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Development of an antigen Enzyme-Linked AptaSorbent Assay (ELASA) for the detection of swine influenza virus in field samples

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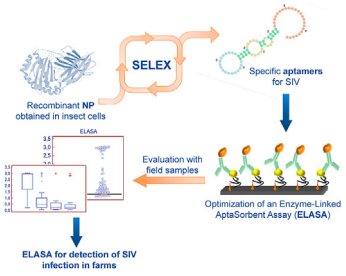
HIGHLIGHTS

- Aptamers are potential tools for the development of diagnostic methods.
- A good diagnostic performance was obtained combining aptamers with monoclonal antibodies.
- The developed ELASA will simplify and reduce costs derived from Influenza surveillance.

GRAPHICAL ABSTRACT



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ABSTRACT

Influenza viruses are highly variable pathogens that infect a wide range of mammalian and avian species. According to the internal conserved proteins (nucleoprotein: NP, and matrix proteins: M), these viruses are classified into type A, B, C, and D. Influenza A virus in swine is of significant importance to the industry since it is responsible for endemic infections that lead to high economic loses derived from poor weight gain, reproductive disorders, and the role it plays in Porcine Respiratory Disease Complex (PRDC). To date, swine influenza virus (SIV) diagnosis continues to be based in complex and expensive technologies such as RT-qPCR. In this study, we aimed to improve actual tools by the implementation of aptamers as capture molecules. First, three different aptamers have been selected using as target the recombinant NP of Influenza A virus expressed in insect cells. Then, these molecules have been used for the development of an Enzyme-Linked AptaSorbent Assay (ELASA) in combination with specific monoclonal antibodies for Influenza A detection. A total of 171 field samples (nasal swabs) have been evaluated with the newly developed assay obtaining a 79.7% and 98.1% sensitivity and specificity respectively, using real time RT-PCR as standard assay. These results suggest that the assay is a promising

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method that could be used for Influenza A detection in analysis laboratories facilitating surveillance labours.

Abbreviations

SIV	Swine Influenza Virus
ELASA	Enzyme-Linked AptaSorbent Assay
ELONA	Enzyme-Linked OligoNucleotide Assay
SELEX	Systematic Evolution of Ligands by Exponential enrichment

1. Introduction

Influenza viruses are highly variable pathogens that infect a wide range of mammalian and avian species. These viruses are enveloped, single-stranded RNA viruses that belong to the *Orthomyxoviridae* family [1]. Influenza viruses are divided into four different genera each containing a single species (A, B, C, and D). Genera classification is carried out according to the internal maintained viral proteins: nucleoprotein (NP) and matrix proteins (M). Type A viruses are the widest spread ones and they are further classified in subtypes according to their hemagglutinin (H) and neuraminidase (N) proteins [2–5]. Swine influenza virus (SIV) causes an acute, highly contagious respiratory disease in pigs and epidemics that can reach a morbidity up to 100%. The prevalence of this virus has been reported to be up to 90%, especially for some of the subtypes in farms with compatible signs [6–8]. During last decades, the pig industry has undergone several changes in herd size and an increase in the movement of animals within farms making Influenza infection an enzootic issue in most of the production countries, with persistent circulation of the virus between animals [9]. This impact leads to high economic loss in the industry due to slow weight gain and, in some cases, reproduction disorders such as abortion [10,11]. Co-infection of influenza viruses and other respiratory pathogens of swine, commonly results in more severe clinical signs and lesions leading to the appearance of porcine respiratory disease complex (PRDC). PRDC is a clinical term used to describe the presentation of respiratory signs and poor growth in pigs, which can be caused by one or several agents and environmental stressors. These shared clinical symptoms hinder proper diagnosis of the causative agent of the respiratory pathology in swine and it delays proper actuation against infection and outbreaks [6,12]. Thus, the early detection of this pathogen within herds may be of great interest to prevent the economic impact of the PRDC. Moreover, swine influenza is of great interest for public health, since virus that are maintained in swine are reassortants with a mix of human- and swine-adapted genes and they can be maintained in pigs and transmitted between them [13].

