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Short communication

Conserved HA-peptides expressed along with flagellin in *Trichoplusia ni* larvae protects chicken against intranasal H7N1 HPAIV challenge

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ABSTRACT

The immunization of poultry where H5 and H7 influenza viruses (IVs) are endemic is one of the strategies to prevent unexpected zoonoses. Our group has been focused on conserved HA-epitopes as potential vaccine candidates to obtain multivalent immune responses against distinct IV subtypes. In this study, two conserved epitopes (NG-34 and CS-17) fused to flagellin were produced in a Baculovirus platform based on *Trichoplusia ni* larvae as living biofactories. Soluble extracts obtained from larvae expressing "flagellin-NG34/CS17 antigen" were used to immunize chickens and the efficacy of the vaccine was evaluated against a heterologous H7N1 HPAIV challenge in chickens. The flagellin-NG34/CS17 vaccine protected the vaccinated chickens and blocked viral shedding orally and cloacally. Furthermore, no apparent clinical signs were monitored in 10/12 vaccinated individuals. The mechanism of protection conferred is under investigation.

1. Introduction

The high pathogenic avian influenza viruses (HPAIV), mainly H5 and H7 subtypes, are devastating pathogens in poultry. On occasions, however, they have spilt over into humans [1] fortunately, with limited evidence of being capable of transmitting between humans. Nevertheless, the risk of an occurring reassortant IV that may cross the human-to-human transmission barrier is threatening the public health.

Controlled vaccination campaigns in some endemic countries may prevent possible outbreaks caused by avian IVs in the poultry. At present, most of the poultry influenza vaccines are inactivated-typed but also there are licensed recombinant and live-attenuated vaccines [2]. Nevertheless, recombinant vaccines overcome many shortcomings from the inactivated-typed vaccines [3–5]. In the present study, Baculovirus were selected as a suitable influenza vaccine technology platform. As biofactories, insect larvae from *Trichoplusia ni* (*T.ni*), were used for recombinant protein production [6]. Besides, a novel baculovirus vector expression cassette, Top-Bac®, was used to optimize the baculovirus expression vector system (BEVS) [7].

Having potent antigenic properties, hemagglutinin (HA) is the most selected recombinant protein from IVs due to its potent antigenic properties favoring antibodies which can prevent IVs infection [8]. Our research group has been dedicated to use conserved HA-epitopes as vaccine candidates [9,10]. Thus, two *in silico* predicted conserved HA-epitopes from H1 (peptide NG-34) and from H5 (peptide CS-17) were selected. Both epitopes were fused to phase 2 flagellin from *Salmonella typhymurium* to improve the immunogenicity of the vaccine [11,12]. The final formulation (flagellin-NG34/CS17) was produced in the Baculovirus system and the soluble extracts obtained expressing flagellin-NG34/CS17 were used as a vaccine against IVs infection in chicken. The final goal of this work was to study the vaccine efficacy of soluble extracts expressing flagellin-NG34/CS17 construct against a heterologous virus challenge in chicken.

Our results indicate that chickens immunized twice with flagellin-NG34/CS17 were protected against a lethal challenge with a heterologous IV (H7 subtype). Immunized animals showed no

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clinical signs of infection and did not shed virus. An increased IgM/IgY was also evident in some of the vaccinated chicken.

2. Materials and methods

2.1. Immunogen, construction of recombinant baculovirus and infection of insect larvae

Table 1 depicts the aminoacidic sequence of the two conserved HA-epitopes (NG-34 and CS-17) used in the vaccine approach, which are connected by the linker sequence: SSGSSGSSGSS. At the N-terminal site of that sequence, the complete sequence of *Salmonella typhimurium* phase 2 flagellin (GenBank: AAC94993.1) was inserted. Moreover, a 6-His tag was added at the C-terminal site of the NG34/CS17 sequence. The whole cDNA was chemically synthesized (GenScript, Piscataway, NJ, USA) (Fig. 1). The codon usage from *Bombyx mori* was utilized to optimize the sequence to improve the epitope expression in the *T.ni* larvae: (http://www.ebi.ac.uk/Tools/st/emboss_backtranseq/). The whole sequence of interest was excised from the pCDNA3.1(+) vector and inserted in the TopBac3.2® vector (Algenex, Madrid, Spain). The resulting donor plasmid was used to generate the corresponding recombinant baculovirus (rBac) by using the Bac-to-Bac® Baculovirus Expression System (Invitrogen, San Diego, CA, USA). Baculovirus obtained were titrated in duplicate by a standard plaque protocol (Invitrogen, No. MAN0000414). Virus titer was determined as plaque-forming units (PFU). *T.ni* larvae in stage of fifth-instar larvae were sedated and injected with 5 μL of the recombinant baculovirus diluted with TNM-FH medium (PAN Biotech GmbH, Aidenbach, Germany) to reach a dose of $5.5x10^6$ pfu/mL.

