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Development and application of an enzyme-linked immunosorbent assay (ELISA) using a soluble recombinant nucleoprotein for the detection of antibodies to avian influenza virus

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Avian influenza (AI) causes significant impact on industrial poultry farming, besides infecting a variety of vertebrates. The detection of antibodies against viral antigens by serological methods is important for the epidemiology, control and prevention of AI because their high simplicity and speed for assaying a large number of samples. Obtaining antigenic preparations used for detection of anti-avian influenza virus (AIV) antibodies usually requires complex and expensive procedures and *Escherichia coli* system expression may be an alternative. The nucleoprotein (NP) of AIV is an ideal antigen candidate because it is highly conserved across AIV strains, resulting in high cross-reactivity and immunogenicity for avian hosts. The NP gene segment was cloned and expressed from AIV isolate H4N6 in *E. coli* fused to a small ubiquitin-like modifier (SUMO) polypeptide and a poly-histidine tag, obtaining a soluble recombinant NP (rNP) containing the most important epitopes. After purification, the rNP was used as an antigen to develop an indirect rNP-enzyme-linked immunosorbent assay (ELISA) to effectively detect anti-AIV antibodies in chicken serum samples. This rNP-ELISA had high sensitivity (95%), specificity (97%), accuracy (96.7%) and agreement (k=0.88) in a comparative analysis with a commercial ELISA kit. The results suggest that rNP-ELISA offers a viable alternative to improve immunodiagnosis of AIV infection in chickens.

Key words: *Escherichia coli* system expression, small ubiquitin-like modifier (SUMO)-peptide, immunodiagnosis, poultry.

INTRODUCTION

Avian influenza (AI) is an infectious disease of domestic and wild birds that causes significant impact on the health of animals and on industrial poultry farming, besides infecting a wide variety of vertebrates, including ducks, chickens, pigs, whales, horses, and seals (Swayne and Halvorson, 2003). Different subtypes of avian influenza virus (AIV) type A cause this disease. Even though molecular techniques are efficient for the direct diagnosis of AIV, the detection of antibodies against specific viral antigens by serological methods is still considered an important tool for the epidemiology, control, and prevention of AI, because they can usually handle a large number of samples and generate results more rapidly at lower costs. The most used serological techniques are agar-gel immunodiffusion (AGID), hemagglutination-inhibition (HI), and enzyme linked immunonosorbent assay (ELISA) (OIE, 2015). ELISA is widely used because it requires smaller amounts of antigen and fewer antibody handlings than either of the other two techniques, whilst also enabling the evaluation of a larger number of samples, in a shorter period (Swayne and Halvorson, 2003; Wu et al., 2007). Antigenic preparations used for detection of anti-AIV antibodies with ELISA are usually obtained through procedures, such as viral propagation in specific-pathogen free (SPF) embryonated chicken eggs followed by purification of virus particles by ultra-centrifugation (OIE, 2015), which are complex and expensive. The expression of heterologous viral proteins is a more practical, simple and economical alternative to these techniques, as recombinant proteins tend to conserve most of the immunochemical properties of the original homologous proteins from viral particles and thus can be effectively used as antigen preparations in different immunodiagnosis methods. Indeed, as NP of AIV is highly conserved across AIV strains and present high cross-reactivity among these viruses and high immunogenicity for avian hosts, it has been expressed as recombinant protein to be used for the detection of AIV-specific antibodies by different ELISA types in chicken sera (Jin et al., 2004; Shafer et al., 1998; Upadhyay et al., 2009; Wu et al., 2007).

Escherichia coli is the most widely used microorganism for the production of recombinant proteins, due to fast growth kinetics, substantial protein production in a short time, ability to reach high cell density cultures in artificial media prepared from readily available and low-cost compounds, and great accessibility for transformation with exogenous DNA (Gopal and Kumar, 2013; Rosano and Ceccarelli, 2014; Sambrook and Russel, 2001). Despite these advantages, the *E. coli* expression system has not been more frequently used to produce AIV recombinant NP because this protein is difficult to express in this system, and must usually be recovered from the insoluble fraction, requiring several purification steps (Jin et al., 2004; Wu et al., 2007).

