**ver1: A prime/boost vaccination with *HA* DNA and *Pichia*-produced recombinant HA protein elicits a strong humoral response in chickens against H5N1**

**ver2: A prime/boost administration of *H5 HA* DNA/H5 HA oligomers elicits a strong humoral response in chickens against H5N1**

**ver3: Evaluation of a prime/booster vaccination with H5 HA DNA/H5 HA oligomers in chickens**

**ver4: A vaccine against H5N1 consisting of a primer/booster administration of *H5 HA* DNA/H5 HA oligomers elicits a strong humoral response in chickens**

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**Highlights**

A prime/boost vaccination strategy with DNA and recombinant protein in chickens was investigated with ELISA and HI test

A DNA plasmid encoding H5 HA and recombinant H5 HA protein produced in *Pichia* *pastoris* have been tested in combination for the first time

Priming of chickens with the DNA vaccine significantly enhanced the immunogenicity of the protein vaccine

**Keywords:** chickens;influenza; DNA vaccine; *Pichia pastoris*; hemagglutinin

**Abbreviations:** HA, hemagglutinin; HPAI,highly pathogenic avian influenza; IMAC, immobilized metal ion affinity chromatography; SEC, size exclusion chromatography; SPF, Specific Pathogen-Free

**ABSTRACT**

Highly pathogenic avian influenza viruses cause severe disease and huge economic losses in domestic poultry and might pose a serious threat to people because of the high mortality rates in case of an accidental transmission to humans. The main goal of this work was to evaluate the immune responses and hemagglutination inhibition potential elicited by a combined DNA/recombinant protein prime/boost vaccination compared to DNA/DNA and protein/protein regimens in chickens. A plasmid encoding hemagglutinin (HA) from the A/swan/Poland/305-135V08/2006 (H5N1) virus, or the recombinant HA protein produced in *Pichia* *pastoris* system, both induced H5 HA–specific humoral immune responses in chickens. In two independent experiments, anti-HA antibodies were detected in sera collected two weeks after the first dose and the response was enhanced by the second dose of a vaccine, regardless of the type of subunit vaccine (DNA or recombinant protein) administered. The serum collected from chickens two weeks after the second dose was characterized by three types of assays: indirect ELISA, hemagglutination inhibition (HI) and a diagnostic test based on H5 antibody competition. Although the indirect ELISA failed to detect superiority of any of the three vaccine regimens, the other two tests clearly indicated that priming of chickens with the DNA vaccine significantly enhanced the protective potential of the recombinant protein vaccine produced in *P. pastoris*.

1. **Introduction**

Vaccination is the major tool for the prevention and control of influenza. Due to the low efficacy of current seasonal influenza vaccines, and by a continuous threat of new pandemic strain outbreaks, there is a pressing need for new generation influenza vaccines. To overcome these difficulties, novel vaccine strategies have been developed (Spackman and Swayne, 2013). Since antibodies that neutralize the influenza virus are mainly directed against the hemagglutinin (HA) protein, much research has focused on this antigen, and a dynamic development of subunit vaccines based on HA currently occurs. Indeed, influenza vaccine candidates utilizing recombinant technology and various expression systems (e.g. bacteria, baculovirus, plant or mammalian cells) are under development or in clinical trials (Buckland et al., 2014; Le Mauff et al., 2015; Moresco et al., 2010; Verma et al., 2012). An additional benefit of using subunit vaccines is their appliance for the vaccination in the buffer zone during H5N1 outbreaks, since they enable the differentiation of infected individuals from those who have been vaccinated. DNA vaccination, which is a variant of the subunit vaccine approach, is a promising new strategy that offers several advantages. The plasmids used for DNA vaccination are usually propagated in *E. coli* and can be easily purified in large scale by ion exchange chromatography, which makes this approach economically attractive. Moreover, it has been suggested that priming with an influenza DNA vaccine received long before pandemic attack could present significant benefits, including reducing the amount of target vaccine needed and conferring some initial immunity level in human populations (Lu, 2011). Numerous researchers have demonstrated that H5N1 DNA vaccines have a protective effect in model animals (Liu, 2011; Meunier et al., 2016; Stachyra et al., 2014a). However, despite the great potential of this type of vaccine, some reports have concluded that DNA plasmids used alone fail to induce significant protection against pathogens in large animals and humans, even with the use of various molecular adjuvants (Lu, 2009).