2.2. Protein extraction, determination and quantification

Total soluble protein (TSP) extract from *T. ni* larvae infected with the rBac was obtained by mixing extraction buffer (PBS 1X, Brij®35 0.1%: BR00171000; PMSF 1 mM and DTT 5 mM) per gram of larvae biomass and homogenizing twice (total protein extract, TP). Sonication, centrifugation and filtration of supernatants with a 22 μM Miracloth filter paper (Calbiochem®, Merck, Darmstadt, Germany) (total soluble protein extract, TSP) was carried out. Samples were resolved in gels both for Coomassie blue staining and Western blots. Transferred membranes were incubated with an anti-His monoclonal antibody (Clontech, Mountain View, CA, USA) diluted 1:2000 in PBST followed by incubation with an anti-mouse IgG horseradish peroxidase (HRP)-labeled conjugate (GE Healthcare, Marlborough, MA, USA) diluted 1:2000 in PBST. All membranes were developed using ECL (Enhance Chemiluminescence) reagent. Images were captured by ChemiDoc™ XRS Gel Imaging System (Bio-Rad, USA) and analyzed using the ImageLab™ Software (Bio-Rad, version 6).

Total protein concentration of the extract was determined by the Bradford method (Bio-Rad, USA) [13] and the quantification of the flagellin-NG34/CS17 expressed in *T. ni* larvae was developed by measurement of band densitometry in Western Blot images using a standard curve with a known quantified 6xHis-tagged protein (ASFV p54 protein).

2.3. Cells, virus and antigen

*Spodoptera frugiperda Sf*21 cell line (Invitrogen, USA) was cultured at 27 °C in TNM-FH medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAN Biotech GmbH, Germany) and gentamicin (50 μg/mL) (PAN Biotech GmbH, Germany).

Madin-Darby Canine Kidney (MDCK, ATCC CCL-34) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine.

A/chicken/Italy/5093/99 H7N1 HPAIV strain was kindly provided by Dr. Moreno (Brescia, Italy) and was propagated in 11-day-old embryonated specific pathogen free (SPF) chicken eggs. Allantoic fluid was collected after 48–72 h of inoculation, filtered (0.45 μM) and diluted ten-fold in PBS for titration in SPF eggs [14].

The H7 protein (A/turkey/Italy/4602/99 (H7N1)) used was purchased from Sino Biological, Beijing, China (cat no. 40169-V08H1).

2.4. Ethics statement, experimental design and pathological assessment

All chickens were handled according to procedures approved by the IRTA Ethics Committee for Animal Experimentation and the Animal Experimentation Commission from the Autonomous Community of Catalonia Government. Additionally, the study was in accordance with the Directive, UE 63/2010; the Spanish Legislation, RD 53/2013; the Catalan Law 5/1995 and Decree 214/1997.

Thirty-four SPF chickens were randomly divided in three different negative isolators with HEPA-filtered air under biosafety level 3 (BSL-3) containment conditions at IRTA-CReSA. Throughout the experiment, chickens were feed with food and water *ad libitum*. One isolator (group A = animals 1–12) was used as negative control and individuals were subcutaneously sham-vaccinated with PBS mixed 70:30 with Montanide™ 71 VG ISA. Chickens in the second isolator (group B = 13–24) were vaccinated at 10 days of age with flagellin-NG34/CS17 and, after 21 days of interval, a boost vaccination followed. In parallel, in the third isolator (group C = 25–34) chickens were immunized only once with flagellin-NG34/CS17 (without receiving boost vaccination). The animals from Group B and C were subcutaneously vaccinated with 0.250 mL/animal of soluble larvae extracts containing 15 μg/mL of flagellin-NG34/CS17 and mixed in a ratio of 70:30 with Montanide™ 71 VG ISA.

Table 1
Amino acid sequences from the HA-epitopes: NG-34 and CS-17, used in the vaccine approach.

HA-epitopes	Aa positions	Consensus virus subtype	GenBank Id
NSDNGTCYPGDFIDYEELREQLSSVSSFERFEIF (NG-34)	59–92	pH1N1	ACS36215
PQRERRRKKRGLFGAIA (CS-17)	337–357	H5N1	AAC32098.1

Aa positions referenced are in accordance with the reference cited from the GenBank database. Abbreviations: aa = amino acid; HA = hemagglutinin; Id = identification.

Fig. 1. Illustration of the sequence of interest and restriction sites inserted in the pCDNA3.1(+) vector.