An approach used to circumvent this limitation is to increase the solubility of expressed recombinant proteins through the use of vectors harboring peptide and tags, such as the small ubiquitin-like modifier (SUMO) peptide (Guerrero et al., 2015; Zuo et al., 2005). Such vectors have been constructed for cloning and expression in *E. coli* systems, but to date this approach has not been used for the expression of AIV proteins.

Influenza A subtype H4N6 is one of the most prevalent subtypes isolated from a large variety of avian hosts (Hinshaw et al., 1981; Olsen et al., 2006). It is circulating around the world and identified in epidemiological surveillance studies with wild and domestic birds in Asia (Deng et al., 2013; Liu et al., 2003; Okamatsu et al., 2013), Europe (Henriques et al., 2011) and North America (Hanson et al., 2003; Scotch et al., 2014). First isolated in Czechoslovakia (1956) (Koppel et al., 1956), H4N6 AIV strains are classified as low-pathogenic avian influenza (LPAI) because animals infected with this subtype generally have the AI asymptomatic form (Olsen et al., 2006), although there are reports of disease and systemic spread in chickens infected experimentally with H4N6 in China (Liu et al., 2003). Influenza A subtype H4N6 has been isolated in Canada since 1999 from pigs with pneumonia (Karasin et al., 2000). In North America, H4N6 was the most isolated subtype in 2007 and 2008 by Wilcox et al. (2011). Kang et al. (2013) found that the predominant subtype in domestic ducks and wild birds in Korea was H4N6 and Latorre-Margalef et al. (2014) also verified that H4N6 was the major subtype found in migratory mallards in Northern Europe between 2002 and 2010.

Interestingly, multiple H4N6 AIV strains co-circulate and recombine with other influenza viruses in live poultry markets and farms (Shi et al., 2016). Since domestic pigs can support recombination of human and avian influenza viruses under natural conditions it is important to increase surveillance for this influenza virus subtype (Karasin et al., 2000).

Considering the positive effect of SUMO fusion technology in the expression of recombinant proteins in the *E. coli* system and the importance of influenza A subtype H4N6, the objectives of this study were to express the nucleoprotein gene of this AIV subtype in a soluble form and to use it as an antigen preparation in an indirect ELISA (rNP-ELISA) to detect anti-AIV antibodies in chicken serum. Additionally, in order to demonstrate

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the efficacy of the method, the performance of AIV-rNP-ELISA was compared with that of a commercial ELISA kit.

MATERIALS AND METHODS

Virus, RNA extraction and reverse transcription

An H4N6 avian influenza A virus isolate was propagated in specific pathogen-free (SPF) chicken embryonated eggs. The eggs were incubated at 37°C for 40 h; next, the allantoic fluid was collected, clarified by centrifugation, and stored at -70°C (Upadhyay et al., 2009). Viral RNA was extracted from allantoic fluid of inoculated eggs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The cDNAs were synthesized according to instructions provided with the superscript reverse transcriptase kit (Invitrogen). The reverse transcription reaction (RT) was performed using the superscript reverse transcriptase kit (Invitrogen) according to the manufacturer's recommendations with the extracted RNA as template. The complementary DNA (cDNA) obtained was used to amplify the NP by PCR.

Amplification of the AIV NP gene

Specific primers for the NP gene (forward + [5'-ATGCACATCATGGCGTCTCAA-3'] and reverse − [5'-TGATGGAGTCCATTGTTCCA-3']) described by Jin et al. (2004) and Yang et al. (2008) were designed with the modifications required for subsequent gene cloning in a pET-SUMO vector (Invitrogen) and used to amplify a 1128-bp region of the NP gene that codes from amino acid 1 to amino acid 376 and includes the major NP antigenic epitopes. The following cycling conditions were used for PCR: 35 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2.5 min, followed by a final extension step at 72°C for 10 min. The PCR product was analyzed by 1% agarose gel electrophoresis.

