Evaluation of serological tests for H5N1 avian influenza on field samples from domestic poultry populations in Vietnam: consequences for surveillance.

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Abstract

In Vietnam, serological post H5N1 vaccination surveillance using the HI test is applied to assess the efficiency of the vaccination in addition to virological monitoring. In this paper we report on the evaluations of the performances of the haemagglutination inhibition (HI) test and of a H5-ELISA, using chicken and duck field samples. The evaluations were conducted by comparison with a pseudotyped-based virus neutralization test (H5pp VNT) performed in a reference laboratory and considered as a “gold standard” and also by using methods developed for imperfect reference test. Their global accuracy and best cut-offs were also estimated. Results from the HI test for several haemagglutinin subtypes and from a commercial type A influenza competition ELISA were also compared.

The results showed that performance of the HI test was very good in comparison with the H5pp VNT. Data also clearly supported the cut-off of $\geq 4\log_2$ used for the HI test for chickens but, a $3\log_2$ positivity cut-off would be more appropriate for ducks. When compared with the VNT, the H5-ELISA showed poor specificity when using the positivity cut-off specified by the manufacturer but could be used as a screening test if confirmed by the HI test or the H5ppVNT which presents some interests for large scale testing (no need for biosafety level 3 conditions and high performance).

A general and highly sensitive pre-screening can also be achieved using the detection of NP-specific antibodies with a competition ELISA. This appears of little interest in a context of high subtypes diversity where only a subtype is targeted for surveillance and control.

Key words: avian influenza, H5N1, vaccination, Vietnam, serology, evaluation tests, influenza pseudotyped lentiviral particles.

Introduction

H5N1 Avian Influenza (AI) virus is a type A influenza virus from the Orthomyxovirus family. The H5N1 strains circulating intensively in domestic poultry in Asia since 2003 are highly pathogenic AI viruses (HPAI) (Peiris, 2009). Observation of poultry immune responses against the AI virus are commonly used either as a way to detect evidence of infection or to evaluate the vaccination efficiency. In order to correctly interpret results of serological tests, it is important (1) to understand the immunology of the population under surveillance or monitoring and (2) to know the performances of the tests being used. The performance of the test is defined here by its sensibility and its specificity.

Influenza viruses type A genome encodes for 10 viral proteins that can be divided into 3 main categories: the surface proteins (haemagglutinin HA, neuraminidase NA and matrix 2 (M2) the internal proteins (3 polymerase proteins PA, PB1, and PB2; the nucleoprotein (NP), the matrix 1 (M1) and the nonstructural proteins 2 (NS2)); and finally, the nonstructural protein 1 (NS1) that is not packaged into the virus particle (Suarez and Schultz-Cherry, 2000). While the surface proteins (HA and NA) are the only antigens capable of inducing neutralizing antibodies and therefore a protective immune response, M2, NP and M1 proteins can also induce antibody response (Aymard et al., 1998; Suarez and Schultz-Cherry, 2000). The NP and M1 antigens have high sequence conservation that allows the detection of antibody from birds infected with any type A influenza viruses (Suarez and Schultz-Cherry, 2000). Several experimental infections conducted in chickens using low pathogenic strains showed that antibodies against HA, NA and NP protein have the same kinetic profile whereas the anti-M2 response showed a different profile by being of shorter duration and disappearing more rapidly (Marche et al., 2010).

The most commonly used serological tests target the NP protein when the objective is to have a non sub-type specific test (*e.g.*: agar gel immunodiffusion (AGID), commercial or in-house

enzyme-linked immunosorbent assay (ELISA)), or the HA protein when a sub-type specific test is required (*e.g*: hemagglutination inhibition (HI), virus neutralization test (VNT) or HA-specific ELISA)(WHO, 2002). Detection of antibodies against subtype-specific NA protein is also used but not routinely. Similarly, detection of antibodies against NS1 and M2 proteins are used to differentiate infected from vaccinated animals (DIVA strategy), but no routine tests are available (Siting et al., 2005).