Two weeks later, all chickens (group A-C) were intranasally inoculated with 100 μL diluted infectious allantoic fluid containing $10^{4.5}$ ELD_{50} H7N1 HPAIV. Two days pre-challenge onwards all the chickens were monitored daily for flu-like clinical signs. The experiment terminated at 10 days post-infection (dpi), when all the remaining chickens were euthanized. Oropharyngeal (OS) and cloacal swabs (CS) samples were collected at 1, 2, 3, 5, 7 and 9 dpi. Blood samples were collected previous to the challenge (33 post-vaccination day (PVD)) and at 10 dpi.

Daily monitoring for clinical signs was carried out according to World Organization for Animal Health (OIE) guidelines [15] and a semi-quantitative scoring: healthy (0), sick (1), severely sick (2), moribund or dead (3) was established. Chickens presenting one of the potential flu-clinical signs were classified as sick (1) chickens manifested two or more of the potential flu-clinical signs were classified as severely sick (2) and were euthanized by inoculating intravenously sodium pentobarbital.

2.5. Quantitative real time (RT-qPCR)

Viral RNA was extracted from OS and CS using the kit NucleoSpin RNA isolation kit (Macherey-Nagel GmbH&CoKG, Düren, Germany), and a fragment of the *M* gene amplified [16] in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA). The detection limit of the technique was 0.84 log_{10}GEC/mL viral RNA copies/sample.

2.6. H7-specific enzyme-linked immunosorbent assays (ELISAs), hemagglutination inhibition (HI) assay and serum neutralization test (SNT)

ELISA plates were coated with 2 μg/mL of H7 protein antigen. Plates were blocked and diluted 1:100 chicken sera were later incubated during 1 h at 37 °C followed with either diluted 1:50000 HRP-conjugated goat anti-chicken IgY (ab6877, Abcam, Cambridge, UK) or diluted 1:50000 HRP-conjugated goat anti-chicken IgM (ab112813, Abcam, UK). After further washing steps, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate solution was dispensed and the reaction was stopped with 1 N H_2SO_4. Plates were finally read at an optical density (OD) of 450 nm.

HI tests were developed according standard protocols [15]. For SNT, plates containing 5 * 10^4cells/well (MDCKs) were seeded and incubated overnight. Chicken inactivated sera samples were serially diluted up to 1: 2560 and incubated with the H7N1 virus (100 $TCID_{50}$/50 μL) during 2 h at 37 °C and in a 5% CO_2 atmosphere. Serum-virus mixtures were then added to PBS1X washed MDCK cells. Cytopathic effect (CPE) was monitored under microscope after 7 days.

In all assays (ELISA, HI, SNT), samples were evaluated in duplicates and a positive H7N1 and negative control anti-serums (GD-Animal Health Service, Deventer, Netherlands) were included in each plate.

2.7. Statistical analysis

R statistical software was used for developing all the statistical analysis (http://cran.r-project.org/). The test used for each technique as well as the significance found among the groups is described at the corresponding figure captions.

3. Results

3.1. Production of recombinant flagellin-NG34/CS17 in T. Ni larvae

Fifth instars *T. ni* larvae were inoculated with 27,500 pfu of the TB3.2 flagellin-NG34/CS17 baculovirus and total soluble protein extract was obtained after 72 h of infection and analyzed by SDS-PAGE and Western blot (Fig. 2A). Flagellin-NG34/CS17 recombinant protein was detected by Western blot as a major band of about 65 kDa. For immunization experiments the amount of flagellin-NG34/CS17 antigen in the soluble larvae extract was quantified by Western blot (Fig. 2B). Concentration was determined in 28.6 μg of flagellin-NG34/CS17 per mL of TSP larvae extract.

3.2. Immunization with flagellin-NG34/CS17 protected chickens against heterologous IV challenge, saved chickens from flu-like manifestations, limited oropharyngeal and cloacal H7N1 viral shedding and induced an increase in IgM and improved IgY response

More than 91% (11/12) of the chickens vaccinated twice with flagellin-NG34/CS17 survived against a challenge with H7N1 HPAIV until the end of the experiment. In contrary, only two animals (16.6%) survived from the unvaccinated group. Animals which received only one vaccination also succumbed to infection and, only two animals (22.2%) remained alive (Fig. 3A) at 10 dpi. The majority of the chickens in the unvaccinated group and chickens which received only one vaccination died between 5 and 8 dpi (Fig. 3A).