Although different tools have been developed for the diagnosis of Influenza infection in humans, including rapid diagnostic tests based on nucleic acid amplification and antigen detection which give results within 10–15 min [14]; the diagnosis in swine continues to be based on more complex techniques such as virus isolation, RT-PCR, fluorescence antibody test or immunohistochemistry. Indeed, nowadays, Influenza diagnosis is carried out by PCR from nasal swabs pooled samples and viruses are further subtyped by H and N sequencing [15]. There are some commercially available antigen-capture ELISAs, however, these tests tend to be less sensitive when compared to other techniques such as RT-PCR [16]. The use of aptamers might improve the application of ELISA technology in antigen detection by replacing or complementing the role of antibodies in these assays [17,18]. Aptamers are short single-stranded DNA or RNA molecules that acquire a 3D-structure and can bind a wide range of molecules with high affinity and specificity through different interactions [19]. Aptamers are selected from a random oligonucleotide library by iterative rounds of affinity purification and amplification by a process called Systematic Evolution of Ligands by Exponential enrichment (SELEX). Since they are selected *in vitro*, aptamers can be exposed to non-physiological conditions during the selection process, and they can be easily modified [20]. In this work, we have selected specific aptamers to the NP of Influenza A virus for the development of an Enzyme-Linked AptaSorbent Assay (ELASA) for the detection of SIV in field samples.

2. Materials and methods

2.1. Biological material

Spodoptera frugiperda clone 9 cell line (Sf9f, CRL 1711, ATCC) was used to propagate recombinant baculoviruses. The cells were grown at 27 °C using Grace's insect tissue culture medium (Gibco, Grand Island, NY, USA) supplemented with 8% fetal calf serum (FCS) (Gibco), 0.2% Pluronic F-68 (Sigma-Aldrich, Steinheim, Germany), and antibiotics. For protein expression a variant of Sf9 cells was used, Sf900, adapted to grow in absence of serum.

Escherichia coli strain DH5α was used for plasmid transformation and amplification.

Influenza A virus subtype H1N1 strain A/Puerto Rico/8/1934 (kindly provided by Dr. Amelia Nieto; CNB, Spain) was propagated over Mardin-Darby Canine Kidney cell line (MDCK cells).

2.2. Cloning and expression of the nucleoprotein (NP) of influenza virus

The codifying gene for the nucleoprotein of the Influenza A, was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from the genomic RNA of Influenza virus using the following primers: forward 5′-*CACCATCACCATCACCAT*ATGGCGTCTCAAGG CACCAAACGATCATAT-3′, and reverse 5′-TCAACTGTCA-TACTCCTCTGCATTGTCTCCGAAGAA-3'. A region codifying for a polyhistidine tag (6xHis) was included in the 5′-end of the gene. RT-PCR products were purified using the AccuPrep® PCR Purification kit (Bioneer, Daedeok, South Korea) and further cloned using the Gateway system (GW; Thermofisher) in the cloning vector pCR™8/GW/TOPO™ (ThermoFisher, Waltham, MA, USA) following the manufacturer's indications. The gene was subcloned into the transfer vector adapted to the Gateway system pAcGP67/GW for protein expression in the Baculovirus-insect cells expression system (BVES).

In order to generate the recombinant baculovirus, the protocol established by Hurtado and cols [21] was followed. Briefly, Sf9 cells were cotransfected with a mixture of linearized BacPak6 DNA (631401 Clontech, Mountain View, CA) and the transfer vector

pAcGP67-NP in presence of Jet PEI® (Polyplus, Illkirch, France) following manufacturer's indications. After 6–7 days, the culture supernatant was collected for baculovirus isolation by plaque assay in presence of X-Gal, white plaques were recovered, and the recombinant baculoviruses were used to obtain a high titter inoculum. A culture of Sf900 cells at $1{\cdot}10^6$ cells$\cdot$mL^{-1} density was infected with a multiplicity of infection (MOI) of 1 ufp$\cdot$cell^{-1}. Culture was harvested at 72 h post-infection and protein was purified from culture medium by affinity chromatography using His GraviTrap columns (GE Healthcare, Chicago, IL, USA) following manufacturer's indications. Protein was quantified by SDS-PAGE densitometry and it was verified by Western blot and ELISA using a NP-specific monoclonal antibody (MAb: 3CG5 for Western blot and 3DH6 for ELISA; INGENASA, Madrid, Spain) as the primary antibodies.