The efficacy of DNA vaccines can be augmented by boosting the vaccinated individuals with either protein or viral vector vaccines. Such combined prime/boost immunizations have been successfully exploited to improve the breadth of the cellular and humoral immune response elicited by various vaccines against different viral pathogens in animal studies, including HIV (Pal et al., 2006), HCV (Li et al., 2006), HPV (Radaelli et al., 2012) and bacterial (Shkreta et al., 2004) or parasitic diseases (Mazumder et al., 2011). Very good effects have been described for DNA vaccines used in combined prime/boost immunization strategies against influenza using inactivated virus (Wang et al., 2008), live-attenuated influenza viruses (Suguitan et al., 2011), virus like particles (Ding et al., 2011; Lin et al., 2012) and recombinant proteins (Luo et al., 2012). Most studies using recombinant antigens were performed in mice, which are a relevant and widespread animal model, but are not a natural host for avian influenza viruses. Studies with natural host species, such as chickens, are needed to evaluate these novel experimental vaccine formulations. Additionally, recombinant proteins produced in human, insect or bacterial cells are usually used for the combined DNA-protein immunizations. Recently, several phase I clinical studies have reported surprisingly good results of approaches with HA-DNA vaccines used as primers and inactivated corresponding viruses as boosters (Crank et al., 2015; Ledgerwood et al., 2015a; Ledgerwood et al., 2015b; Ledgerwood et al., 2013). The results of these experiments confirm the potential of the combined DNA/protein immunization approach and emphasize the need to develop novel components for such an approach. However, the production methods mentioned above possess some limitations, e.g. vaccine antigen produced on cell lines have to be thoroughly screened for viruses and/or potential cancerogenic agents and/or protein contamination, which might cause adverse effects. One of the systems used for recombinant influenza vaccine manufacturing is baculovirus expression system based on insect cells. However, this solution involves high manufacturing costs connected with the demand for high qualified staff and expensive reagents. Therefore, new, inexpensive and safe methods of producing vaccine antigens are still being explored. Despite the existing solutions that have emerged so far, there is a constant need to develop a new method of vaccine antigen and new antigen production.

The *Pichia pastoris* system has been extensively utilized as an industrial platform to produce various biopharmaceuticals, including vaccines (Shanvac™, Elovac™, Gavac™), however we are not aware of any reports on using it for the DNA/protein prime/boost strategy. *Pichia* cells are able to carry out post-translational modifications, offer the possibility of producing even gram amounts of recombinant protein per liter of culture, and in addition in a secretory fashion which greatly facilitates subsequent purification of desired protein.

In this study we evaluated the effects of application of the subunit prime/boost vaccine strategy against HPAI H5N1 in chickens. Although each of the subunit vaccines applied in this work has been tested individually against HPAI H5N1 viruses, the combination of both vaccines in a prime/boost strategy has not been previously reported. Here we primed chickens with a DNA plasmid encoding H5 HA from the HPAI virus (A/swan/Poland/305-135V08/2006 (H5N1)) and boosted once with H5 oligomers from the same strain, produced in *P. pastoris*. A comparison of the antibody levels and HI titers elicited with DNA/DNA, DNA/protein and protein/protein prime/boost strategies indicated that the level of antibodies capable of hemagglutinin inhibition was significantly higher in DNA/protein than in protein/protein vaccinated animals, while the difference between the DNA/DNA and DNA/protein groups was not significant.

1. **Materials and methods**
   1. **Plasmid used for DNA vaccination**

A plasmid containing the cDNA encoding full length (except the 341-RRRKKR-347 residues, a proteolytic cleavage site between HA1 and HA2 subunits) hemagglutinin (HA) from A/swan/Poland/305-135V08/2006 (H5N1); clade 2.2 and optimized to the domestic chicken codon bias was described earlier (Stachyra et al., 2014b; Stachyra et al., 2016)