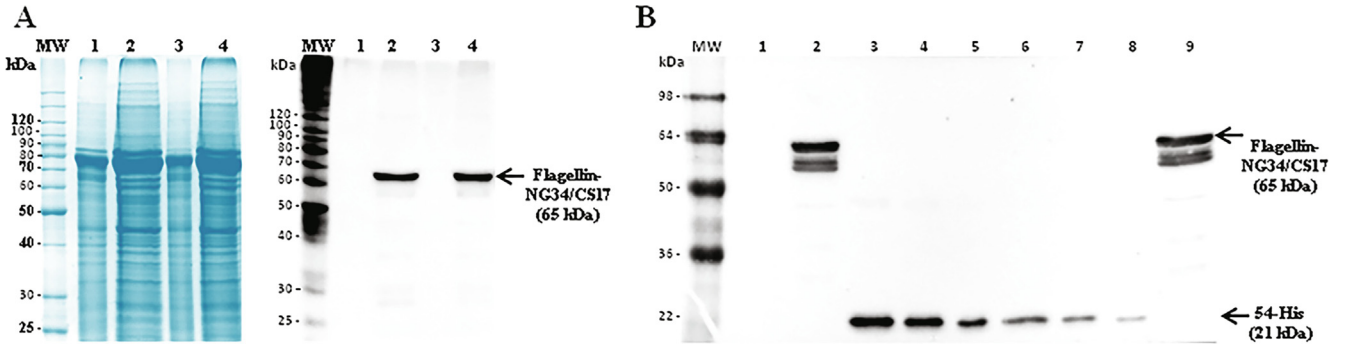
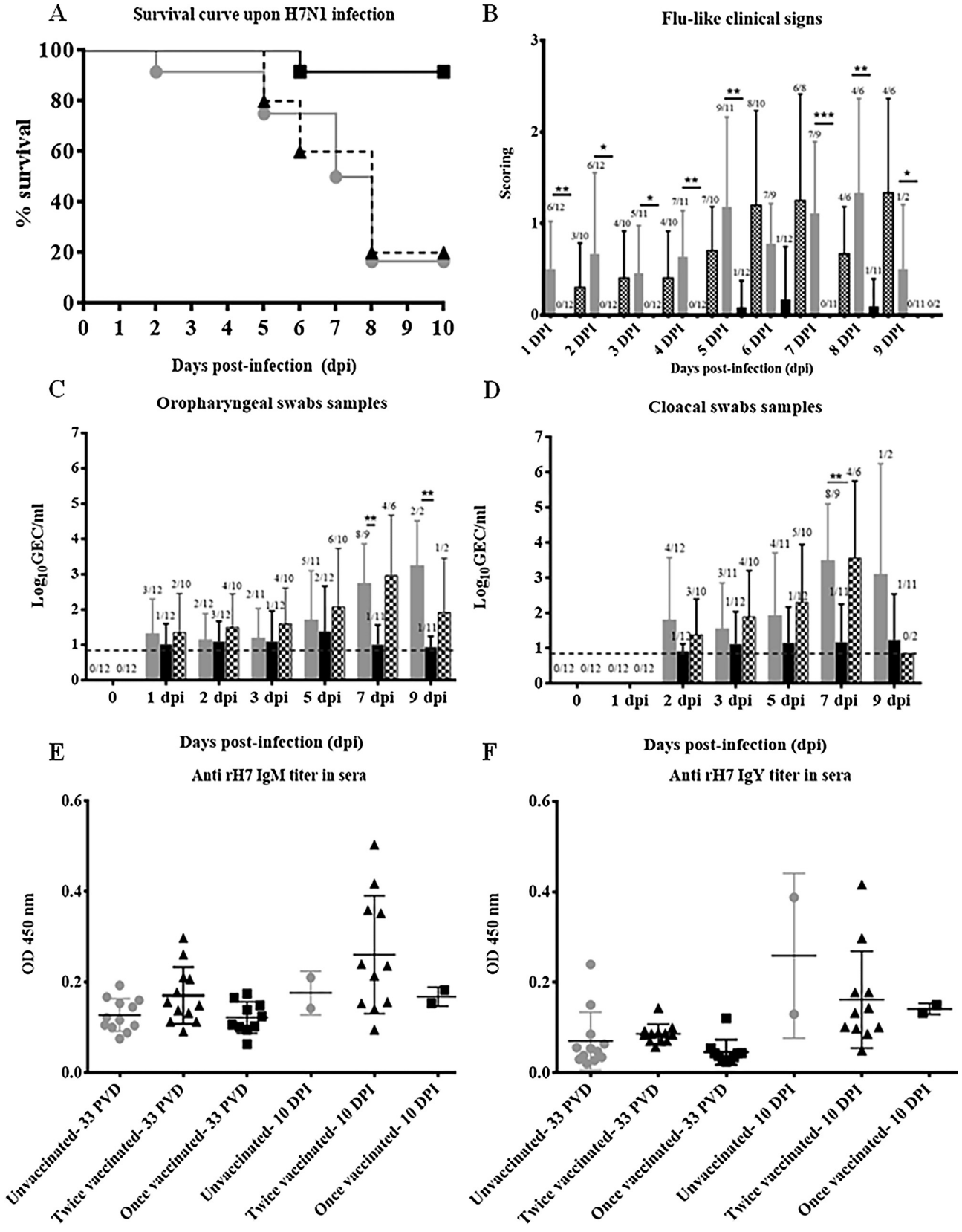