Neutralizing antibodies are participating to protection; those directed towards HA are the more potent (Garcia et al., 2010; Suarez and Schultz-Cherry, 2000). In contrast, irrespective of their neutralizing activity, antibodies against HA, NA and NP are marker of infection. Some authors even indicate that detection of antibodies against NP protein provides a more sensitive test than detection of antibodies again HA protein (Marche et al., 2010).

Vietnam experienced severe epizootics of HPAI H5N1 from 2003 to 2005 before adopting a mass vaccination strategy to control the number of outbreaks in domestic poultry and to limit the number of human cases. With implementation of the vaccination, serological post-vaccination surveillance became an important tool to assess the efficiency of vaccination. Serological surveillance currently applied in Vietnam uses the HI test and aims at evaluating the immunity induced by the H5N1 vaccine on vaccinated birds and in some circumstances at detecting the circulation of H5N1 virus on non vaccinated ones. In addition, virological monitoring in market places and in non vaccinated population is also being applied. The use of sentinel birds in vaccinated flocks to detect virus circulation was not adopted in the country.

In this study, antibodies against HA were used as a marker for both infection and vaccination since we collected samples from partially vaccinated domestic poultry in Vietnam. Because the vaccine used in Vietnam is generated from a genetically modified reassortant H5N1 low pathogenic virus (referred to as Re-1) (Qiao et al., 2006), distinction between infected and

vaccinated birds is not possible when serological response against HA antigen is measured. In this paper we report on the evaluations of the performances of several diagnostic techniques under field conditions considering the two main species present in the country: chicken and duck. In particular, we have evaluated the performance of the HI test as well as of an H5-ELISA for its rapidity and easiness of implementation compared to the HI test. Results from the HI test for several haemagglutinin subtypes and from a commercial type A competition ELISA (detecting the NP antibodies) were also used for our evaluation. The evaluation of these tests were conducted by comparison with an influenza H5 pseudotyped based VNT performed in a reference laboratory as a reference assay given true serological status and also by comparing results of the different tests using methods developed for imperfect reference test . The neutralization assays are considered to be a sensitive and specific test for both animals and humans (WHO, 2002). The VNT applied in our study uses a H5-pseudotyped lentiviral particle for the neutralization-based assay (H5pp VNT assay) (Garcia et al., 2010) and was used instead of the conventional neutralization assay because it is recognized this method is at least as sensitive as the conventional method (Garcia and Lai, 2011; Tsai et al., 2009), does not need biosafety 3 level conditions, and is less labor intensive. Evaluation of the sensitivity and specificity of serological tests using field samples will be valuable for routine AI surveillance and post-vaccination evaluation in Vietnam.

2. Materials and methods

2.1 Field data

Four repeated cross sectional surveys were conducted over one year (2008-2009), in order to study the H5N1 HPAI seroprevalence in the domestic poultry population of the Red River Delta (Northern Vietnam). Around 1000 birds were sampled during each campaign with the farms (for farm poultry) or villages (for backyard poultry) being randomly selected in the study area. Fifteen birds were sampled from each selected epidemiological unit providing a

total of 4356 sera. The population was known to be partially immunized against H5N1 virus with the Re-1 vaccine produced by Weike Biological Company of the Harbin Veterinary Research Institute (Chinese Academy of Agricultural Sciences, Harbin, People's Republic of China). This vaccine derives its HA and NA genes from GS/GD/96 virus (belonging to H5N1 clade 0) (Qiao et al., 2006).

Influenza H5 seroprevalence was estimated on the 4356 sera by the HI test specific for the H5 subtype performed at the National Institute of Veterinary Research (NIVR) in Hanoi, Vietnam (results not presented nor discussed in this paper). Our sera were classified by species and production types (broiler and breeder) and other serological tests were also applied on different subsets of those sera.

One subset of sera was used for the evaluation of HI and ELISA tests as follows:

- 406 sera randomly selected from the chicken and duck breeder and broiler populations were tested using the H5pp VNT performed at HKU-Pasteur Research Centre.
- From those 406 sera, a subsample of 230 from the chicken and duck breeder' populations (96 and 134 respectively) was also tested using an H5-ELISA kit performed at the NIVR.