Cloning of NP-AIV gene and recombinant NP expression (rNP)

The purified PCR product of NP gene was inserted into a pET SUMO vector (Invitrogen) following the manufacturer's indications after cloning in TOP10F' *E. coli* competent cells (Invitrogen). This plasmid construction was used to transform competent BL21 *E. coli* cells, from which the plasmids were analyzed after purification, by nucleotide sequencing to confirm the presence of the gene insert. The *E. coli* BL21 cells transformed with the recombinant pET SUMO vector containing the NP gene were grown in Luria Bertani medium and protein expression was induced using 1.0 mM isopropyl-β-d-thiogalactopyranoside (IPTG) at 37°C for 16 h. The cell pellets were retrieved after centrifugation of *E. coli* culture at 12,000 × *g* for 10 min, diluted in lysis buffer (50 mM K_2HPO_4/KH_2PO_4, 400 mM NaCl, 100 mM KCL, 10% glycerol, 0.5% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and 10 mM imidazole pH 7.8), and sonicated. The soluble fraction of this culture containing expressed rNP was separated after centrifugation at 12,000 × *g* for 10 min and purified in nickel-agarose resin (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's recommendations. Fractions (1-ml) were collected from the column and their protein concentrations were determined by the Bradford method (Bradford, 1976). Non-purified and purified preparations of recombinant NP were characterized by SDS-PAGE and western blot according to protocol followed by Mahmood and Yang (2012).

In silico analysis of recombinant AIV-NP antigenicity and epitope prediction

The deduced amino acid sequence from the rNP nucleotide sequence cloned in this study was analyzed for potential epitopes and compared with other deduced AIV-NP amino acid sequences deposited in GenBank, including GU052384 Czechoslovakia/1956 (H4N6), M22579 A/swine/Germany/2/1981 (H1N1), CY067273 A/mallard/Postdam/178-CIP046-qa6rnd2/1983 (H2N2), CY005555 A/duck/Hong Kong/7/1975 (H3N2), CY092164 A/duck/Western Australia/8108/1984 (H4N6), CY005570 A/duck/Hong Kong/365/1978 (H4N6), CY015084 A/chicken/Scotland/1959 (H5N1), CY005614 A/duck/Hong Kong/d134/1977 (H6N2), CY130153 A/turkey/England/1963 (H7N3), and CY005634 A/duck/HK/784/1979 (H9N2). The Hopp and Woods method (Hopp and Woods, 1981) included in BioEdit Sequence Alignment Editor Version 7.0.2 was used to evaluate the hydrophilicity profiles of rNP amino acid sequences expressed in this study and other NP amino acid sequences from other AIV subtypes. Recombinant NP epitope prediction was performed using the Bepipred Linear Epitope Prediction method (Larsen et al., 2006) (http://tools.immuneepitope.org/bcell/).

Chicken serum samples

A pool of 10 serum samples from specific-pathogen free (SPF) chickens was used as negative sera. The positive serum samples were provided by the National Veterinary Services Laboratories of the United States Department of Agriculture (NVSL, USDA). An additional set of 121 chicken serum samples was provided by the National Laboratory for Agriculture, São Paulo (LANAGRO/SP), which is the national reference laboratory for poultry diseases in Brazil.