Fig. 2. Production of recombinant flagellin-NG34/CS17 protein in *T. ni* larvae (A) Coomassie blue staining (left) and anti-6xHis-tag Western Blot (right) of protein extracts (10 μL loaded/lane) obtained from empty rBac TB3.2-Ni (a recombinant TopBac baculovirus without a gene of interest) (lane 1: TP, lane 3: TSP) or rBac flagellin-NG34/CS17 (lane 2: TP, lane 4: TSP) infected *T. ni* larvae run in a 10% SDS-PAGE gel. Arrow indicates the band corresponding to flagellin-NG34/CS17 protein in the Western Blot image. MW: BenchMark™ Unstained Protein Ladder (Invitrogen, USA). (B) Quantification by anti-6xHis-tag Western Blot of the amount of flagellin-NG34/CS17 protein contained in the soluble larvae extract. Lanes: (1) TSP extract from empty rBac TB3.2-Ni infected *T. ni* larvae (5 μL loaded), (2 and 9) TSP extract from rBac TB3.2 flagellin-NG34/CS17 infected *T. ni* larvae (5 μL loaded), (3–8) p54-His standard curve (150, 100, 50, 40, 20, 10 ng/lane, respectively). Arrows indicate the bands corresponding to flagellin-NG34/CS17 and p54-His proteins. MW: See Blue Plus2 Prestained Ladder (Invitrogen, USA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

No clinical signs of disease were observed in majority of chickens twice flagellin-NG34/CS17 vaccinated (group B) (Fig. 3B). Only one chicken showed apathy and was euthanized at 6 dpi. One more chicken from that group also manifested signs of sickness at 8 dpi (ruffled feathers and mucus secretions) but recovered. In contrast, all animals from the unvaccinated group (group A) and from the



once flagellin-NG34/CS17 vaccinated group (group C) were found sick with signs of apathy right from the first day of challenge. Statistically significant differences were found in nearly all timepoints when comparing the unvaccinated (group A) with the twice flagellin-NG34/CS17 vaccinated group (group B). Apathy in chickens from both A and B groups appeared more severe as one animal died at 2 dpi and other 2 at 5 dpi regarding the unvaccinated (group A), and two chickens died at 5 and at 6 dpi concerning the once flagellin-NG34/CS17 vaccinated (group C). Signs of flu-like sickness were more apparent and severe that obliged to sacrifice most of the unvaccinated and once flagellin-NG34/CS17 vaccinated chickens at 7 and 8 dpi [15].

Significant differences were observed in the viral shedding both from oropharynx and cloaca of chickens twice vaccinated with flagellin-NG34/CS17 (Fig. 3C and 3D). While the chickens twice vaccinated with flagellin-NG34/CS17 had viral RNA detection values almost at the basal level, the unvaccinated group and the flagellin-NG34/CS17 once vaccinated group showed variations that peaked at 7 dpi (both orally and cloacally). Regarding OS, statistically significant differences in the mean values of $\log_{10}$ GEC/mL among twice vaccinated and unvaccinated groups were noticed at 7 dpi at 9 dpi ($P < 0.01$) (Fig. 3C). Similarly, cloacal viral shedding from unvaccinated and once vaccinated animals was in higher number than the twice vaccinated ones. At 7 dpi, significant differences ($P < 0.001$) in the mean values among unvaccinated and flagellin-NG34/CS17 twice vaccinated groups were detected (Fig. 3D). It should be noted that only two chickens remained alive from unvaccinated challenged and once flagellin-NG34/CS17 vaccinated groups at 9 dpi. Concerning the unvaccinated group, viral secretion in OS, as recorded by RT-qPCR, was observed in both chickens, while viral secretion from CS was detected only from one of the two animals.

Flagellin-NG34/CS17 twice vaccinated chicken (group B) displayed a higher mean of IgM antibodies against H7 in sera at 33 PVD and 10 dpi than the unvaccinated and once flagellin-NG34/CS17 vaccinated chickens (Fig. 3E). IgY antibody levels, on the other hand, were accelerated slightly in the flagellin-NG34/CS17 twice vaccinated chickens at 33 PVD though this tendency was not maintained at 10 dpi. In fact, higher mean of IgY antibodies at 10 dpi were observed in the unvaccinated chickens rather than in the once or twice flagellin-NG34/CS17 vaccinated (Fig. 3F). This tendency may attribute to the fact that only two unvaccinated chickens remained alive on 9 dpi and one of them showed higher amount of antibodies.