2.3. Aptamer selection. SELEX

DNA aptamers were selected from a synthetic random oligonucleotide library (RND40) by iterative rounds of selection following the SELEX methodology [22,23]. RND40 was composed of ssDNA molecules of 76 nucleotides length containing a central random region of 40 nucleotides flanked by two conserved 18-nucleotides regions named F3 and R3, respectively and further used as primers: 5′-GCGGATGAAGACTGGTGT-40N-GCCCTAAATACGAGCAAC-3' (IBA Life Sciences, Göttingen, Germany). Thus, a total of 10^{23} aptamers combinations could be obtained. For the initial SELEX round, 25 μg of RND40 were structured (95 °C for 10 min and then cooled on ice for 10 min), adding a total of 10^{15} different ssDNAs in a final volume of 300 μL of selection buffer (PBS, 1 mM $MgCl_2$). Structured aptamers were added over three wells of a 96-well plate coated with 1 μg/well of the MAb 3DH6 with the recombinant NP captured at 1 μg/well. Wells were incubated for 1 h at room temperature and 260 rpm, then they were washed three times with selection buffer supplemented with 0.05% (v/v) Tween20. Bounded aptamers were recovered by adding 50 μL of PCR mix and heating at 100 °C for 5 min. PCR mix was prepared under the conditions of 0.8 μM of each primer, 200 μM dNTPs, 2 mM $MgCl_2$, and 6 U$\cdot$μL^{-1} Taq polymerase (Biotools, Madrid, Spain). Bounded population was amplified by PCR following the described conditions: 2 min at 95 °C/10X (30 s at 95 °C/20 s at 57 °C/30 s at 72 °C). For the next selection rounds, 1 μg of the previously selected population was structured as described before and incubated with the same amount of protein. In addition, for rounds 4 to 6, incubation time was reduced to 30 min. Contraselection against wells coated with antibody, in absence of NP, was performed after rounds 2 and 5. A total of 6 selection rounds were carried out for the obtaining of specific aptamers.

2.4. Enzyme-linked OligoNucleotide assay (ELONA)

To assess the enrichment of the selected population's affinity for the target protein, aptamers were labelled by PCR using 5′ digoxigenin-labelled F3 and R3 primers (IBA Life Sciences). Recombinant NP was diluted to 2 ng μL^{-1} in 1X KPL Coating Solution (SeraCare, Milford, MA, USA), 100 μL of the solution were added to a 96-well microtiter plate (NUNC, Rochester, NY) and incubated at 4 °C overnight. The plate was washed four times with selection buffer supplemented with 0.05% Tween20 and blocked with 200 μL/well of PBS-5% BSA for 1 h at room temperature and 260 rpm. As a negative control, wells were coated in the same conditions using a different protein obtained in insect cells. Digoxigenin-labelled aptamers were structured in selection buffer at 1 ng/μL, and 100 μL of the solution were added per well in triplicate. The plate was incubated at room temperature for 1 h and 260 rpm. The same number of wells were incubated in absence of aptamers, only with selection buffer, to have a background signal which was subtracted from the wells' signal. Then, the plate was washed three times to remove unbound ssDNA. A conjugated anti-digoxigenin antibody with horseradish peroxidase (HRPO) (Roche, Basel, Switzerland) was added to wells at a 1/1000 dilution and incubated for 1 h at room temperature and 260 rpm. The plate wase washed four times and the assay was developed with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) solution (Roche) according to the manufacturer's indications. Results were read out as A_{405} after 20 min of incubation in dark.

2.5. Aptamers characterization

After the sixth round of SELEX, the DNA pool was cloned using the pGEM-T Easy vector kit (Promega, Madison, WI, USA) and transformed into chemically competent *E. coli* JM-109 cells by heat shock treatment. White colonies were picked and expanded in liquid culture; the plasmid DNA was purified by miniprep kit (Promega, Madison, WI, USA.) and amplified by PCR to determine the presence of aptamers within the vector. Finally, the plasmid DNA was sequenced by the Sanger method.

To assess the affinity of the individual aptamers, they were labelled by PCR from isolated plasmids using 5′ digoxigenin-labelled F3/non-labelled R3 and non-labelled F3/5′ digoxigenin-labelled R3 primers for the labelling of forward chain or reverse chain, respectively. These labelled aptamers were used as primary molecules in an ELONA following the previously described protocol.

Once best candidates were selected, they were synthetically produced and labelled with digoxigenin (IBA Life Sciences, Göttingen, Germany) and their affinity parameters were determined: equilibrium binding constant (Kd) and maximum specific binding capacity measured in A_{405} (Bmax). Briefly, an ELONA was carried out using different concentrations of aptamers (25 nM, 50 nM, 100 nM, 200 nM and 400 nM) in triplicate. A_{405} was determined every 10 min during 1 h of incubation at room temperature in the dark. Results were analysed by One Site-Specific binding analysis using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, USA).

Structure prediction was carried out using the centroid structure prediction of the free software RNAfold (Accessible on-line at: http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi). The capacity of forming G-quadruplex structures was tested with the QGRS mapper software (Accessible on-line at: http://bioinformatics.ramapo.edu/QGRS/index.php).