Indirect ELISA (AIV-rNP-ELISA)

AIV-rNP-ELISA was performed according to the general protocol developed by Silva et al. (2014). A checkerboard titration of four rNP concentrations (2, 4, 8, and 16 µg/ml) and six dilutions (1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600) of the positive and negative serum controls was performed to determine the optimum antigen concentration and the ideal serum dilution. The microplate wells were coated with 50 µl of the purified recombinant antigen diluted in carbonate-bicarbonate buffer (0.05M, pH 9.6) and the reaction was incubated for 16 h at 4°C. Next, the microplates were washed four times with PBS (pH 7.4) + 0.05% Tween 20 (PBST) and the non-specific binding sites were blocked with 10% skim milk powder in PBST (100 µl per well) (blocking buffer) followed by incubation for 45 min at 37°C. Following another washing cycle, the chicken serum samples were diluted in blocking buffer, added to the microplates (50 µl per well), and incubated for 1 h at 37°C. After this step, the microplates were washed and treated with 50 µl per well of rabbit anti-chicken IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MI, USA) diluted 1:1000 in blocking buffer for 1 h at 37°C. The substrate-chromogen solution (0.05 M citrate-phosphate buffer pH 5.0; 0.04% σ-phenylenediamine and freshly added 0.006% H_2O_2) was added and the colorimetric reaction was stopped by adding 2M HCL after 15 min, and the optical densities (ODs) were determined at 490 nm using ELISA reader (Bio-Rad, Hercules, CA, USA). For each test serum sample, the mean OD (ODMTS) was expressed in relation to the positive reference serum mean OD (ODMPRS) and the negative reference serum mean OD (ODMNRS), as a sample to positive ratio (S/P), according to the formula S/P = ODMTS − ODMNRS/ODMPRS − ODMNRS. The cutoff point was the mean S/P value + 3 standard deviations calculated from 10 AIV-negative chicken serum samples (Gibertoni

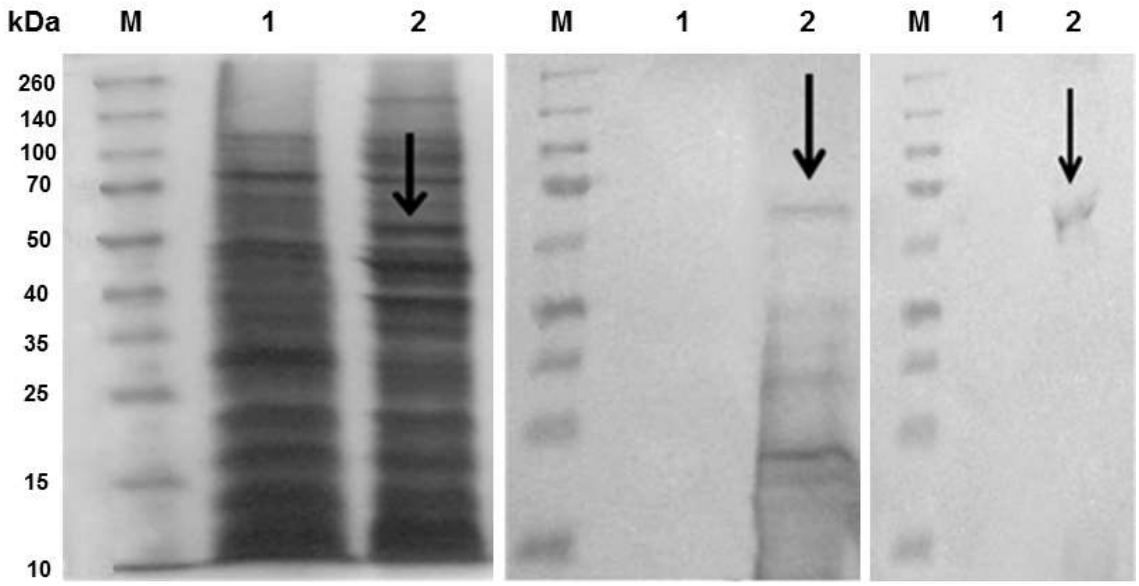

Figure 1. SDS-PAGE and western blot analysis of the AIV rNP expressed in *E. coli*. (A) SDS-PAGE. Column M: molecular weight marker; lane 1: non-induced fraction of *E. coli* culture (negative expression control); lane 2: *E. coli* induced fraction containing the rNP expressed. (B) Western blot of crude extract of rNP expressed in *E. coli* probed with anti-His monoclonal antibody. Lanes M, 1, and 2 received the same reagents as described for (A) except the samples were not purified. (C) Western blot of affinity-purified rNP fraction; lane M: molecular weight marker; lane 1: non-induced fraction of *E. coli* culture after chromatography in nickel-agarose resin; and lane 2: purified fraction of rNP after chromatography in nickel-agarose resin, probed with anti-His antibody. Black arrows indicate rNP.

et al., 2005).