3.3. Flagellin-NG34/CS17 failed to induce HI and SNT titers against H7N1

HI and SNT titers were negative in the three groups. An HI titer of 1:80 was only observed in only one flagellin-NG34/CS17 twice vaccinated chicken that also showed flu-like clinical symptoms and recovered later.

4. Discussion

Immunization of poultry is vital as a prophylactic method when there is risk of IVs incursion and when the areas are endemic to such viruses. In the present study, two conserved HA-epitopes (NG-34 and CS-17) selected by *in silico* prediction were linked and used as vaccine candidates. The NG-34 peptide belongs to a highly conserved region of the HA, the E domain [17]. The CS-17 peptide, corresponds to the cleavage site of the HA from H5 HPAIVs. Previous immunization studies using polypeptide covering the cleavage site from IV subytpes A and B have elicited neutralizing antibody responses and conferred protection in mice [18,19]. Additionally, flagellin was fused to HA-epitopes (NG-34 and CS-17) in order to enhance their immunogenicity. Similar studies were also carried out previously with flagellin ligand in IV vaccines as effective adjuvants for poultry [20,21].

More than the 80% of chickens vaccinated twice survived the IVs challenge reducing significantly the virus load in oropharyngeal and cloacal mucosal tissues. This is in accordance with the standard set by OIE [15]. We are aware that a higher viral dose must be used to achieve a 90% of the mortality in the unvaccinated challenged animal group. However, it should be taken into account that one of the two survivors in the unvaccinated group showed deteriorated signs of sickness and most probably needed euthanization.

Protection against IVs is generally attributed to anti-HA antibodies, determined by ELISA, HI and SNT [22]. In this study, hemaglutination-inhibiting/neutralizing antibodies were either very low or below detection levels. Elevated IgM antibody levels in the flagellin-NG34/CS17 twice vaccinated group were recorded. We assume that simple ELISA test detect also lower affinity antibodies that are missed in HI or neutralization assays [23]. There is however, no consensus of the ELISA titers required in order to achieve protection [24]. Protection without the need of high HI and/or neutralizing titers against H7 subtype IVs has been described previously [25,26]. Reports indicate that candidate

Fig. 3. (A) Survival rates (%) of the different treatment groups are after challenge with 0.1 mL of $10^{4.5}ELD_{50}$ of a H7N1 HPAIV. Group A (animals 1–12; unvaccinated) is depicted by grey circles, Group B (animals 13–24; flagellin-NG34/CS17; vaccinated twice with flagellin-NG34/CS17) by black squares and Group C (animals 25–34; flagellin-NG34/CS17; vaccinated once with flagellin-NG34/CS17) by black triangles. The significance between the survival curves was determined by Kaplan-Meier survival analysis with log-rank (Mantel-Cox) test. Statistically significant difference concerning survival among Group A and Group B ($P < 0.001$) is depicted as ***: The significant difference ($P < 0.01$) among Group B and Group C is depicted as: **. (B) Mean scoring of the flu-like clinical signs of group A (unvaccinated animals 1–12; grey bars), group B (animals 13–24; vaccinated twice with flagellin-NG34/CS17; black bars) and group C (animals 25–34; flagellin-NG34/CS17; vaccinated once with flagellin-NG34/CS17; by black and white checker bars) plotted from 1 to 10 dpi. Dpi, days post-inoculation. Rates above the bars indicate the relation between positive chickens and the total of chickens examined. Error bars represent the mean ± SEM. One-way ANOVA followed by Dunnett's multiple test was employed (assigning as control group: group A, the unvaccinated). Statistically significant difference concerning OD values among groups (P value < 0.05) is marked as *: $P < 0.05$. (C) Mean of genomic equivalent copies (GEC) per mL obtained from oral swabs (D) and cloacal swabs; sampled at 0, 1, 2, 3, 5, 7 and 9 dpi from Group A (animals 1–12; unvaccinated; grey bars), Group B (animals 13–24; vaccinated twice with flagellin-NG34/CS17 vaccinated; black bars) and Group C (animals 25–34; flagellin-NG34/CS17; vaccinated once with flagellin-NG34/CS17; black and white checker bars) detected by RT-qPCR. Dpi, days post-inoculation. GEC, genomic equivalent copies. Rates above the bars indicate the relation between positive chickens and the total of chickens examined. Dashed line marks the detection limit of the technique: 0.84 $\log_{10}$GEC/mL. Error bars represent the mean ± SEM. One-way ANOVA followed by Dunnett's multiple test was employed (assigning as control group: group A, the unvaccinated). Statistically significant differences concerning shedding among groups (P value < 0.05) are depicted as *** $P < 0.001$, **:$P < 0.01$. (E) Individual and group mean of IgM HA-specific and (F) of IgY antibody levels detected in sera samples at 33 PVD and 10 DPI from Group A (animals 1–12; unvaccinated; grey circles), Group B (animals 13–24; vaccinated twice with flagellin-NG34/CS17; black squares) and Group C (animals 25–34; flagellin-NG34/CS17; vaccinated once with flagellin-NG34/CS17; by black and white checker bars) by ELISA test. OD, optical density. PVD, post-vaccination days and DPI, days post-inoculation. Error bars indicate the mean ± SEM. One-way ANOVA followed by Dunnett's multiple test was employed (assigning as control group: group A, the unvaccinated).

