Vaccine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Can genotype mismatch really affect the level of protection conferred by Newcastle disease vaccines against heterologous virulent strains?

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ARTICLE INFO

Article history:
Received 8 March 2018
Received in revised form 10 May 2018
Accepted 17 May 2018
Available online xxxx

Keywords:
Newcastle disease
Vaccine
Genotype mismatch
Viral shedding
Protection

ABSTRACT

Newcastle disease (ND), caused by virulent class II avian paramyxovirus 1 (Newcastle disease virus, NDV), occurs sporadically in poultry despite their having been immunized with commercial vaccines. These vaccines were all derived from NDV strains isolated around 70 years ago. Since then, class II NDV strains have evolved into 18 genotypes. Whether the vaccination failure results from genotype mismatches between the currently used vaccine strains and field-circulating velogenic strains or from an impaired immune response in the vaccination remains unclear. To test the first hypothesis, we performed a heterologous genotype II vaccine/genotype XI challenge in one-day old specific pathogen free (SPF) chicks and reproduced viral shedding. We then produced two attenuated strains of genotype II and XI by reverse genetics and used them to immunize two-week old SPF chickens that were subsequently challenged with velogenic strains of genotypes II, VII and XI. We found that both vaccines could induce antibodies with hemagglutination inhibition titers higher than 6.5 $\log_2$. Vaccination also completely prevented disease, viral shedding in swabs, and blocked viral replication in tissues from different genotypes in contrast to unvaccinated chickens that died shortly after challenge. Taken together, our results support the hypothesis that, in immunocompetent poultry, genotype mismatch is not the main reason for vaccination failure.

1. Introduction

Newcastle disease (ND) is one the most fatal diseases of birds, and thus a major threat for the poultry industry owing to its sanitary and economic impacts around the world [1,2]. The causative agent is a virulent Newcastle disease virus (NDV), an enveloped virus that belongs to the *Avulavirus* genus, *Paramyxoviridae* family [3]. The genome of NDV is a ca. 15 kilobase (Kb) negative-sense single-strand RNA molecule structured as 3′-Leader-NP-P-M-F-H N-L-Trailer-5′ [4]. This genome encodes six structural proteins, nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), large protein (L) and two non-structural proteins, V and W from P gene editing [5,6]. The host of NDV is avian, although virus has occasionally been isolated from other animals, such as pig and mink [7–9].

ND vaccines have been used to control the disease since the 1950s, but ND outbreaks still occur in poultry under the pressure of vaccination, leading to the conclusion that current vaccines may not be efficient enough to prevent ND [1,10]. However, the basis for reduced protection is unclear. Some authors suggest that mutations in the viral antigenic F and HN proteins may help NDV to escape vaccination. Indeed, different teams have shown that ND vaccine is more potent to prevent disease in chickens challenged with a homologous genotype than with a heterologous genotype [11–14]. Other authors suspect that immunocompromised chickens in the field can become vaccine non-responders in flocks that are still susceptible to the infection and can thus develop disease [1,15]. So far, NDV, is recognized as only one serotype [16]. In addition, it has already been established that the

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https://doi.org/10.1016/j.vaccine.2018.05.074
0264-410X/

hemagglutination inhibition (HI) antibody titer correctly relates to protection and prevention of viral shedding, irrespective of the genotypes of the vaccine and challenge strains [15,17,18].

To check whether genotype mismatches could be responsible for the failure of ND vaccination in immunocompetent chickens, we first conducted a preliminary protection assay to evaluate if two currently used vaccines are able to provide sufficient protection for chicks against a NDV strain isolated from poultry in Madagascar in 2008 and classified as genotype XI [19]. Second, we generated a new attenuated vaccine strain by reverse genetics to match the genotype XI virulent virus circulating in Madagascar, even in vaccinated flocks. This recombinant vaccine was based on the vaccine strain, LaSota, which is used worldwide, and in which the F and HN genes were previously replaced by those of NDV/chicken/Madagascar/MG-725/2008 strain (MG-725). In order to modify the virulence, the F protein cleavage site of MG-725 was mutated towards a lentogenic motif (RRRRRF to GRQGRL). This candidate vaccine was tested and compared to the parental LaSota strain in a vaccine/challenge experiment in chickens involving different velogenic strains of genotype II, VII and XI.

2. Material and methods

2.1. Ethics statement

All animal experiments were conducted in agreement with the European Directive 2010/63/UE. A preliminary protection assay was carried out at ANSES-Ploufragan, France after approval by the ethical committee C2EA-16 : "*Comité d'éthique ComEth* ANSES/ENVA/UPEC". The second protection assay was performed by the "*Centre de Recerca en Sanitat Animal* – IRTA-CReSA", Barcelona, Spain according to the Standard Operating Procedures (SOPs) of IRTA-CReSA and after approval by the ethical committee of Animal Experimentation (CEEA) of the institution (IRTA). Certificates of authorization are available from the authors upon request.

2.2. Cells and viruses

Baby hamster kidney cells (BHK-21) were cultured in Eagle's minimum essential medium (Gibco) with 10% fetal bovine serum (PAN-Biotech) and 5% CO_2 at 37 °C. Chemically competent *E. coli* 10-beta cells were purchased from New England Biolabs. The virulent MD/TA/1322 strain, belonging to genotype XI, was isolated in Madagascar [19]. The virulent genotype IV strain Herts/33 was provided by ANSES, Unité VIPAC, Ploufragan, France. The rMG-725 strain was produced by reverse genetics based on the full genome of the MG-725 strain [20]. The virulent NDV/EG/CK/104/12 strain belonging to genotype VII was kindly provided by Patti J. Miller from Southeast Poultry Research Laboratory, USA. The GB Texas strain, kindly provided by Bénédicte Lambrecht from CODA-CERVA, Belgium, is a virulent genotype II strain. The rLaSota strain and rLaSota/M-Fmu-HN were rescued in this study. All these viruses were grown in 10-day old specific pathogen free (SPF) chicken embryos (Couvoir de Cerveloup, France). After three days of infection or egg death, allantoic liquid was harvested, filtered through 0.22 μm filters, titrated and stored at −80 °C.