Commercial indirect ELISA for the detection of anti-AIV antibodies

The IDEXX Avian Influenza Antibody Test kit (IDEXX Laboratories, Westbrook, ME, USA) was used according to the manufacturer's instructions to test the set of 121 chicken serum samples. The results were compared with those of rNP-ELISA.

Statistical analysis

The rNP-ELISA results were compared with results from a commercial indirect ELISA (AI Ab Test kit, IDEXX Laboratories) that uses whole virus as antigen. Sensitivity, specificity, accuracy, and agreement (κappa coefficient) values for rNP-ELISA were determined in comparison to the IDEXX kit according to Mohan et al. (2006). For the kappa coefficient (k), $k < 0.2$ indicates low agreement, $0.2 < k < 0.4$ indicates weak agreement, $0.4 < k < 0.6$ indicates moderate agreement, $0.6 < k < 0.8$ indicates good agreement, and $k > 0.8$ indicates a high level of concordance between tests (Landis and Koch, 1977).

RESULTS

Construction and expression of rNP in *E. coli*

A 1128-bp fragment was amplified and cloned from an H4N6 AIV isolate, encoding NP amino acids 1 to 376, into a pET SUMO vector. Nucleotide sequencing of the recombinant vector confirmed the presence and correct insertion of the NP gene, which had 99.72% identity with the nucleotide sequence deposited in GenBank (GU052384 of A/duck/Czechoslovakia/1956 [H4N6]) – a single amino acid change (T373A) in the deduced amino acid sequences. It was also confirmed that the NP gene fragment was cloned in frame with the SUMO peptide and poly-histidine tag genes. The expressed NP was a soluble recombinant protein with a molecular weight (MW) of approximately 56 kDa, by SDS-PAGE and western blot analysis (Figure 1). Line 2 of Figure 1B shows bands corresponding to SUMO peptide plus the poly-histidine tag (~13 kDa) and other proteins from *E. coli* due to the fact that this sample was not purified. However, after purification in nickel-agarose resin, only the rNP band was detected in the western blot (Figure 1C, line 2). The MW observed (~56 kDa) corresponds to a NP fragment (~43 kDa) fused to the SUMO peptide and poly-histidine tag (~13 kDa). A 1 mg/ml yield of rNP was obtained from 1000 ml of transformed BL21 *E. coli* culture, after purification of the protein on nickel-agarose resin.

In silico analysis of rNP antigenicity

The Bepipred Linear Epitope Prediction method

Table 1. Amino acid residues and respective positions and sizes for the 15 AIV recombinant NP epitopes predicted by Bepipred Linear Epitope Prediction.

Epitope	AA start	AA end	Peptide	Size
1	1	25	MASQGTKRSYEQMETGGERQNATEI	25
2	73	74	ER	2
3	76	99	NKYLEEHPSAGKDPKKTGGPIYRR	24
4	102	103	GK	2
5	123	131	ANNGEDATA	9
6	144	149	NDATYQ	6
7	159	160	MD	2
8	171	184	TLPRRSGAAGAAVK	14
9	206	214	FWRGENGRR	9
10	231	233	QTA	3
11	243	252	RESRNPGNAE	10
12	289	293	YDFER	5
13	318	325	PNENPAHK	8
14	352	360	VVPRGQLST	9
15	364	374	QIASNENMEAM	11
AA: amino acid		-	-	-