* 1. **Recombinant H5 HA protein expression, purification and analysis**

The same H5N1 virus strain used as the source of the DNA vaccine was also used as the source of cDNA encoding HA cloned into the *P. pastoris* expression vector. A DNA fragment corresponding to extracellular domain of HA (residues 17-531, ∆RRRKKR), was ligated into the pPICZαC vector (Invitrogen, USA) using the *Cla*I and *Sac*II restriction sites. The HA antigen was subsequently overexpressed and secreted from the *P. pastoris* KM71 strain. The yeast cells were grown in 400 ml of BMGY medium at 30 °C on a plate agitator at 160 rpm agitation. The induction of HA expression was carried out in 1/4 of the original volume of BMMY medium at pH 7.8 using 5% methanol. Subsequent incubation at 26 °C with vigorous shaking was continued for 7 days. The culture medium was cleared of yeast cells by centrifugation at 3000 x g at 4 °C for 6 min and the supernatant was collected for further HA purification. The protein was purified in a one step procedure using Ni-NTA agarose (Qiagen, Germany), and its purity was analyzed using 4–12 % SDS-PAGE (Bio-Rad, Poland). After dialysis against PBS, pH 7.4, the protein was lyophilized and stored at -20 **°**C. Oligomerization of H5 HA protein was analyzed on a Superdex-200 10/300 GL column (GE Healthcare, Poland) pre-equilibrated with 10 mM Tris pH 7.8 with 200 mM NaCl. Fraction of oligomers were collected and only oligomers were used in further steps. Molecular weight marker standards (Bio-Rad, Poland) were used for column calibration providing the generation of standard curves to identify the molecular weights of the proteins present in the samples. The protein elution was monitored at 280 nm.

* 1. **Immunization experiment**

Broiler chickens Ross 308 were purchased from a commercial brooder on the day of hatching and maintained at an experimental poultry house in standard bedding conditions. The details of two independent experiments are specified in Table 1. Chickens were divided into four groups: DNA/DNA, DNA/protein, protein/protein and control. The DNA vaccine was applied with Lipofectin (Invitrogen, Germany) carrier in a final volume of 100 µl via intramuscular injection, while the protein (recombinant H5 HA) was delivered with alhydrogel adjuvant in a final volume of 200 µl via subcutaneous injection. The empty pCI vector (Promega, Poland) was used as a negative control. The experiments were approved by the Second Local Ethical Committee for Animal Experiments at the Medical University of Warsaw, Permit Number 17/2009.

* 1. **ELISA**

The one-dilution indirect ELISA test was performed essentially as described earlier (Stachyra et al., 2014b), using homologous HA produced in a baculovirus system (Oxford Expression Technologies, UK) and anti-chicken IgY secondary antibodies (Thermo, Germany).

The competitive ELISA test using ID Screen® Influenza H5 Antibody Competition (ID-Vet, France) was performed, according to the manufacturer’s protocol with sera collected at day 35. The results were expressed as S/P values using the cutoff recommended in the manual (S/P = [optical density of sample (S)/optical density of positive control (P)] × 100). The test detects subtype–specific antibodies in sera and was performed to obtain additional information about anti-HA antibodies level; the manufacturer recommends the following interpretation: S/P < 35%, positive; S/P > 40%, negative; and S/P 35–40%, doubtful result.

* 1. **Hemagglutination inhibition (HI)**

The HI test was performed with binding procedures as described previously (Thayer and Beard, 1998) using either distinct heterologous antigen from A/chicken/Belgium/150/1999(H5N2); clade EA-nonGsGD (DG Animal Health, Netherlands), which is commercially available H5 antigen for HI tests and it is routinely used in our lab, or homologous antigen prepared from A/turkey/Poland/35/07(H5N1); clade 2.2 (National Veterinary Research Institute lab stock). The HI titer was defined as the reciprocal of the highest dilution of sera that completely inhibited hemagglutination.

* 1. **Statistical analysis**

Statistica 12 software (StatSoft, Poland) was used in all analyses. Non-parametric tests (Kruskal-Wallis for comparison of multiple groups or Mann-Whitney U for comparison of two groups) were employed to evaluate the statistical differences. A value of p < 0.05 was considered significant.