2.6. Field samples

A total of 171 field samples (nasal swabs) were used for the evaluation of the assay. 118 samples belonging to 23 different respiratory outbreaks detected in different pig farms were characterised as positive by the technique used as reference in this work, RT-qPCR [24]. The remaining 53 samples belonged to nasal swabs obtained in previous longitudinal studies [25,26] carried out in pig farms from randomly sampled animals without clinical signs and characterised as negative by the same RT-qPCR.

2.7. ELASA

The plates were coated with aptamers using two different approaches: modification of the aptamers with a biotin group or with an amino group by a C6-link, both at the 5′-end (IBA Life Sciences, Göttingen, Germany). The aptamers were structured at 100 μM in PBS, 1 mM $MgCl_2$ as described for the selection, and they were diluted for the coating of the plate. For the first approach (biotin

labelling), the aptamers were diluted in carbonate buffer, pH 9.6 at different concentrations, and 100 μL were added to each well on a Pierce Streptavidin Coated Plate (ThermoFisher, Waltham, MA, USA). Whereas, for the second approach (amino labelling), the aptamers were diluted in sciSPOT Oligo B2 buffer (Scienion, Berlin, Germany), and added to a Polystyrene High Binding Stripwell microplate (Corning, New York, MA, USA). In both cases, plates were incubated at 4 °C overnight. Plates were washed 5 times with PBS, 0.05% Tween20, 0.005% Kathon and they were stabilized for 1 h at room temperature.

For the development of the assay, samples were diluted 2-fold in PBS, 135 mM NaCl, 0.05% Tween20 and 100 μL were added per well. The samples were incubated 3 h at room temperature, washed 5 times and the MAb 3DH6-HRPO was added at a 1/5,000 dilution in the same assay buffer. The plates were incubated for another hour at room temperature, and, after another washing step, the assay was performed with 3, 3′,5,5′-Tetramethylbenzidine (TMB) for 30 min at dark. Results were read out in a plate reader as A_{450}. For the calculation of the limit of detection (LoD), wells were incubated with serial dilutions of the recombinant NP diluted in assay buffer, selecting as LoD the last concentration that gave a two-fold signal when compared with the negative control.

2.8. Statistical analysis

For the statistical evaluation, samples were classified as positive or negative according to the reference technique used in this study, RT-qPCR. Data was statistically analysed by receiver operating characteristic (ROC) curves using the MedCalc® 10 software (MedCalc Software, Ostend, Belgium) to establish the optimal cut off value and to determine the sensitivity and specificity of the test. Positive samples were clustered according to their Ct value, and these clusters were analysed by a box- and whisker-plot using the same software. In addition, the results of ELASA were compared with RT-qPCR results to estimate the diagnostic sensitivity and specificity, positive and negative predictive values and positive and negative likelihood ratios (WinEpi software 2.0, Faculty of Veterinary, University of Zaragoza, Spain). Agreement between RT-qPCR and ELASA results was assessed using Cohen's kappa coefficient (κ) ($\kappa \leq 0$ no agreement; $0.01 < \kappa < 0.20$ slight agreement; $0.21 < \kappa < 0.40$ fair agreement; $0.41 < \kappa < 0.60$ moderate agreement; $0.61 < \kappa < 0.80$ substantial agreement; and $0.81 < \kappa < 1.00$ almost perfect agreement) (WinEpi software 2.0, Faculty of Veterinary, University of Zaragoza, Spain).

3. Results

3.1. Expression of recombinant NP

The NP of SIV was obtained with high yields in insect cells. After the purification step, two bands were observed, one of 59 kDa approximately, which was the expected molecular weight, and the other one of 63 kDa approximately. They both were specifically recognised by the monoclonal antibody 3CG5 α-NP Influenza type A (Fig. 1). The NP was soluble and, considering that both bands were specifically recognised by the monoclonal antibody, the purity was determined as 99%.

3.2. Aptamers selection

Using the produced protein, specific aptamers were selected. After six rounds of SELEX, affinity was evaluated by ELONA with the digoxigenin-labelled populations of RND40, and those from Round 3, and Round 6 (Fig. 2).

ELONA analysis of the different populations shows enrichment in the specific molecules against the NP. The population obtained after the sixth SELEX round produced a signal for the target protein more than 24 times higher than the signal obtained with the initial population, RND40, and 6 times higher when compared with the non-related protein included as control in the assay. Furthermore, this pool of DNA molecules had a specificity enough for the characterization of aptamers. It was cloned and sequenced as described before to select the best candidates for the development of a diagnostic assay.

