/3/E6	9/11	None	8/11	None	9/11	0/1
21/03/D2	8/11	None	5/11	None[c]	9/11	0/1
157/1/F4	9/11	None	8/11	None	10/11	0/1
132/03/D8	7/11	None	5/11	None[c]	8/11	1/1
22/03/D9	6/11	None	6/11	None[c]	9/11	0/1

Given are the number of virus isolates detected by the mAb and the number of viruses tested, respectively. mAb = monoclonal antibody.

[a] Capture Ab = rabbit anti-H5N1 polyclonal antibody, detector Ab = biotinylated mAb.
[b] See Table 1.
[c] Reactions with other influenza A viruses were only analysed for subtypes H1, H3, and H7.

Table 4. Optimal antibody combinations for detection of H5 influenza viruses by sandwich ELISA.

Capture Ab	Detector Ab	Detection of		
		HPAIV H5N1	H5N2 and H5N6	Other Influenza A virus subtypes
mAb 140/1/D9	pAb H5N1	9/9	1/2	18/19
pAb H5N1	mAb 10/1/D6	8/9	0/2	0/19
pAb H5N1	mAb 157/1/F4	7/9	1/2	0/19
mAb 140/1/D9	mAb 10/1/D6 + mAb 157/1/F4	9/9	0/2	0/19

Numbers indicate the number of virus isolates detected by the mAb and the number of viruses tested, respectively. mAb = monoclonal antibody; pAb H5N1: rabbit anti-H5N1 polyclonal antibody.

Table 5. Characterisation of mAbs raised against inactivated H5N1 virus A/Whooper Swan/Germany (R65-2/06) by isotyping, immunofluorescence test (IFA), and microtitre neutralisation test (MNT).

mAb no.	Mouse strain used for fusion	Isotype	IFA	MNT
20/1/E6	BALB/c	$IgG_{2a\ kappa}$	–	–
107/1/E4	BALB/c	$IgG_{1\ kappa}$	–	–
140/1/D9	BALB/c	$IgG_{1\ kappa}$	–	–
10/1/D6	BALB/c	$IgG_{1\ kappa}$	–	–
1/03/F2	C57BL/6	$IgG_{2b\ kappa}$	–	+
23/3/G5	C57BL/6	$IgG_{2b\ kappa}$	–	+
492/3/E6	BALB/c	$IgG_{1\ kappa}$	(+)	–
21/03/D2	C57BL/6	$IgG_{2b\ kappa}$	–	–
157/1/F4	C57BL/6	$IgG_{2b\ kappa}$	–	–
132/03/D8	C57BL/6	$IgG_{2\ kappa}$[a]	–	–
22/03/D9	BALB/c	$IgG_{1\ kappa}$	+	–

[a] The heavy chain was not definitely determinable.

Table 6. Neutralisation capacity of mAb 1/03/F2 and mAb 23/3/G5 with different H5N1 strains.

H5N1 strains	mAb 1/03/F2 (100 ng/ml)	mAb23/3/G5 (100 ng/ml)
H5N1 A/Whooper Swan/Germany (R65-2/06)	+++	+++
H5N1 A/Hong Kong/213/03/28	+++	–
H5N1 A/Common Buzzard/T206-91823	+++	+++
H5N1 A/Thailand (KAN-1)/2004	++	–
H5N1 A/Chicken/Scotland/59	++	–
H5N1 A/Fulica atra/G/655/06	++	+++
H5N1 A/Bubo bubo/G/R1166/06	+++	+++
H5N1 A/Cygnus olor/G/R1349/07	+++	+++

+++: 3/3 wells without CPE; ++: 2/3 wells without CPE; +: 1/3 wells without CPE; −: 3/3 wells with CPE.

Figure 1. ELISA: Determination of detection limit (LoD) for H5N1, A/Whooper Swan/Germany (R65-2/06) used for immunisation. Capture mAb was coated to the wells in concentrations of 0.5, 1.0, or 5.0 µg/ml. SA-POD: peroxidase-conjugated streptavidin; Poly-HRP: peroxidase-conjugated streptavidin with HRP as homopolymer.