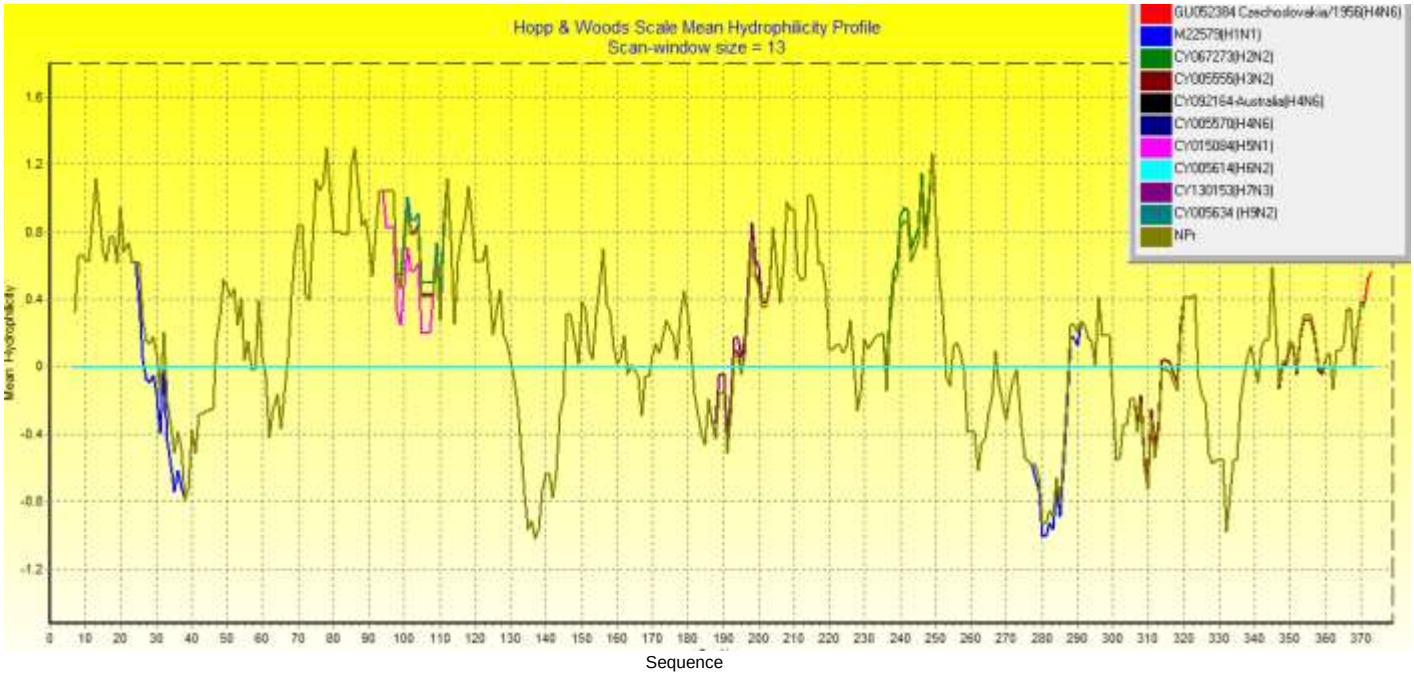

Figure 2. Comparison of hydrophilicity profiles by the Hopp and Woods method between amino acid sequences from AIV recombinant NP predicted epitopes and deduced amino acid sequences from AIV NP genes deposited in GenBank. Each amino acid sequence is represented by one color and identified in the plot legend.

determined fifteen epitopes from the deduced amino acid sequence of rNP (Table 1). To confirm this, the Hopp and Woods method was used (Hopp and Woods, 1981) to generate a hydrophilicity profile analysis by aligning the rNP deduced amino acid sequences in this study with amino acid sequences deduced from the NP gene from other AIV strains deposited in GenBank (Figure 2). This analysis predicted nearly the same 15 potential epitopes of these proteins, corresponding to the oligopeptides encompassing the amino acid residues in the

