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Short communication

EVOLUTION OF AVIAN INFECTIOUS BRONCHITIS VACCINE VIRUSES REISOLATED FROM VACCINATED BROILERS

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Summary

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Genetic and phenotypic diversity among infectious bronchitis virus (IBVs) is a key element of the epidemiology of avian infectious bronchitis. Virtually, all studies have focused on the evolution of wild viruses. Live-attenuated vaccine strains deserve a special attention. Two strains of IBV, 2T/17 and 16T/17, detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in samples from H120 vaccinated chickens showing respiratory signs, were genetically analysed targeting the *S1* gene. The total RNA was extracted by the classical Trizol method. The whole *S1* gene was amplified in an end-point RT-PCR using a specific primer pair, before being sequenced. The obtained sequences were compared to those of vaccine strains and reference viruses and finally subjected to a phylogenetic analysis. The two isolates turned out to be vaccine strains as evidenced by the monophyletic cluster they formed with the H120 vaccine strain (100% bootstrap). Also, they shared with this vaccine strain 99.60% and 99.47% of nucleotides (nt) for strain 2T/17 and 16T/17 respectively. The sequence alignment revealed, for each of the two strains, one non-synonymous nt substitution outside the hypervariable regions: C56T and T39G leading to two amino acid (aa) substitutions: Alv19Val and Cys13Trp, respectively for the strain 2T/17 and 16T/17. The second hypervariable region of strain 2T/17 displayed 2 nt substitutions at positions 345 and 347, resulting in an aa substitution Lys116Thr. As for 16T/17 strain, it harboured two non-synonymous nt substitutions (T353G and T380G), leading to two aa substitutions: Val118Gly and Met127Arg. The chromatogram analysis revealed minor and major peaks at all positions where nt changes were observed. Nucleotides associated with minor peaks matched with those observed in the sequence of the H120 vaccine strain at the same positions, indicating subpopulation selection. Only the substitution T353G results in a mutation as evidenced by the absence of minor peak at this position. Viral subpopulations contained in IBV live-attenuated vaccines are susceptible to be in vivo selected and emerge as persistent pathotype with reversion to virulence. This draws attention to the need of monitoring the evolution of IBV vaccine strains.

Key words: IBV, mutations, selection, vaccines, viral subpopulations

The infectious bronchitis virus (IBV), against which we vaccinate since the early 1950s, continues to cause heavy economic losses to the poultry industry worldwide. This grim observation is largely related to the genetic and phenotypic diversity of the

virus. The situation is aggravated by the lack of cross-protection between commercial vaccines and some of the newer emerging variants, which calls into question the efficacy of the vaccination (Jackwood *et al.*, 2012; Wang *et al.*, 2012; Toro *et al.*, 2012). Live-attenuated IBV vaccines have been used worldwide since the 1950s. The existing vaccines are out of phase with the genetic diversity of the wild viruses, which often leads to outbreaks in correctly vaccinated birds.

Using the virus neutralisation test, antigenic differences between isolates originating from Massachusetts and those obtained in the state of Connecticut in the USA detected for the first time by Jungherr *et al.* (1956) Currently, genetic typing by sequencing of the spike gene has enabled identification of several serotypes worldwide.

The IBV genome consists of a positive single-stranded and non-segmented RNA of 27.6 kb in length. Two-thirds of this large viral genome encodes nonstructural proteins (NSP). The rest of the genome encodes four structural proteins including the spike (S), envelope (E), membrane (M), and nucleocapsid (N). The spike is the largest protein encoded in the genome and is post-translationally cleaved into two non-covalently linked subunits termed S1 and S2. The spike glycoprotein is the most protruding from the surface of the virion and is therefore the most exposed to the host immune system. While S2 subunit function is a fusion between viral and cellular membranes (Belouzard *et al.*, 2012), the S1 subunit is responsible for the attachment to the host and contains epitopes for virus-neutralising antibodies (Lai & Cavanagh, 1997; Casais *et al.*, 2003). It is therefore understood that mutations in the *S1* gene result in the emergence of new genotypes and serotypes that can partially escape neutralisation by antibodies produced in response to active immunisation using existing vaccine strains.

Coronaviruses have the largest RNA genome (Lai & Holmes, 2001). They are also the only RNA viruses to encode an exoribonuclease activity with a proofreading function (Snijder *et al.*, 2003), allowing for correcting transcription errors and limiting the emergence of large number of genotypes. Therefore, coronaviruses in mammals occur as only one or a few different serotypes. This contrasts with the circulation of countless different serotypes of avian coronaviruses, making IBV unique among all other coronaviruses (Jackwood, 2012). This may be linked to low fidelity of the IBV exoribonuclease or may be the virus has unidentified mechanisms of genetic diversification. Anyway, the proofreading function of the IBVs seems to be adjusted in such a way as to guarantee a subtly tuned balance between genome stability and the diversity required for virus adaptation and survival (Smith & Denison, 2012).

The evolution of IBDVs involves point mutations, insertions, deletions and also homologous RNA recombination resulting from their unique random template switching during RNA replication (Cavanagh *et al.*, 1992). These same events are responsible for the viral attenuation process (Liu *et al.*, 2007).

This study aims at characterising two vaccine strains reisolated from vaccinated broilers experiencing respiratory disease and variable mortality rates. The birds were sampled during the acute outbreaks that occurred in the Northeast of Algeria in 2017. All of the twenty sampled flocks were positive to avian influenza virus (Barberis *et al.*, 2020) and two of them were also positive for infectious bronchitis

virus. All the studied flocks were vaccinated with the IBV H120 strain, a chicken embryo origin (CEO) vaccine.