Another subset of sera was used to explore the possible cross reactivity of the H5-ELISA between HA subtypes. Initially 1103 sera randomly selected were tested by an influenza type A ELISA test kit, and from the positive samples, a subset of 260 sera were further tested by the H5-ELISA and by the HI test for H5 and other available subtypes (H3, H4, H6 and H9).

2.2 Serological tests

The HI test was used to estimate the H5N1 seroprevalence on all sera samples collected considering that the main H5 subtype in Vietnam is the H5N1 HPAI and that the only vaccine being used is generated from a H5N1 virus. The analyses were performed at NIVR in Hanoi,

153 Vietnam. The test used a HA clade 1 antigen (A/Dk /Vietnam/6/03 H5N1) following the
154 protocol described in the OIE manual. All sera were first heated-inactivated at 56°C for 30
155 min. This method uses the ability of influenza virus to agglutinate red blood cells and
156 measures inhibition of this process by anti-HA antibodies specific to the viral strain. Serum
157 titers were expressed as $\log_2$ values of the highest reciprocal dilution that showed complete
158 inhibition of haemagglutination. All sera with a titer $\geq 4\log_2$ were initially defined as positive
159 following the most commonly used cut-off (OIE, 2008). The HI test was also used for 4 other
160 AI subtypes commonly infecting the domestic poultry in the region: H9, H3, H6 and H4
161 (A/Dk/HK/Y280/97 H9N2; A/Dk/Vietnam/12/03 H3N2; A/Teal/HK/W312/97 H6N1;
162 A/Dk/Siberia/378/01 H4N6).
163 A subtype specific ELISA (ID-Screen ® Influenza H5 Antibody Competition) was also
164 applied on a selection of sera in order to evaluate the performances of this test. This test
165 detects anti-H5 antibodies. Under the manufacturer's instructions, a sample is considered to
166 be positive if it gives a result less than or equal to 50% competition and negative if it gives a
167 result more than or equal to 60 % competition. The competition percentage was determined
168 by the following formula: (OD of the sample divided by the OD of the mean value of the
169 negative control) x 100, but results were presented using the inhibition percentage (100 -
170 competition percentage).
171 A competition ELISA kit based on a blocking procedure and detecting antibodies against the
172 internal nucleocapsid (NP) of influenza A virus (ID-Screen® Influenza A Antibody
173 Competition) was used to estimate the Influenza A seroprevalence. Under the manufacturer's
174 instructions, a result is considered positive if it displays a result lower or equal to 45% of
175 competition and negative if it gives a result more than or equal to 50 % competition. The
176 competition and inhibition percentages were calculated as described above.

Finally, 406 randomly selected sera (out of 4357) were also tested using as reference test, an influenza A (H5) pseudotyped lentiviral particle-based (H5pp) VNT performed at HKU-Pasteur Research Centre (Du et al., 2010; Garcia et al., 2010; Nefkens et al., 2007). The H5pp VNT was performed as described by Garcia et al. (2010). Briefly, two-fold serial dilutions of sera were incubated for 1 hour with luciferase encoding H5 pseudotyped lentiviral particles before transfer to a monolayer of Madin-Darby canine kidney (MDCK) cells and incubated at 37 °C in 5% CO_2. After 48h infection, Steady-Glo substrate (Promega) was added and luminescence read on a Microbeta luminometer (Perkin-Elmer). H5 antigen was derived from the HA clade 1 A/Cambodia/408008/2005 virus. The neutralization titer was determined as the dilution of serum that results in the inhibition of 50% of signal [as compared to negative (absence of virus) and positive (absence of sera) controls considered as 100% & 0% neutralization, respectively].