Individual clones were evaluated by ELONA labelling the forward or reverse chain respectively (data not shown) and best candidates were synthetically obtained. Fig. 3 shows the predicted secondary structure for the three most promising aptamers, whose affinity parameters were determined as described in materials and methods (Table 1). High-affinity parameters were obtained, with values of Kd as low as 2 nM. The best candidate appears to be the 6.2F, even though, all aptamers selected were tested for the development of a diagnostic assay. The most stable molecule according to secondary structure predictions is the 6.2F, which can form a G-quadruplex structure.

3.3. ELASA optimization

The three selected aptamers were synthetically produced and labelled with biotin or with an amino group through a C6-link as described before. The plates were coated at 500 nM, a saturating concentration of aptamer, for the selection of the best conditions.

From the observation of Fig. 4, it can be determined that for this assay, the modification of the aptamers with an amino group exhibits higher signals of A_{450} than the modification with biotin for all the molecules tested. Moreover, the aptamer 6.2R shows a higher sensitivity than the rest of the molecules tested with both modifications, even though it was the one exhibiting the highest Kd value (Table 1). Therefore, for the development of a diagnostic assay, the coating of high binding polystyrene plates with the 6.2R-amino aptamer was the approach selected. Using these conditions, a LoD of 62.5 $ng \cdot mL^{-1}$ of recombinant protein was obtained. No signal was observed with the non-related protein used as negative control.

Next, coating conditions were optimised by titration of the capturer aptamer modified by an amino group in the proper coating buffer. The NP was added at 62.5 $ng \cdot ml^{-1}$, which is the last point detected by the assay, to determine the minimum quantity of aptamer needed to obtain no variations at the A_{450} signal. No significant changes in A_{450} were observed over 62.5 nM of capturer aptamer, whereas under this concentration a significant decrease in this signal is obtained (Fig. 5). Thus, the optimal concentration for the coating of the plates was selected as 65 nM, which produced minimal background, as well as the highest sensitivity and accuracy for the detection of the recombinant NP.

Once the assay was optimised, the capacity of recognising the native antigen in field samples was confirmed with a high titre inoculum of Influenza A. Moreover, its robustness was evaluated with positive and negative controls after performing interlab, interperson, interplate and intraplate evaluations, obtaining variance coefficients under 5 for all the determinations. The best conditions for the analysis of broader collections of samples were established at the 2-fold dilution of the sample in assay buffer.

3.4. Samples analysis

Once assay was optimised, a total of 171 field samples were analysed with the preliminary developed assay to establish the diagnostic sensitivity and specificity of the assay. A cut-off value of 0.2 A_{450} was established to obtain the best diagnostic parameters.

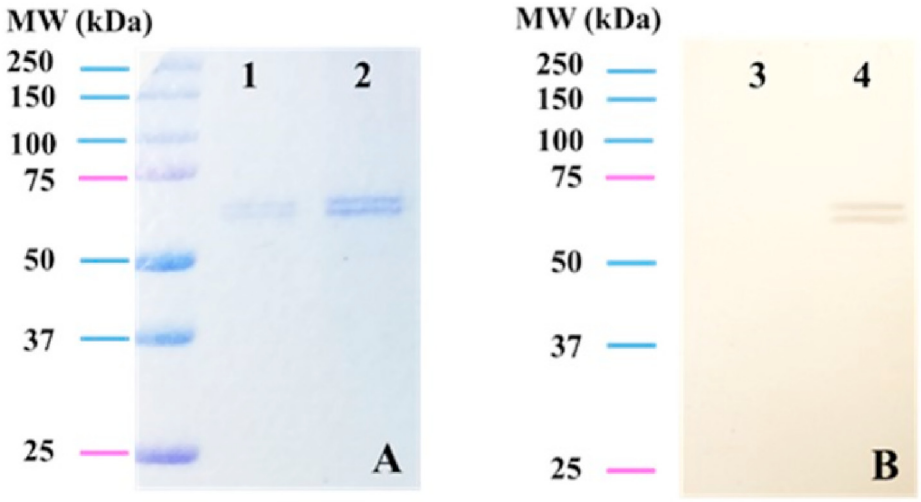

Fig. 1. SDS-PAGE of purified NP of SIV expressed in insect cells using the Baculovirus expression system and further analysed by A. Coomassie staining of 250 ng (1), 500 ng (2) and 1 μg (3) of the purified protein. And B. Western blot of 500 ng of purified protein (4) and (3) negative control.