1. **Results**
   1. **Characterization of recombinant hemagglutinin protein used as a subunit vaccine**

Recombinant H5 HA protein was produced in *P. pastoris* cells as a secreted protein. Such an approach proved to be efficient, convenient and economically justified since a high level of purity was achieved after one-step purification via Ni-based chromatography (Fig. 1A). The apparent size of the protein (about 70 kDa) was consistent with the molecular weight of recombinant glycosylated HA. Purified antigen fractions produced by the immobilized metal affinity chromatography (IMAC) procedure were further polished using size exclusion chromatography (SEC). This analysis showed that the recombinant protein formed a mixture of monomers and oligomers. The H5 antigen is presumably secreted as a monomer and subsequently it spontaneously oligomerizes into dimers, trimers and higher oligomers. To characterize the stability of the oligomers, we collected the high molecular fraction and re-analyzed it by SEC after a lyophilization/resuspension cycle. To characterize the stability of the oligomers, we collected the high molecular fraction and re-analyzed it by SEC after a lyophilization/resuspension cycle. Fig. 1B shows that the SEC–purified fraction remained as stable HA oligomers. We previously demonstrated that H5 HA protein comprising a mixture of mono- and oligomeric forms was highly immunogenic and protected from lethal challenge with the homologous virus (Pietrzak et al., 2016). In this work, only the fraction containing the oligomers was used for immunization. The final yield of the purified oligomeric form of H5 antigen was 40 mg from 1L of culture medium.

* 1. **Difference in humoral response of chickens to the vaccine variants**

Immunological effects of the combined DNA/protein versus DNA/DNA and protein/protein prime/boost strategies were verified in two independent experiments (Table 1). Sera collected from the immunized chickens were examined for the presence of HA–specific antibodies by ELISA and further characterized using the HI test. The indirect ELISA indicated the presence of anti-H5 HA–specific antibodies in sera from all three groups of chickens and failed to indicate the apparent superiority of any one of the three vaccine regimens (Fig. 2A and 2B). In Experiment 1, the levels of anti-H5 HA antibodies were higher in the DNA/DNA and DNA/protein groups, while in Experiment 2 the antibody levels were higher in the DNA/protein and protein/protein groups.

In contrast to the results of the indirect ELISA, the results of the HI test indicated that priming with a DNA vaccine significantly improved the efficacy of the recombinant HA protein used as a subunit vaccine. In Experiment 1, the HI test using a distinct heterologous antigen (H5N2) detected the HI-positive individuals only in the DNA/DNA (median log2 = 3, corresponding to the serum dilution of 1/8) and the DNA/protein (median log2 = 2, corresponding to the serum dilution of 1/4) groups (Fig. 2C). In Experiment 2, two types of viruses were used for HI assessment. The HI test with heterologous H5N2 antigen (the same as in Experiment 1) indicated a lack of production of antibodies capable of effectively blocking hemagglutination in the case of protein/protein immunization, while 88% of chickens were HI-positive in the DNA/DNA and DNA/protein groups (median log2 = 3 and 4, respectively). Interestingly, the HI test using homologous H5N1 virus produced 100% positive results both in the DNA/protein and the DNA/DNA groups and 44% of HI-positive chickens immunized twice with the HA protein (the protein/protein group). The median log2 = 6 (corresponding to the serum dilution of 1/64) was equal for the DNA/protein and DNA/DNA groups (Fig. 2D and 2E).

The results of the HI test were supported by the results of the diagnostic competitive ELISA (Fig. 3). This test is recommended for the detection of potentially highly-pathogenic strains of H5N1 in the serum of infected chickens and is moderately suitable for analysis of the efficiency of vaccination, due to its rather low sensitivity in such cases. However, in our study the test aimed to provide additional information about anti-HA antibody levels after the used vaccination strategies. The results of this test indicated that the serum samples from the protein/protein groups in both experiments were strongly negative, with the mean % of competition around 80–90%, while slightly better results were observed in the DNA/protein group. Despite the fact that most samples were negative (as expected) the observed trends in the level of antibodies seem to be in concordance with the results of ELISA and HI tests presented in Fig.2. Result of this test indicated a significant difference between the protein/protein group and the DNA/protein group in both experiments and between the protein/protein group and the DNA/DNA group in Experiment 1.