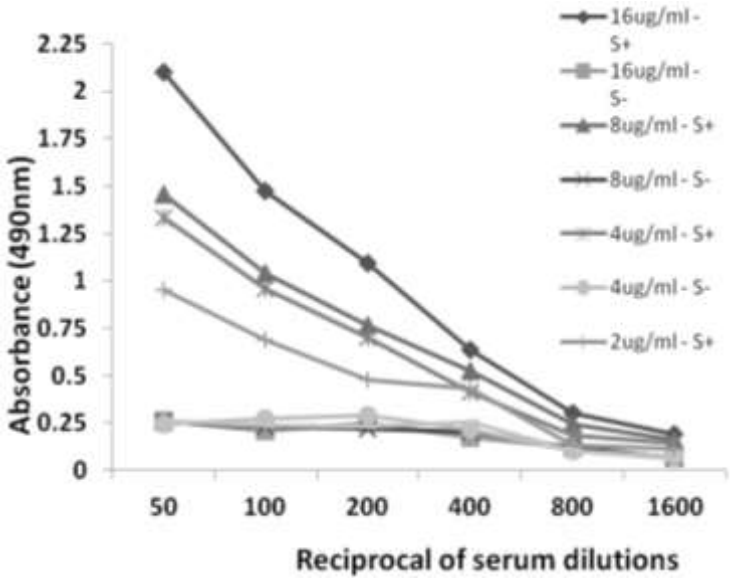

Figure 3. Checkerboard titration results of indirect ELISA for different concentrations of the rNP antigen against different dilutions of AIV-positive (S+) and AIV-negative (S-) reference chicken sera.

hydrophilicity peaks above the threshold value (green line = 0). The great similarity between the hydrophilicity peaks of the rNP amino acid sequences and those of the other NP sequences analyzed indicated that most potential rNP epitopes are conserved in NP sequences from different AIV subtypes (Figure 2).

Indirect AIV-rNP-ELISA

The checkerboard titration showed that, in indirect AIV-rNP-ELISA, AIV-positive serum reacted in a dose-dependent manner as rNP concentration increased from 2 to 16 µg/ml and, conversely, declined as the AIV-positive serum was diluted (Figure 3). An rNP concentration of 8 µg/ml and a single serum dilution of 1:100 were selected as ideal for the analysis of chicken test sera in AIV-rNP-ELISA (Figure 3) because, under these conditions, the highest specific reactivity between the chicken antiserum against AIV was found and difference was observed in the optical density values obtained from AIV-positive and AIV-negative serum controls. Analysis of 10 AIV-negative serum samples defined a cutoff S/P value of 0.125 for rNP-ELISA.

Comparative analysis between AIV-rNP-ELISA and commercial ELISA (AI Ab Test, IDEXX)

Table 2 compares the performance of rNP-ELISA in detecting anti-AIV antibodies with that of IDEXX ELISA (IDEXX Laboratories), in a set of 121 chicken serum samples. The sensitivity, specificity, and accuracy of rNP-ELISA were 95.0, 97.0, and 96.7%, respectively. The agreement of κ = 0.88 indicated a high similarity in the performance of the two ELISA tests.

Table 2. Comparison of AIV-rNP-ELISA and IDEXX AI Ab test for the detection of anti-AIV antibodies.

AIV-rNP-ELISA	IDEXX AI Ab Test		
	Positive	Negative	Total
Positive	19	3	22
Negative	1	98	99
Total	20	101	121

Sensitivity = 95.0%; specificity = 97.0%; accuracy= 96.7%; agreement (kappa index) = 0.88.

DISCUSSION

The pET SUMO vector-*E. coli* cloning and expression system used in this study resulted in the expression of a relevant quantity of a soluble form of AIV rNP that conserved most of the antigenicity of the original viral NP. One of the reasons for this result may be the effect of the SUMO peptide fusion in enhancing the solubility of viral recombinant proteins expressed in *E. coli* (Guerrero et al., 2015; Shafer et al., 1998; Zuo et al., 2005)

Recombinant NP epitope prediction confirmed that most antigenic sites of this recombinant protein were conserved compared to the NP of homologous (H4N6)

and heterologous (H1N1, H2N2, H3N2, H5N1, H6N7, and H9N2) AIV subtypes. In addition, some amino acid residue stretches predicted as rNP epitopes in this study, such as 71 to 96 and 290 to 353, have been previously confirmed as target epitopes of monoclonal antibodies to NP from AIV subtype H1N1 (Varich and Kaverin, 2004; Yang et al., 2008). These findings are further supported by the reactivity of rNP to chicken AIV-specific antibodies in the indirect ELISA developed in this study.