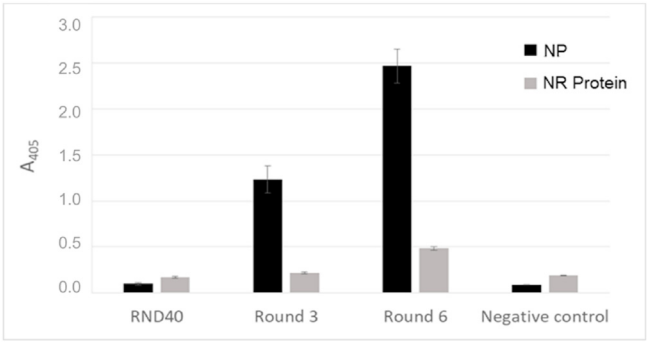

Fig. 2. ELONA analysis of the different populations obtained during the SELEX process. X-axis shows the population analysed. Y-axis shows the media A_{405} of the three wells analysed for each population with error bars showing their standard deviation. A non-related protein (NR protein) was incubated with different aptamer populations to assess the unspecific signal. Three wells were incubated in absence of aptamers as negative control.

Table 1
Affinity parameters for the selected aptamers (mean ± SD).

	6.2F	6.2R	6.14F
Kd (nM)	2.6 ± 0.9	4.4 ± 1.9	3.7 ± 0.9
Bmax	0.9 ± 0.03	1.3 ± 0.08	1.1 ± 0.03

Ninety-four out of the 118 samples classified as positive by the reference technique were detected as positive by the newly developed test, showing a sensitivity of 79.7% (95% CI: 72.4–86.9%) for the ELASA. Moreover, only one sample among the 53 samples classified as negative with the reference technique gave a positive signal with the developed ELASA, exhibiting a specificity of 98.1% (95% CI: 94.5–101.8%) (Fig. 6). The positive predictive value and negative predictive value were 98.9% (95% CI: 96.9–101.0%) and 68.4% (95% CI: 58.0–78.9%), respectively. In addition, the positive likelihood ratio and negative likelihood ratio were 42.0 and 0.21, respectively. Finally, the concordance or level of agreement

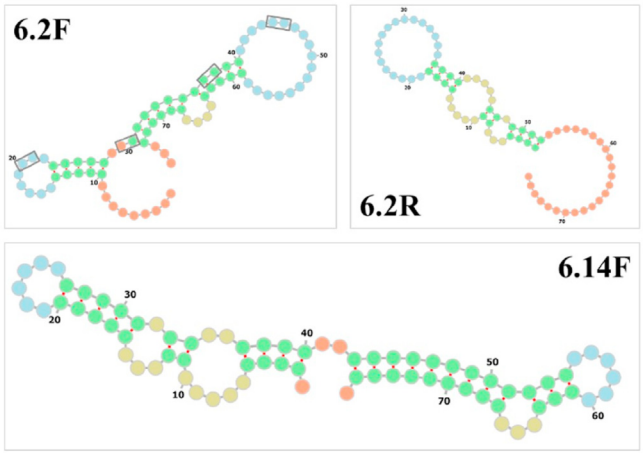

Fig. 3. Secondary structure prediction for the best candidates selected by SELEX. Quadrangles show the parts of the different sequence (6.2F) which are able to form a G-quadruplex structure between them.

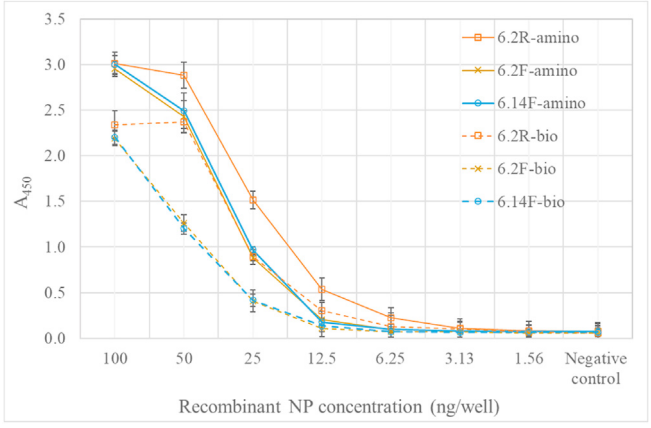

Fig. 4. NP titration with the different combinations of aptamers available. X axis shows the amount of recombinant protein (ng) added per well. Y axis shows the A_{450} obtained at each point (logarithmic scale). The different markers show the aptamer used for the coating of the plate (□ 6.2R, x 6.2F, and ○ 6.14F), whereas the line code shows the modification strategy of the aptamer used for coating plates: (dashed line) biotin modification and (solid line) amino modification. Error bars show standard deviation for each point of the measurements (triplicates).