2.3 Data analysis

2.3.1. General methodology for evaluating the Se and Sp

Sensitivity (Se) is the proportion of diseased animals correctly identified by the test. Specificity (Sp) is the proportion of healthy animals correctly identified by the test. Se and Sp were evaluated separately for chicken and ducks in order to take into account possible differences in the tests' performance. Those differences are expected because of species specific natural inhibitory substances in the samples (a known source of trouble in the HI assays) or because the diversity of virus that could infect duck (and other aquatic birds) is theoretically much higher than for chicken and therefore may affect the match between the antigen used in the assays and the antigens that triggered the antibodies in the case of infection.

We calculated the Se and Sp of HI test using 3 methods: (1) Se and Sp and their 95% exact binomial Confidence Intervals (CI) were calculated using results from the H5pp VNT at a positivity cut-off of titer $\geq$80 as the true status; (2) adjustment on the Se and Sp were made using Staquet equations (Enoe et al., 2000; Staquet et al., 1981) assuming that the reference test is imperfect but with known Se and Sp and that the test to be evaluated and the reference test are conditionally independent given the true disease status (we fixed the Se and Sp of H5pp VNT using the cut-off titer of 80 at 0.90 and 0.99 respectively following the estimations made by Garcia (Garcia et al., 2010); and (3) we estimate the Se and Sp with their 95% probability interval by a Bayesian analysis for 2 dependent tests and 2 populations using code developed by Branscum et al (Branscum, 2003; Branscum et al., 2005). The 2 populations were either chicken broilers and chicken breeders; or duck broilers and duck breeder.

The Se and Sp of the H5-ELISA test were calculated using frequentist methods only (non Bayesian methods). Doubtful results from the ELISA test were not included into the Se and Sp calculation.

2.3.2 Bayesian inference

Bayesian analyses were performed on OpenBUGS (Spiegelhalter et al., 2007). Beta prior distributions were defined using informative prior information for the Se and Sp of the H5pp VNT test (based on Garcia and al, 2010) and the prevalence of the 2 populations (unpublished data from author Desvaux) (see Table 1 for details). Non informative priors were used for the Se and Sp of HI test and the correlation between tests (beta distributions (1,1) equivalent to uniform distributions (0,1)). A large sample of the posterior distributions was generated by a Markov Chain Monte Carlo (MCMC) algorithm, and the median of this sample is presented as a Bayesian estimate of our parameters. We presented the median together with the 2.5 and 97.5 percentile points that define the 95% probability interval of our parameters.

2.3.3 Receiver Operating Characteristic (ROC) analysis

ROC analysis was used to globally assess the accuracy of the tests to be evaluated and to define their optimal cut-off points. ROC analyses were performed using *roctab* command in Stata (non-parametric ROC analyses). ROC curves were plotted using empirical data and the Area Under the Curve (AUC) was calculated. The AUC is a global (i.e. based on all possible cut-off values) summary statistic of diagnostic accuracy that is independent of the prevalence. A ROC curve is obtained by calculating the sensitivity of the test at every possible cut-off point, and plotting sensitivity against 1-specificity (Akobeng, 2007); the greater the AUC, the better the test. An AUC of 0.5 or less means the test is not able to differentiate cases and non cases (Akobeng, 2007). The best cut-off was then calculated using the "closest-to-(0,1)" criterion which is the cut-off that gives minimal value for $(1\text{-Se})^2+(1\text{-Sp})^2$.

3. Results

3.1 Evaluation of the HI test

3.2.1 Evaluation of HI performances using defined cut-off

Using H5pp VNT at a cut-off of ≥80 as a reference test, we evaluated the HI performance for detecting H5 neutralizing antibodies at a cut-off of ≥4 Log_2. We estimated that the Se of the HI test performed in Vietnam for chickens and ducks varies between 83% and 88% when both species are considered. However, when evaluating chicken and duck samples separately, we found that Se for H5 antibody detection in chickens was higher, whatever the calculation method used (between 91% to 100 % for chickens and between 74% to 81% for ducks) (Table 2).

The AUC of the ROC curves (Table 2) were greater than 0.9 indicating high accuracy of the HI test when compared to the H5pp-based assay performed in the reference laboratory.