The functionality of AIV-rNP-ELISA was evaluated by analysis of 121 field chicken serum samples. Results showed high sensitivity (95%), specificity (97%), accuracy (96.7%), and good agreement (k = 0.88). compared to a commercial ELISA kit. Although, there are no optimum definitive values of sensitivity, specificity and agreement of a serological test (OIE, 2015), the sensitivity, specificity and agreement values of the current study are in the range from those found for these parameters in the analysis of commercial ELISAs and other indirect ELISA methods using recombinant NP preparations from different AIV strains. Upadhyay et al. (2009) found 98% sensitivity and 97% specificity compared to the commercially available ProFLOK® AIV Plus ELISA kit (Synbiotics, Kansas City, MO, USA). However, the concentration of yeast-expressed recombinant NP used to coat the ELISA microplate wells (80 μg/ml) was ten-fold higher than in the present study (8 μg/ml). Similarly, Wu et al. (2007), comparing the results of an indirect ELISA developed with an insoluble fraction of a recombinant NP expressed in another *E. coli* system with HI, AGID, and commercial ELISA kit IDEXX FlockChek™ (IDEXX Laboratories), reported a concordance of 92.0, 83.3, and 96.2%, respectively, but these authors used a higher concentration (50 μg/ml) of recombinant NP.

Overall, our findings indicate that the cloning and expression of a partial NP fragment in a pET SUMO-*E. coli* system yielded a soluble form of a recombinant viral antigen containing the most important epitopes of the protein. This approach contributed to the development and application of rNP-ELISA, with a performance similar to that of a commercial ELISA kit. Thus, the ELISA developed here using a soluble form of rNP offers a viable alternative to improve the immunodiagnosis of AIV infection in chickens, as it was able to analyze rapidly, simply and inexpensively a large number of serum samples with a low concentration of rNP compared with others studies and commercials kits. In addition, the rNP-ELISA has the potential to detect chicken antibodies against different AIV subtypes, because this technique uses a highly conserved antigen (NP) among the different subtypes of AIV, as demonstrated the epitope prediction analysis of this study and the results from previous studies reported by (Jin et al., 2004; Upadhyay et al., 2009; Wu et al., 2007). Despite the current rNP-ELISA was not tested for the detection of anti-AIV antibodies of other avian species, the rNP can be used also as coat-antigen in other ELISA methods such as blocking-ELISAs to react with anti-NP monoclonal antibodies (Jensen et al., 2013) to replace the purified AIV particles obtained from AIV-infected SPF embryonated chicken eggs that are submitted to purification by ultra-centrifugation in sucrose gradient.

The diagnosis of AI is achieved either by direct methods such as viral isolation, detection of genomic RNA, and viral proteins in biological samples, or by indirect methods involving the detection of AIV-specific antibodies (OIE, 2015) as performed in this study. The different diagnostic procedures available to diagnosis AI have to be able to handle a large number of samples and generate prompt and accurate results. Thus, the availability of appropriate reagents is of great importance, especially when serological tests are used for this purpose. Nowadays, most of the commercial kits for the detection and monitoring of chicken AIV-specific antibodies with ELISA use antigenic preparations from purified viral particles obtained by ultra-centrifugation, a very time-consuming and expensive method. Molecular cloning and expression of viral recombinant proteins, as described here, can provide a more refined and accessible antigen preparation such as AIV recombinant NP for use in serological techniques for the diagnosis of infection caused by this virus.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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