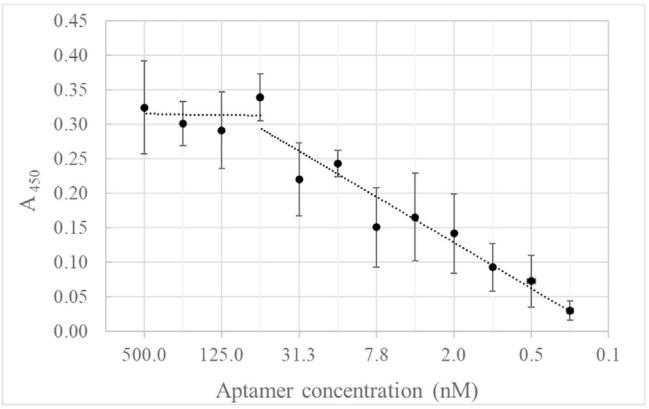

Fig. 5. Titration of capturer aptamer in ELASA. X axis shows the concentration of aptamer 6.2R used for the coating of the plates. Y axis shows the A_{450} signal obtained at each concentration of capturer aptamer with a constant concentration of 300 ng/μL of recombinant NP (logarithmic scale). Error bars show standard deviation for each point of the measurements (duplicates).

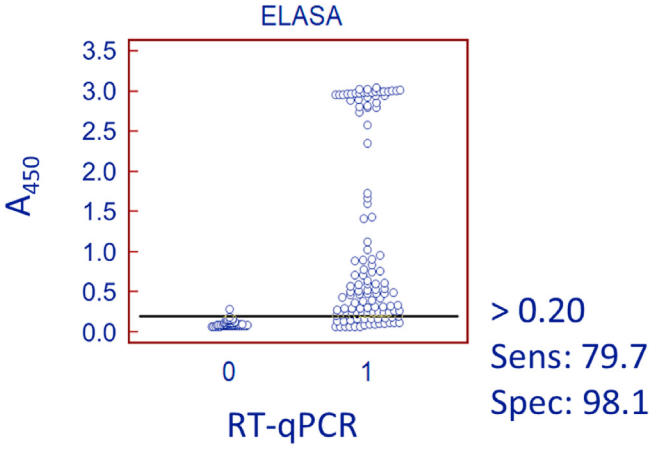

Fig. 6. ROC curve analysis of the evaluated samples with the ELASA developed. Samples classified as negative are indicated with a "0" whereas positive samples are indicated with a "1". Y axis show the A_{450} for each sample.

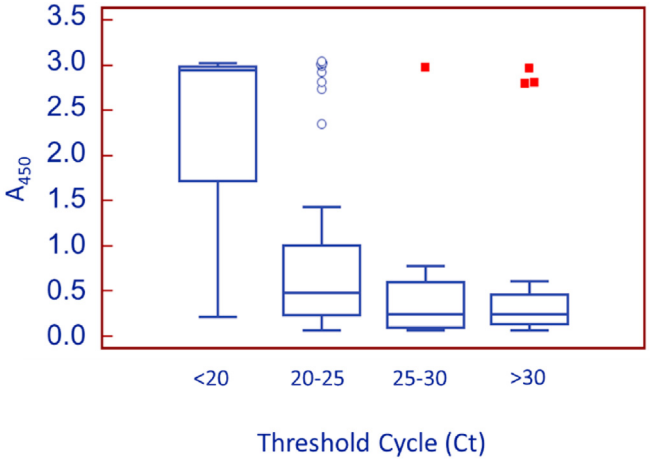

Fig. 7. Box and whisker plot of samples clustered according to Ct value obtained by RT-qPCR. X axis shows different Ct clusters whereas Y axis shows absorbance value obtained by ELASA.

between both diagnostic assays for tissue samples was substantial ($\kappa = 0.695$).

No correlation was observed between Ct (RT-qPCR) and absorbance signal (ELASA) with a correlation coefficient of −0.5166. For a more detailed evaluation of assay parameters, samples were clustered according to the Ct value in four different groups: <20, 20−25, 25−30 and > 30 and analysed by box and whisker plot.

As shown in Fig. 7, samples within different groups exhibit different intensity signals of absorbance. However, an increase in absorbance median is observed as Ct decreases, especially observed for samples under Ct 20, where also, an accumulation of samples is observed over A_{450} 3, the upper limit of the detection of the reader. None sample with Ct value under 20 gave a negative signal with the developed ELASA, only one weak positive sample was obtained of A_{450} 0,324. For samples classified within the group of Ct between 20 and 25, more than 80% of samples (35/43) were detected as positive with the developed ELASA. This percentage was reduced when analysing samples within the group of Ct between 25 and 30 to a 63% of positives (12/19) and to a 55% (11/20) among samples in the group of Ct over 30. Considering this fact, although no direct correlation was observed between ELASA and the reference technique, it was easier to detect NP in samples with higher viral loads, as it would be expected. One sample within the 25−30 Ct group and 3 samples within the over 30 Ct group, gave Absorbance signals around 3, which were high signals for the viral load expected. Moreover, it should be mentioned that, within samples analysed in the present study, we analysed several subtypes of SIV (H1N1, H3N1, H3N2, H1avN1, H1pdmN1 …) not observing any kind of relationship with the detection of the virus.