3.2.2 Best cut-off estimation

When applying the "closest-to-(0,1)" criterion in the ROC analysis, we confirmed that the cut-off $\geq 4\log_2$ is well suited for chickens in our population, but a cut-off of $\geq 3\log_2$ for ducks would be more appropriate (Figure 1). For this $\geq 3\log_2$ cut-off, the HI Se increases from 78% to 88% and the HI Sp decreases from 99% to 94.23%.

3.3 Evaluation of the H5 ELISA

3.3.1 Evaluation of H5 ELISA performance using defined cut-off

Using H5pp VNT at a cut-off of ≥80 as a reference test, we evaluated the H5-ELISA performance for detecting H5 antibodies at the cut-off defined by the manufacturer (Table 3 and 4). We estimated that the Se of the H5-ELISA was 100% but the Sp varied from 58% to 70% according to the species and calculation methods used. The Sp value for ducks was lower than for chickens (between 55% to 58% and 69% to 70 respectively) (Table 4). Despite, low agreement (Kappa < 0.5) between both tests using the manufacturer's cut-off for H5-ELISA, the AUC of the ROC curves were superior to 0.9 indicating a global high accuracy of this ELISA test when compared to the H5pp-based assay performed in the reference laboratory. This indicates that different cut-offs may give better agreement for this ELISA as described below.

3.3.2 Best cut-off estimation

When applying the "closest-to-(0,1)" criterion in the ROC analysis, we found that a different cut-off than the one proposed by the manufacturer should be selected. When both species are considered together, a positivity cut-off of≤18% which gives a Se of 90% and a Sp of 82%, should be applied. A slightly different cut-off could be applied for chickens and ducks (21% and 16% respectively) to get a Se of 100% for chickens and 84% for ducks and a specificity of 86% for chickens and 89% for ducks. This cut-off, defined in comparison with H5pp VNT on field samples, is very different from the one proposed by the manufacturer (50%).

3.3.3 Supporting data from influenza type A Elisa

Of the 1103 samples randomly selected from our total number of sera, the overall type A seroprevalence on all species was estimated at 43% (95% CI: 40%-45%).

Among those 1103 samples, 12% (23/185) of the sera positive by HI test for H5 were negative for the ELISA A, giving indication of a possible higher sensitivity of the HI test. Those 23 discordant sera presented an average mean H5 HI titer of 5.5 $\log_2$.

From the subset selection of 230 samples also tested by the H5-ELISA, less than 1% of the H5-ELISA positive sera were negative for the ELISA A, giving indication of good concordance between the 2 ELISA tests for the positive results (Table 5).

The comparison between HI test for different subtypes and H5-ELISA on 260 samples of ELISA A positive sera is detailed in Table 6. In this sample, 56% of the ELISA A positive sera were not identified by the HI test using H5, H6, H9, H3 or H4 antigens. Furthermore, from those 260 samples of ELISA A positive sera, around 10% of the H5-ELISA positive sera were positive by the HI test for HA subtypes other than H5.

4. Discussion

The aim of this study was to evaluate the performance of two H5 antibody detection methods based on field samples collected from a partially immunized population in Vietnam in comparison with a more sensitive and specific neutralization test used as reference.

We found that performance of the HI test performed at NIVR was very good in comparison with an H5pp-based assay at the influenza reference laboratory in Hong Kong. The globally lower Se for ducks might be explained by the use of an inappropriate positivity cut-off for that species. Data clearly supported the cut-off of $\geq 4\log_2$ used for the HI test for chickens but, a $3\log_2$ positivity cut-off would be more appropriate in the domestic duck population in comparison with the reference test used. By changing the cut-off of the HI test for ducks we

increase the Se of this test on that population but as a consequence, we slightly reduce its specificity.

The Bayesian analysis, evaluating the HI test with some uncertainty related to the H5pp VNT's performance, also confirmed the global tendency of a higher Se for chickens compared to ducks.