2.3. Preliminary protection assay: Evaluation of cross-protection between commercial genotype II vaccines and virulent genotype IV and XI strains

A total of 50 one-day-old SPF chicks were immunized with a combination of a commercial inactivated ND LaSota (strain clone 30) vaccine (genotype II, 0.5 ml per one-day old chick, via the intramuscular route) and living ND Hitchner B1 vaccine (genotype II, 1 dose per 2-day old chick, via the intranasal and eye route). The two vaccines were combined to provide the best protection against the virus challenge [21]. Three weeks after vaccination, the chickens were randomly split into two groups (25 chickens per group). Groups 1 and 2 were challenged with the virulent NDV Herts/33 strain (genotype IV) and Madagascar MD/TA/1322 strain (genotype XI), respectively. All the chickens received 10^5 50% egg infective doses (EID_{50}) via the intramuscular route. After challenge, the chickens were observed daily for clinical signs. In addition, oral and cloacal swabs were collected in 1.5 ml of Eagle's minimal essential medium (EMEM) at 3, 5, 7, 10 and 14 days after challenge, frozen at −80 °C. Five swabs were pooled and extraction was performed with the MagAttract Virus Mini M48 kit (Qiagen). RNA was eluted in 100 μL of elution buffer and qRT-PCR, based on the M gene, was performed according to Wise et al. [22]. For some positive pools by M-gene qRT-PCR, the sequence of the virus in swabs was further determined as follows. Viral RNA was reverse transcribed using hexanucleotides as primers. The resulting cDNA was subjected to a partial F-based PCR with primers NDV-158F (ACACCTCATCCCAGACAG) and NDV-527F (TCTTCCCAACTGCCACTG), generating a fragment of 358 nucleotides including the cleavage site. Sequences of purified PCR products were determined using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and the same primers as those used in PCR, on an automatic DNA sequencer ABI3130 (Applied Biosystems). The resulting sequences were compared to the MD/TA/1322 challenge virus sequence.

2.4. Second protection assay using recombinant vaccines

2.4.1. Generation of a new antigenic/genotype matching vaccine

Based on the results we obtained in cross protection in previous *in vivo* assays, we decided to generate an antigenic/genotype match vaccine against the virulent genotype XI strains. This recombinant vaccine is constituted of the LaSota vaccine strain backbone and the F and HN genes of the genotype XI MG-725 strain. For cloning, the Fmu gene was first amplified from the plasmid containing the complete genome of the MG-725 strain with the F protein cleavage site mutated to that from the LaSota strain (pMG-725/Fmu. in-house) using M-F-change-F (5′-TCCCCGCGGGCAAGATGGGCTC TAAATCTTCTAC-‘3) and M-F-change-R (5′-ACCGGCCGGCCTCATCT GTGTTCATATTCTTGTGGTGGCTC-‘3) primers. Next, the F gene of the pLaSota plasmid was replaced by the Fmu gene using SacII and FseI restriction enzymes to obtain pLaSota/M-Fmu plasmid. The HN gene of the MG-725 strain was amplified from pMG-725/Fmu plasmid using M-HN-change-F (5′-ACCGACAACAGTCCTCAAT CATGGACCATGTAGTTAGCAG-‘3) and M-HN-change-R (5′-CCTTAAT TAATCAAGTCCTGCCATCCTTGAGAATCTCCACT-‘3) primers. The non-coding region between F and HN genes of the LaSota strain was generated by M-HN-change-L-F (5′-GCACATCTGCTCTCAT TACCT-‘3) and M-HN-change-L-R (5′-GATTGAGGACTGTTGTCGGT-‘3) primers. The two fragments were then assembled by overlap PCR and inserted in the place of the corresponding region in pLaSota/M-Fmu by FseI and PacI enzymes to finally obtain the pLaSota/M-Fmu-HN plasmid. Fragments bearing NP, P and L genes of the LaSota strain were generated from the wild LaSota strain by RT-PCR using LaSo-NP-F (5′- CCGCTCGAGATGTCTTCCGTATTT GATGAGTA-‘3) and LaSo-NP-R(5′- ATTTGCGGCCGCTCAATACCC CCAGTCG-‘3), LaSo-P-F (5′- CCGCTCGAGATGGCCACCTTTACAGAT GC-‘3) and LaSo-P-R(5′- ATTTGCGGCCGCTTAGCCATTTAGAGCAAG-‘3), and LaSo-L-F (5′- GGACTAGTATGGCGAGCTCCGGTCCTG-‘3) and LaSo-L-R (5′- ATTTGCGGCCGCTTAAGAGTCACAGTTACTG-‘3) primers, respectively. These fragments were cloned into pCI-neo plasmid as previously described [20]. All plasmids were purified using the EndoFree Plasmid Maxi kit (QIAGEN), aliquoted, stored at −20 °C and sequenced. The recombinant rLaSota/M-Fmu-HN

Please cite this article in press as: Liu H et al. Can genotype mismatch really affect the level of protection conferred by Newcastle disease vaccines against heterologous virulent strains? Vaccine (2018), https://doi.org/10.1016/j.vaccine.2018.05.074