4. Discussion

Taking into consideration the high impact that swine influenza and PRDC have in farms, the need to have reliable and fast tools for the detection of the initial agents causing this complex disease is essential to prevent high economic loss. Nowadays, there are several molecular tools available for the identification of these agents, the most acute and rapid diagnosis is obtained by RT-qPCR [27]. This kind of tests are expensive, need highly qualified staff, and can be inhibited by some compounds present in the sample whereas ELISA-based methods are faster, cheaper and easier to perform since they do not need extraction steps nor complicated incubation cycles [28]. Among many bioreceptors used for the development of ELISA-based assays, nucleic acid aptamers have received great attention, because they have unique features such as high stability, low production costs and inherent amplification abilities [29] which give them the capacity to replace or complement the role of antibodies. Aptamers are widely applied in human health within different areas ranging from diagnosis to treatment of multiple pathologies, however, this technology is poorly applied in veterinary sciences. In this study, three different aptamers have been selected with the ability to specifically recognise the NP of Influenza A virus exhibiting great affinity parameters and the ability to recognise not only the recombinant protein used for the selection, but also the native hole virus.

The pAcGP67 vector used in our study is designed for the extracellular expression of the recombinant target proteins in insect cells. The protein sequence expressed in this vector is preceded by the gp67 secretion signal which is eliminated by the cell machinery after protein expression. In this sense, the double band observed for NP expression in the present study can be explained by a miss processing of the NP after its expression, since the difference between the two bands matches with the size of the cleavage peptide, 4.1 kDa. However, since the protein was properly recognised by the available monoclonal antibodies obtained

against the whole virus, and characterised as specific for the NP, the obtained recombinant protein was used with great results for the selection of specific aptamers against Influenza A NP.

Empirical results showed that the 6.2F aptamer was the greatest candidate for the development of a diagnostic assay, exhibiting the lowest Kd and the putative presence of a G-quadruplex structure within its sequence, that gives to it higher stability and protection against degradation [30]. However, during the optimization of the assay, 6.2R aptamer exhibited better parameters in the ELASA format, which might be due to the higher Bmax obtained with this molecule. Since 6.2R aptamer renders higher A_{450} signals with lower amount of NP, it can detect lower amounts of virus within the samples than the aptamers with higher affinities to the target protein. Consequently, our results highlight that for the development of the assay both parameters, Kd and Bmax, are equally important for obtaining a sensitive test. In this case, the higher Bmax obtained for the 6.2R than for the 6.2F makes the former a better candidate for the detection of small amounts of antigen in field samples.

With the newly obtained aptamers, an ELASA was developed. This assay exhibited a limit of detection of 62.5 ng μL^{-1} of recombinant NP. When tested with field samples, the assay showed great diagnostic parameters with almost 80% sensitivity and more than 98% of specificity which are over the values obtained by other commercially available methods, whose sensitivity usually range from 67% to 71% and which has only been evaluated for human screening [31]. No correlation was observed between the absorbance signal in ELASA and the threshold cycle (Ct) in RT-qPCR, although a tendency was observed with greater detection percentages in samples with lower Ct values. NP has been demonstrated lately to be highly maintained within several Influenza A isolates [25], hence NP variability cannot account for the losing of some positive samples with the developed ELASA, since all samples analysed were characterised as Influenza A. Since ELASA avoid purification steps when compared with PCR, the non-detection of samples with good viral loads (Ct values between 20 and 30) may be explained by the presence of some contamination in the nasal swabs that leads to the interference for NP detection.

Considering that the technique used as reference in this study is the RT-qPCR, diagnostic parameters indicate that the new ELASA may be a great candidate for its use in field. It is shown to detect any sub-type of Influenza A virus infection with greater sensitivity than other immunological assays currently available for swine Influenza virus detection. The application of the developed ELASA in the first steps of surveillance campaigns followed by the selection of one positive sample for the lineage determination, would reduce costs and it would facilitate the accurate diagnosis of the respiratory pathology in swine.

Author statement

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Data availability statement

The data that support the findings of this study is available from the corresponding author upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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