When compared with the H5pp VNT, the H5-specific ELISA showed a major specificity problem at the manufacturer's positivity cut-off. Several hypotheses can be put forward to explain this difference. One of them could be that the H5-specific ELISA has a better cross-reactivity than the H5ppVNT to detect a variety of H5 strains. This hypothesis cannot be excluded but is also not fully supported by our data, since we can increase the agreement between the H5-specific ELISA and the reference test just by adapting the cut-off of the Elisa (Kappa increasing from 0.41 to 0.58 for the cut-off determined by the best-cut-off estimation, data not shown). We also increased the agreement between both tests by using a different cut-off (40 instead of 80) for the H5pp VNT (data not shown) indicating that disagreement between the two techniques mainly occurs for the sera with low titer considered to be non specific by the reference method. Furthermore, the observation that 10% of the H5-ELISA positive are actually positive to subtypes other than H5 by the HI test, supports the hypothesis that H5-specific ELISA cross-reacts, to a certain extent, with other HA subtypes. In conclusion, the best cut-off estimations for the H5-specific ELISA would be in the high positive range in comparison to the manufacturer's recommendations, so the test could only be considered to be accurate in identifying birds giving a high positive reaction. To date, no other studies are available on the assessment of H5-specific ELISA test either under experimental or field conditions.

The HI testing with selected subtypes on a subset of type A ELISA positives showed around 55% of the sera could not be subtyped by HI test when the most common HA subtypes for

poultry in the region were used. Either the type A blocking ELISA is more sensitive for detecting birds exposed to influenza viruses than the HI test for specific subtypes or there are other HA subtypes circulating that were not tested for. This difference of results between samples tested with a competitive or blocking type A ELISA detecting NP antibodies and the HI test, suggesting an apparent higher sensitivity of the ELISA method, was described and discussed previously for studies using field samples from different bird species (Perez-Ramirez et al., 2010; Starick et al., 2006). Experimental studies also indicated that competitive type A ELISA tests were able to detect an antibody reaction earlier than the HI test (Song et al., 2009; Starick et al., 2006). Those findings are supported by the observation of the NP antibody kinetic profile after infection using the same type A ELISA kit (Marche et al., 2010). Therefore, to assess those observations it would have been necessary to sample the birds at a later date or to conduct HI tests using all the other AI subtypes as well as representatives of the main H5 clades. Nevertheless, it is difficult to justify that around 12% (23/185) of the H5 HI positive sera (out of the 1103 sera tested by the type A ELISA) were negative for the type A ELISA. This would either suggest a higher sensitivity of the HI test compared to the type A ELISA as described for a blocking ELISA on an experimental trial on ducks (Spackman et al., 2009), or this would indicate a lower specificity for the HI test (perhaps some sera with a low HI titer were false positives).

5. Conclusion

The strategy currently applied in Vietnam that uses the H5 HI test on sera samples for estimating the proportion of birds responding to vaccination against H5N1 or exposed to the virus, proved to be good in comparison with a H5ppVNT using the same HA clade. Nevertheless the cut-off for ducks needs to be changed to obtain a non biased estimation of

the proportion of seropositive birds. Differentiation between vaccinated and non vaccinated birds remains an issue but can be by-passed by appropriate record of vaccination status and regular virological monitoring.

From the study it can also be concluded that a H5 ELISA with a good sensitivity could be used as a screening test in a surveillance programme aiming at determining the proportion of birds having significant antibody titers to H5N1 viruses as a result of prior infection or H5N1 vaccination as long as positive sera are being re-tested by a more specific method. The H5 HI and/or the H5ppVNT could be suitable options for confirmation. The H5pseudotyped based VNT, even if more costly than the HI test, presents the advantages of having a less subjective reading as well as better performances, and does not need biosafety level 3 conditions. This test could be particularly interesting for large scale testing in the context of highly pathogenic strains surveillance where a specific subtype is targeted. Furthermore, there is a need to validate the manufacturer positivity cut-off for the H5-ELISA and possibly to adapt it to the study population. In complement to H5 HI or H5ppVNT, a N1-specific ELISA could be an interesting option to support the identification of the strains circulating on non vaccinated birds but needs to be validated on the poultry population of interest.