inactivated vaccines against potential pandemic viruses (H5/H7) have induced low immunogenicity, required two doses as well as adjuvant in their composition. Possible explanations of these low inhibiting/neutralizing titers are focused on the levels of glycosylation of the HA protein [27,28].

Previous reports also suggest that non-neutralizing antibodies may confer protection against IVs [22,29]. These antibodies either interact with the complement [30] or recruit NK cells/monocytes leading to kill IV-infected cells by antibody-dependent cellular cytotoxicity (ACDD) [31]. IgM antibodies, on the other hand, could neutralize IVs as efficiently as IgY antibodies in the presence of complement [32]. Experiments with passive serum transfer from vaccinated to naïve chicken may unravel the mechanism of antibody induced protection. Possible involvement of cellular immune response in flagellin-NG34/CS17-associated protection in chicken against IVs challenge is under investigation. In our previous work we showed that NG-34 peptide included in the vaccine formulation induces strong T cell response [9].

In conclusion, flagellin-NG34/CS17 may serve as a potential vaccine to protect against heterologous IV infections in chicken. It further limits IVs transmission by blocking virus shedding from vaccinated animals. Further experiments are needed to understand completely the immune mechanisms associated to the protection conferred. Whether this vaccine would protect animals from other HPAIV infections is currently under investigation.

5. Author's contributions

MSO, SMP obtained the recombinant Baculovirus and the infected larvae extracts, VV predicted the HA-epitope sequence, MSO, AD prepared vaccine formulation; MSO and AD contributed to the design of the animal experiments, MSO, SLS, LC contributed in the sample analysis, MSO, DS and IC were involved in animal handling; MSO, SMP and AD analyzed the data, MSO, SMP, JME and AD wrote the manuscript. Authors' analytically read and reviewed this manuscript, being in accordance with its final version.

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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References