1. **Discussion**

The traditional influenza vaccine production is a lengthy procedure. It requires the use of eggs and requires the generation of virus reassortants, which can effectively multiply in hen‘s eggs to obtain a sufficient yield of vaccine product. Moreover, the production process depends on a continuous supply of eggs, which has always represented a bottleneck in this traditional approach. The next generation vaccines, such as recombinant HA protein–based or DNA–based subunit vaccines, have become leading alternatives for influenza vaccine manufacturers. Since these two types of subunit vaccines utilize different mechanisms to elicit antigen–specific responses, after vaccination they could be used as complementary approaches that may overcome each of their respective shortcomings (Kardani et al., 2016; Woodland, 2004). Thus, the combination of DNA and recombinant protein approaches, where the DNA vaccines serve as a priming agent to significantly increase the immunogenicity of a vaccine based on a recombinant protein, appears an attractive proposition for the production of highly effective influenza vaccines.

We previously confirmed the protective activity of the DNA vaccine against H5N1 using SPF White Leghorn chickens. Experimental infections (challenge experiments) indicated that 100% of vaccinated chickens were protected against H5N1 of the same clade as vaccine strain and that 70% were protected against H5N1 influenza virus of a different clade. Moreover, the DNA vaccine significantly limited (or even eliminated) transmission of the virus to contact control chickens (Stachyra et al., 2014b). Recombinant influenza H5 protein produced in *P. pastoris* (using a simple and efficient one-step purification) has also been shown to induce a strong immunological response in mice (Kopera et al., 2014). Further experiments by Pietrzak and colleagues showed that immunization with a subunit vaccine based on the extracellular region of H5 hemagglutinin, with deletion of the multibasic cleavage site, elicited serum HA–inhibiting antibodies and fully protected chickens from lethal infections by the homologous highly pathogenic H5N1 virus (Pietrzak et al., 2016). Therefore, we tested the combined DNA/protein prime/boost strategy using both of these vaccines. In contrast to our previous studies, we applied a suboptimal immunization schedule for protein/protein vaccination and a suboptimal dose of plasmid for DNA/DNA vaccination in attempts to more accurately pinpoint the differences between the three types of regimens. In our previous studies we defined the optimal dose of DNA vaccine in the applied schedule (two doses, two weeks apart) as 125 µg of plasmid DNA per chicken, while the sub-optimal dose (60 µg) was chosen for this study in an attempt to tease out potential differences in the responses in the three groups. Such a dose could be less effective in induction of immune responses because of transfecting insufficient number of cells. The use of the suboptimal dose of the DNA vaccine could explain the differences in responses observed between individuals within the group of chickens that received the same treatment and between the similar (corresponding) groups in two independent experiments; however, the vaccine efficacy was still good. Similarly, the results of the HI test showed a lower efficacy of protein/protein vaccine variants, which likely resulted from the suboptimal immunization schedule (a two-week interval between doses). Previous results using the recombinant H5 HA produced in a *P. pastoris* system demonstrated that only 50% of the immunized animals were HI positive two weeks after the first vaccination, whereas two weeks after the booster (applied four weeks after the primer) the immunological response had increased and 9/10 chickens were HI positive (Pietrzak et al., 2016). This is consistent with the results of other avian H5 DNA vaccine prime/boost studies in which a long interval was associated with improved immunogenicity, as compared to a short interval (Ledgerwood et al., 2011; Ledgerwood et al., 2013). However, the need to administer two doses of a vaccine, with long intervals between doses, might be inconvenient in the case of short-lived animals such as broilers. Therefore, from this point of view, the combined DNA/protein vaccine might be more suited for use in these animals.

Nevertheless, given the immunization schedule used in this study (two-week interval between the doses), the DNA vaccine acted perfectly as a priming agent to significantly augment and broaden the level of humoral response to the protein–based vaccine. In HI and in competitive ELISA tests, the DNA/protein immunization produced a better response than the protein/protein regimen. The second expectation, that protein boosting could augment DNA efficacy and make the mixed vaccine more effective than the usage of DNA vaccine alone, was not completely clarified by our study, although it has been previously reported in chickens and mammals (Gao et al., 2013; Golshani et al., 2015; Gupta et al., 2015; Li et al., 2013; Luo et al., 2012; Mazumder et al., 2011; Peng et al., 2016). Another interesting aspect of the prime/boost immunization experiment used in this study is that, despite having the same protein sequence, the glycosylation pattern of HA produced in chickens and *P. pastoris* cells is different. Recent studies revealed that HA glycosylation is a significant factor affecting induction of cross-reactive antibody responses and that it can focus humoral immunity towards different regions of HA; therefore, introduction of glycosylation sites in the HA head domain elicits broader polyclonal responses (Medina et al., 2013) and can redirect antibody responses towards the conserved stalk domain of HA (Eggink et al., 2014). This aspect of the DNA/protein regimen was not sufficiently investigated in our work; however, it represents a tempting direction for future studies examining the development of a universal influenza vaccine based on DNA/protein prime/boost treatment.