In addition, in a context where the diversity of subtypes is known to be low, a general and highly sensitive pre-screening can be achieved using the detection of NP-specific antibodies with a competition ELISA. It also presents the advantages of being less subject to reader interpretation and can be implemented in an ordinary laboratory (no need to work on a biosafety level 2 or 3 conditions). In the epidemiological context of Vietnam with a high seroprevalence of type A influenza virus resulting from the circulation of a diversity of avian influenza subtypes, this type of test appears of little interest because the surveillance needs to specifically targets the sub-types involved in the national disease surveillance and control programme.

Finally, to adequately fit the antigens being used for serological surveillance, regular virus detection and characterisation, as this is being done in Vietnam, is an essential component of the surveillance programme.

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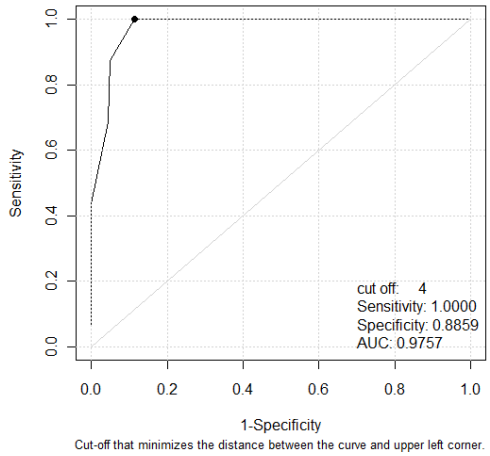
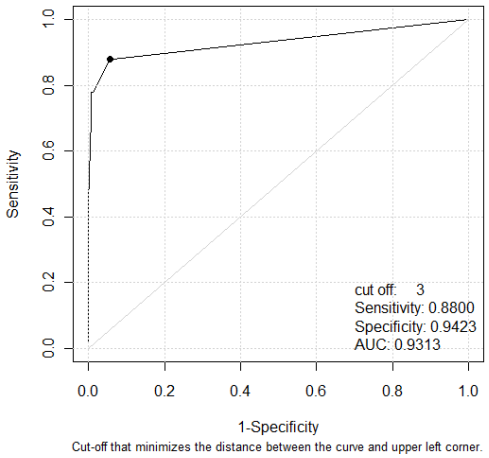
Figures caption

Figure 1. Determination of the optimal cut-off for HI test using “closest-to-(0,1)” criterion

Chicken	Duck
	

Tables

Table 1. Input information used to define beta prior distributions of the 2 Bayesian models

Bayesian analysis for chicken population			Bayesian analysis for duck population		
Parameters	**95% sure the parameter is**	**Mode**	**Parameters**	**95% sure the parameter is**	**Mode**
Prevalence of chicken breeders population	> 15%	25%	Prevalence of duck breeders population	> 25%	30%
Prevalence of chicken broilers population	< 30%	10%	Prevalence of duck broilers population	< 30%	10%
Se H5pp VNT	> 85%	90%	Se H5pp VNT	> 85%	90 %
Sp H5pp VNT	> 95%	99%	Sp H5pp VNT	> 95%	99%

480 Table 2. HI test performances using H5pp VNT at a cut-off of ≥80 as a reference test

	All species (n=406)	**Chickens (n=200)**	**Ducks (n=206)**
	Value (95% CI)	**Value (95% CI)**	**Value (95% CI)**
Se (1)	**83%** (72.1% - 91.4%)*	**100%[a]** (79% - 100%)*	**78%[b]** (64% - 89%)*
Sp (1)	**94%** (90% - 96%)*	**89%[a]** (83% - 93%)*	**99%[b]** (97% - 100%)*
PPV	71% (60% - 81%)	43% (27% - 61%)	98% (87%-100%)
NPV	97% (94%- 98%)	100%	93% (89%-97%)
Kappa	0.72 (0.62-0.82)	0.55 (0.43-0.68)	0.83 (0.69-0.96)
AUC	0.94 (0.90-0.96)*	0.98 (0.94-0.99)*	0.93 (0.89-0.96)*
Se adjusted (2)	**88%**	**100%**	**81%**
Sp adjusted (2)	**80%**	**82%**	**79%**
Se adjusted (3)	na	**91%** (83%-93%)**	**74%** (60%-87%)**
Sp adjusted (3)	na	**88%** (83%-93%)**	**98%** (95%-100%)**