[1] Malik Peiris JS. Avian influenza viruses in humans. Rev Sci Tech 2009;28:161–73.
[2] Jadhao SJ, Suarez DL. New approach to delist highly pathogenic avian influenza viruses from BSL3+ Select Agents to BSL2 non-select status for diagnostics and vaccines. Avian Dis 2010;54:302–6.
[3] Cox MM. Cell-based protein vaccines for influenza. Curr Opin Mol Ther 2005;7:24–9.
[4] Gerdil C. The annual production cycle for influenza vaccine. Vaccine 2003;21:1776–9.
[5] Liu F, Wu X, Li L, Ge S, Liu Z, Wang Z. Virus-like particles: promising platforms with characteristics of DIVA for veterinary vaccine design. Comp Immunol Microbiol Infect Dis 2013;36:343–52.
[6] Liu Y, DeCarolis N, Beek N. Protein production with recombinant baculoviruses in lepidopteran larvae. Methods Mol Biol 2007;388:267–80.
[7] Gómez-Sebastián S, López-Vidal J, Escribano JM. Significant productivity improvement of the baculovirus expression vector system by engineering a novel expression cassette. PLoS One 2014;9:e96562.
[8] Waffarn EE, Baumgarth N. Protective B cell responses to flu-no fluke!. J Immunol 2011;186:3823–9.
[9] Vergara-Alert J, Argilaguet JM, Busquets N, Ballester M, Martín-Valls GE, Rivas R, et al. Conserved synthetic peptides from the hemagglutinin of influenza viruses induce broad humoral and T-cell responses in a pig model. PLoS One 2012;7:e40524.
[10] Sisteré-Oró M, Vergara-Alert J, Stratmann T, López-Serrano S, Pina-Pedrero S, Córdoba L, et al. Conserved HA-peptide NG34 formulated in pCMV-CTLA4-Ig reduces viral shedding in pigs after a heterosubtypic influenza virus SwH3N2 challenge. PLoS One 2019;14:e0212431.
[11] Huleatt JW, Jacobs AR, Tang J, Desai P, Kopp EB, Huang Y, et al. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine 2007;25:763–75.
[12] Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 2008;26:201–14.
[13] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[14] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoint. Am J Hyg 1938;27:493–7.
[15] World Organization for Animal Health (OIE). Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 3.3.4. Avian influenza (infection with avian influenza viruses). 2012. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf (accessed February 12, 2019).
[16] Busquets N, Segalés J, Córdoba L, Mussá T, Crisci E, Martín-Valls GE, et al. Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. Vet Res 2010;41:1–14.
[17] Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, et al. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol 2000;74:8502–12.
[18] Bianchi E, Liang X, Ingallinella P, Finotto M, Chastain MA, Fan J, et al. Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. J Virol 2005;79:7380–8.
[19] Horváth A, Tóth GK, Gogolák P, Nagy Z, Kurucz I, Pecht I, et al. A hemagglutinin-based multipeptide construct elicits enhanced protective immune response in mice against influenza a virus infection. Immunol Lett 1998;60:127–36.
[20] Song L, Xiong D, Song H, Wu L, Zhang M, Kang X, et al. Corrigendum: mucosal and systemic immune responses to influenza H7N9 antigen HA1-2 co-delivered intranasally with flagellin or polyethyleneimine in mice and chickens. Front Immunol 2018;9:1846.
[21] Astill J, Alkie T, Yitbarek A, Taha-Abdelaziz K, Shojadoost B, Petrik JJ, et al. A comparison of toll-like receptor 5 and 21 ligands as adjuvants for a formaldehyde inactivated H9N2 avian influenza virus vaccine in chickens. Viral Immunol 2018;31:605–12.
[22] Kamal RP, Blanchfield K, Belser JA, Music N, Tzeng WP, Holiday C, et al. Inactivated H7 influenza virus vaccines protect mice despite inducing only low levels of neutralizing antibodies. J Virol 2017;91:e01202–e1217.
[23] Wilson JR, Guo Z, Tzeng WP, Garten RJ, Xiyan X, Blanchard EG, et al. Diverse antigenic site targeting of influenza hemagglutinin in the murine antibody recall response to A(H1N1)pdm09 virus. Virology 2015;485:252–62.
[24] Reber A, Katz J. Immunological assessment of influenza vaccines and immune correlates of protection. Expert Rev Vaccines 2013;12:519–36.
[25] Cox RJ, Major D, Hauge S, Madhun AS, Brokstad KA, Kuhne M, et al. A cell-based H7N1 split influenza virion vaccine confers protection in mouse and ferret challenge models. Influenza Other Respi Viruses 2009;3:107–17.
[26] Krammer F, Albrecht RA, Tan GS, Margine I, Hai R, Schmolke M, et al. Divergent H7 immunogens offer protection from H7N9 virus challenge. J Virol 2014;88:3976–85.

[27] Ushirogawa H, Naito T, Tokunaga H, Tanaka T, Nakano T, Terada K, et al. Re-emergence of H3N2 strains carrying potential neutralizing mutations at the N-linked glycosylation site at the hemagglutinin head, post the 2009 H1N1 pandemic. BMC Infect Dis 2016;16:380.
[28] Abe Y, Takashita E, Sugawara K, Matsuzaki Y, Muraki Y, Hongo S. Effect of the addition of oligosaccharides on the biological activities and antigenicity of influenza A/H3N2 virus hemagglutinin. J Virol 2004;78:9605–11.
[29] Manicassamy B, Medina RA, Hai R, Tsibane T, Stertz S, Nistal-Villán E, et al. Protection of mice against lethal challenge with 2009 H1N1 influenza a virus by 1918-like and classical swine H1N1 based vaccines. PLoS Pathog 2010;6:e1000745.
[30] Co MD, Cruz J, Takeda A, Ennis FA, Terajima M. Comparison of complement dependent lytic, hemagglutination inhibition and microneutralization antibody responses in influenza vaccinated individuals. Hum Vaccin Immunother 2012;8:1218–22.
[31] Henry Dunand CJ, Leon PE, Huang M, Choi A, Chromikova V, Ho IY, et al. Both neutralizing and non-neutralizing human H7N9 influenza vaccine-induced monoclonal antibodies confer protection. Cell Host Microbe 2016;19:800–13.
[32] Skountzou I, Satyabhama L, Stavropoulou A, Ashraf Z, Esser ES, Vassilieva E, et al. Influenza virus-specific neutralizing IgM antibodies persist for a lifetime. Clin Vaccine Immunol 2014;21:1481–9.