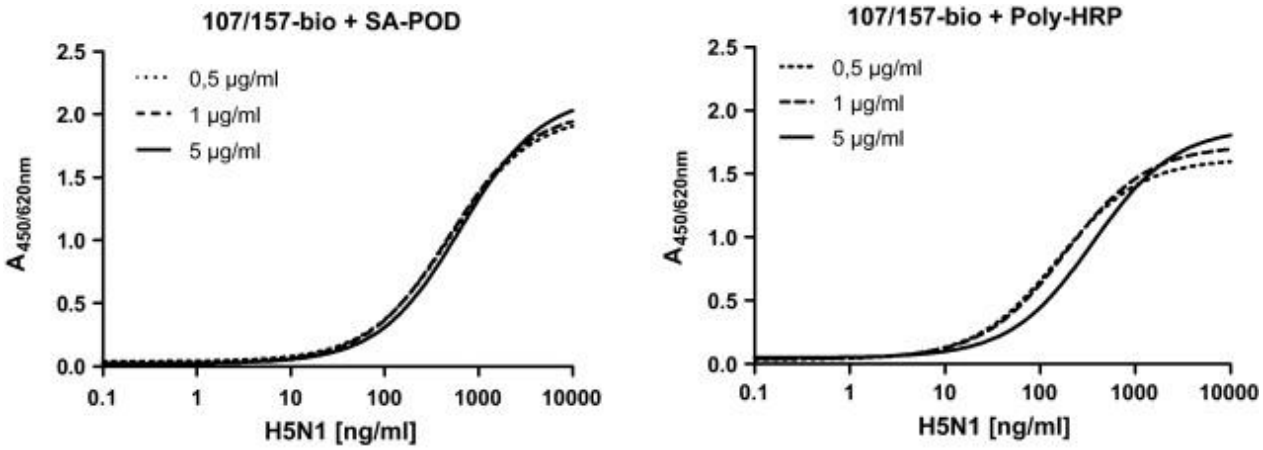
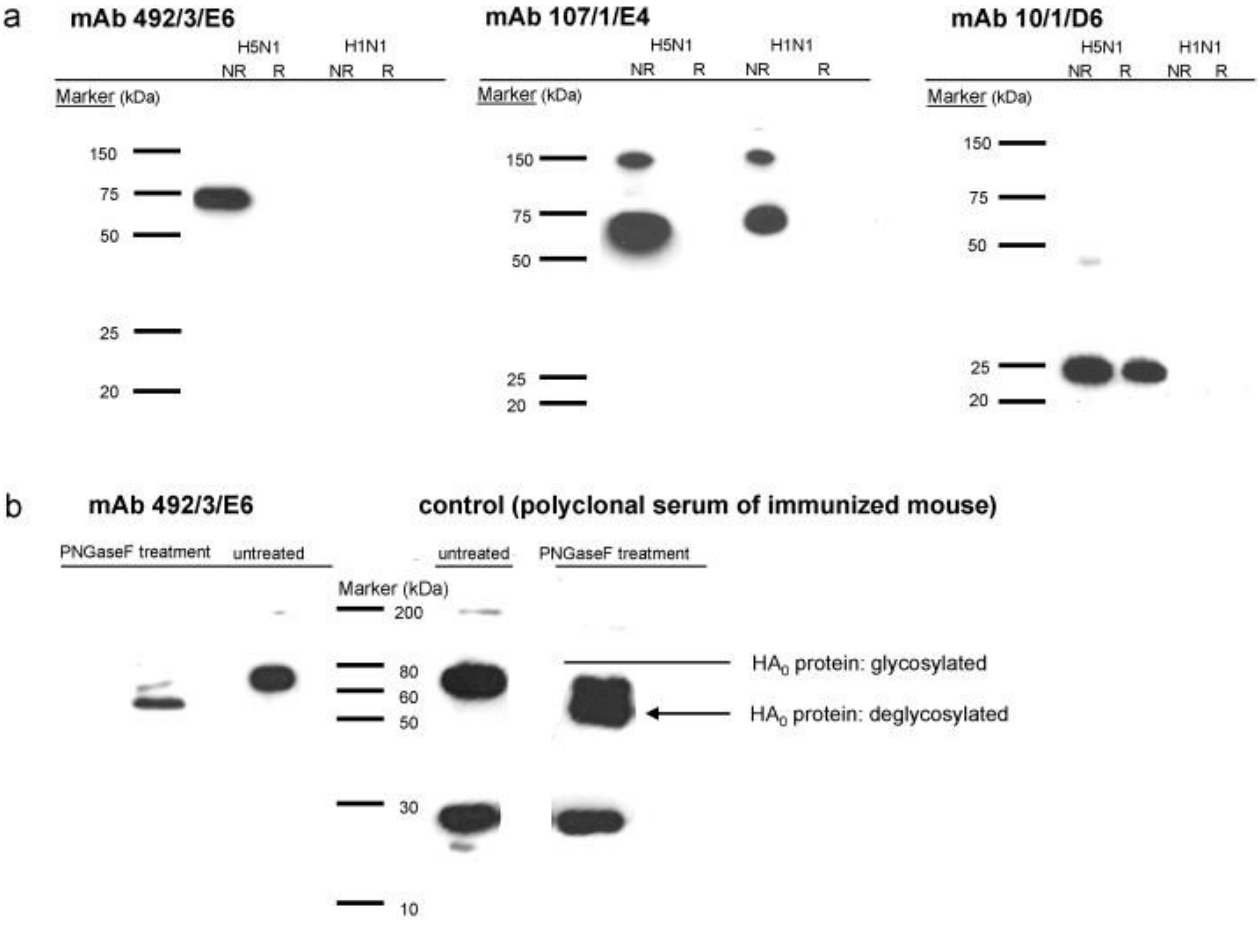


Figure 2. Western blot analysis of selected mAbs and H5N1 A/Whooper Swan/Germany (R65-2/06). (a) Western blot analysis was performed with H5N1 A/Whooper Swan/Germany (R65-2/06). The blots are representative of all 11 mAbs presented here. 107/1/E4 reacted with influenza HA protein (~77 kDa) of subtypes H5 and H1, 10/1/D6 bound to M1 protein (28 kDa), and 492/3/E6 detected H5 HA exclusively. (b) Western blot analysis with deglycosylated HA-protein of H5N1, A/Whooper Swan/Germany (R65-2/06), using mAb 492/3/E6 and polyclonal mouse serum (control) as primary antibodies.



NR: non-reducing conditions R: reducing conditions