481 * Exact Binomial CI
482 ** Probablity interval
483 (1) Estimation of Se and Sp using H5pp VNT as a reference test given true serological status
484 (2) Adjustment using equations for Se and Sp proposed by Staquet et al
485 (3) Adjustment using Bayesian analysis assuming conditional dependence
486 [a, b] Different lower-case superscript letters indicate a significant ($p<0.05$) difference between groups
487 (per row) with the use of a Student—t-test with unequal variance

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490 Table 3. Contingency table for the comparison between H5-ELISA and H5pp VNT assays
491 including both chicken and ducks species

	H5pp VNT positive	H5pp VNT negative	Total
H5-ELISA positive	48	66	114
H5-ELISA negative	0	107	107
H5-ELISA doubtful	0	9	9
	48	182	230

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494 Table 4. H5-ELISA test performances using H5pp VNT at a cut-off of ≥80 as a reference
495 test given true status.
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	All species (n=221) Value (95 % CI)	**Chickens (n=92) Value (95% CI)**	**Ducks (n=129) Value (95% CI)**
Se (1)	100% (93%- 100%)*	100% (72%-100%)*	100% (91%- 100%)*
Sp (1)	62% (54%- 69%)*	69%[a] (58%- 79%)*	55%[b] (45%- 66%)*
PPV	42% (33%- 52%)	31% (16%- 48%)	47% (36%- 59%)
NPV	100% (97%- 100%)	100% (94%-100%)	100% (93%-100%)
Kappa	0.41 (0.31-0.52)	0.35 (0.19-0.50)	0.42 (0.28-0.56)
AUC	0.92[1] (0.88-0.95)*	0.94[2] (0.87- 0.98)*	0.91[3] (0.85-0.95)*
Se adjusted (2)	100%	100%	100%
Sp adjusted (2)	64%	70%	58%

*95% exact Binomial CI
(1) Estimation of Se and Sp using H5pp VNT as a reference test given true serological status
(2) Adjustment using equations for Se and Sp proposed by Staquet et al
[1] n = 230, [2] n =96, [3] n=134
[a, b] Different lower-case superscript letters indicate a significant ($p<0.05$) difference between groups (per row) with the use of a Student—t-test with unequal variance

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499 Table 5. Concordance between the ELISA-A and the H5-ELISA positive results

		H5-ELISA			
		Negative	**Positive**	**Doubtful**	**Total**
ELISA A	**Negative**	57	**2**	0	59
	Positive	46	**111**	9	166
	Doubtful	4	**1**	0	5
	Total	107	**114**	9	230

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Table 6. Results of the HI tests applied on 260 ELISA type A positive samples and comparison with H5-ELISA results

	H5-ELISA			Total	%
HI results	**Negative**	**Positive**	**doubtful**		
H3	0	1	0	1	0.4
H3 H4	0	2	0	2	0.8
H4	1	2	0	3	1.2
H4 H9	2	0	0	2	0.8
H5	0	58	2	60	23.1
H5 H4	0	2	0	2	0.8
H5 H4 H6	0	1	0	1	0.4
H5 H4 H9	0	1	0	1	0.4
H5 H6	0	2	0	2	0.8
H5 H9	0	5	2	7	2.7
H6	2	0	0	2	0.8
H6 H9	1	0	0	1	0.4
H9	19	10	2	31	11.9
Not identified	68	68	9	145	55.8
Total	**93**	**152**	**15**	**260**	100%