In conclusion, at least two types of assays, namely the HI test and the competitive ELISA, indicated the superiority of the combined DNA/protein prime/boost approach over the regimen consisting of two doses of protein alone for vaccination against H5N1; however, further experiments, such as virus neutralization or challenge, are needed to confirm this observation. The DNA/protein regimen can prime T cells (with the DNA vaccine) to generate elevated secondary responses to the booster in the form of a protein vaccine, or to produce high-affinity antigen–specific T cells whose numbers are increased following boosting with the protein antigen. Therefore, the DNA/protein strategy might be a good approach for further development of an effective influenza vaccine.

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**Authors’ contribution**

ASt, AP and AG-S prepared the DNA vaccine and conducted the immunization experiments, MP, AM and EK prepared the recombinant protein, MO, KŚ and ZM designed and performed the HI experiments. All authors participated in study design and data analysis, manuscript and figures preparation, have read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Ethics approval and consent to participate**

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The experiments with chickens were approved by the Second Local Ethical Committee for Animal Experiments at the Medical University of Warsaw, Permit Number 17/2009.

Table 1. Details of the immunization experiments.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Group** | **Dose of the vaccine** | | | **Day of the treatment** | | | |
| **Prime** | | **Boost** | **Immunization** | | | **Blood collection** |
| ***Experiment 1*** | | | | | | | |
| DNA/DNA  (n=12) | 60 µg DNA +lipofectin | 60 µg DNA +lipofectin | | | 8 and 22 | 22 and 35 | |
| DNA/protein  (n=12) | 60 µg DNA +lipofectin | 25 µg rHA+AlOH | | |
| protein/protein  (n=12) | 25 µg rHA+AlOH | 25 µg rHA+AlOH | | |
| ***Experiment 2*** | | | | | | | |
| DNA/DNA  (n=9) | 60 µg DNA +lipofectin | 60 µg–DNA +lipofectin | | | 7 and 20 | | 20 and 35 |
| DNA/protein  (n=9) | 60 µg DNA +lipofectin | 25 µg rHA+AlOH | | |
| protein/protein  (n=9) | 25 µg rHA+AlOH | 25 µg rHA+AlOH | | |

**Figure legends**

**Figure 1**. **Characterization of the recombinant HA antigen produced in *P. pastoris* cells.** A.SDS-PAGE analysis of recombinant HA after a one step purification process using Ni-NTA chromatography. Molecular weight marker, wash fraction and elution fraction samples were electrophoresed in 4–12% SDS-PAGE gel and stained with Coomassie Blue. A band representing recombinant hemgglutinin (HA) is visible in elution fraction lane. B.Characterization of recombinant HA by Size Exclusion Chromatography; upper plot – chromatography profile of the antigen fractions after one-step purification; middle plot – chromatography profile of the HA oligomers after a lyophilization/resuspension cycle, lower plot – profile of protein molecular weight marker (Biorad); O – oligomers, M – monomers.

**Figure 2. Humoral response of chickens the DNA/DNA, DNA/protein and protein/protein vaccination.** The results of one-dilution indirect ELISA for sera collected from the immunized chickens two weeks after the first dose (A) and two weeks after the second dose (B); values for each individual (Raw Data), medians and the 10th and 90th percentiles are shown for each group. The HI titers in sera collected two weeks after the second dose with the homologous (H5N1) or heterologous (H5N2) antigens are presented for the Experiment 1 (C) and Experiment 2 (D,E); values for each individual (Raw Data) and medians are shown for each group using the log2 values of the reciprocal of the highest dilutions producing the positive result in the test. Sera with undetected HI levels were assigned an arbitrary value of 1. Statistically significant differences (p < 0.05) are marked by asterisks.

**Figure 3. Results of the ID Screen® competitive ELISA.** The values (% of competition) for each individual (Raw Data), medians and the 10th and 90th percentiles are shown for each group. Statistically significant differences (p < 0.05) are marked by asterisks. The cutoff value suggested by the manufacturer (35%) is marked as a dashed line.

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