Total RNA extraction from organ pools (trachea, kidneys, liver, and intestines) was performed by a Trizol method (Invitrogen, Carlsbad, CA, USA) conforming to the instructions of the manufacturer. To amplify and detect a 143-bp fragment of the IBV 5′-UTR gene, a TaqMan real-time RT-PCR was carried out using AgPath-ID™ One-Step RT-PCR Kit Mix (Applied Biosystems) and a specific probe and primers previously described by Callison *et al.* (2006). The gene amplification reaction conditions were as follows: 1 cycle of 45 °C for 10 min (RT reaction), 95 °C for 10 min (initial PCR activation), and 40 three-step cycles of 95 °C for 15 s and 60 °C for 1 min (annealing, extension step, and fluorescence data collection).

The extracted viral RNA from the two IBV positive samples was subsequently submitted to a conventional one-step RT-PCR in order to amplify the whole *S1* gene, using EasyScript® One-Step RT-PCR kit (TransGen Biotech). The forward S1oligo 5' (TGAAACTGA ACAAAAG ACA) and the reverse S1oligo 3' (CAT AACTAACATAAGGGCAA) primers were used to specifically amplify the target glycoprotein gene. The RT-PCR was performed in T100 Thermal Cycler (Bio-Rad), where viral RNA was retrotranscribed at 45 °C for 30 min and stopped at 94 °C for 5 min followed by 35 three-step cycles of 94 °C for 30 s, 49 °C for 30 s and 72 °C for 3 min. The cycling program ended with a final extension at 72 °C for 10 min. The amplified 1.7 kb product was used as a template for subsequent sequencing reactions.

Before sequencing, PCR amplicons were subjected to an enzymatic cleanup, using shrimp alkaline phosphatase and exonuclease I (ExoSAP-IT®, Affymetrix) to remove excess primers, nucleotides, and enzymes. The purified PCR products were then sequenced, using the BigDye Terminator version 3.1 Cycle Sequencing Kit in an ABI Prism 3100 sequencer (Applied Biosystems) according to the manufacturer's protocol. The down and upstream primers used in the sequencing reaction were the same used to amplify the target gene. Consensus DNA sequences were generated using DNA Baser v4.12.0. Nucleotide and amino acid sequence alignment was done by ClustalW algorithm in BioEdit Sequence Alignment Editor v7.2.5. Phylogenetic analysis was performed in MEGA v6.0.5 and the tree, constructed using a neighbor-joining method, was a consensus of 1000 bootstrap replicates.

In this study, molecular comparison between the H120 vaccine strain and the viruses isolated from H120 vaccinated chickens, namely 2T/17 and 16T/17, focused on the S1 subunit of the spike glycoprotein which is considered as the major site for genetic changes during the viral adaptation to the host system (Liu *et al.*, 2007). The results of the sequencing analysis showed that the two studied IBVs were two vaccine strains reisolated from broilers vaccinated with the H120 strain (or from contact-exposed birds) as evidenced by the high percentage of nucleotides they shared with the H120 vaccine strain (99.60% and 99.47% for 2T/17 and 16T/17 respectively) and by their position in the phylogenetic tree, where they were monophyletic with the H120 vaccine strain in 100% of the replicates generated by the bootstrap (Fig. 1).

In the second hypervariable region (HVR) of the *S1* gene, the 16T/17 strain, displayed one synonymous mutation

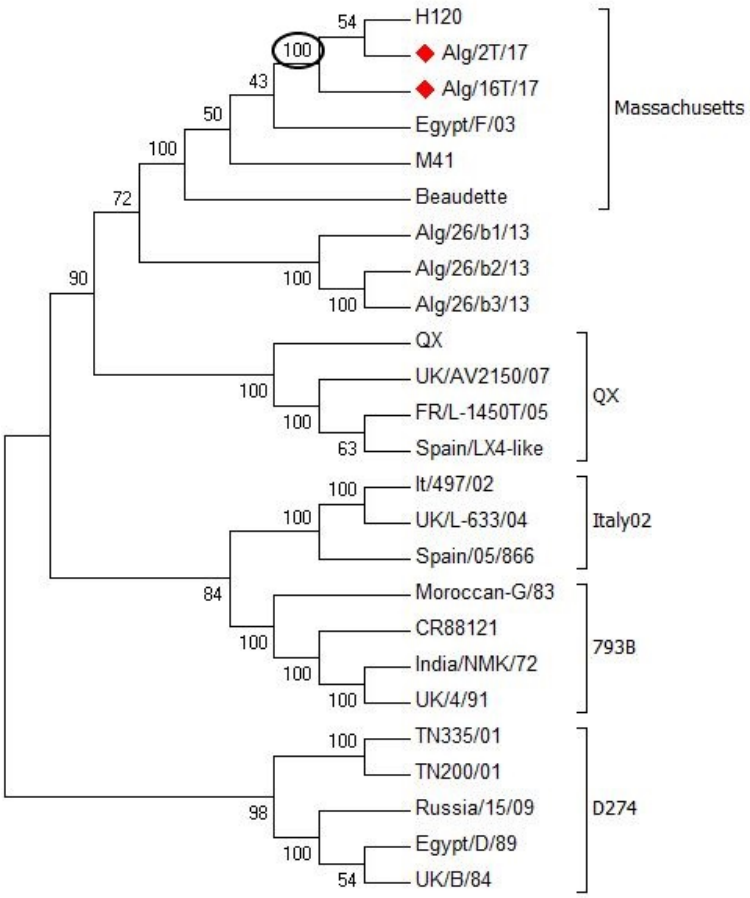

Fig. 1. Phylogenetic consensus tree based on nucleotide sequences of the S1 glycoprotein gene, showing the relationship between the studied isolates (diamond) and the reference IBV strains. Numbers at the nodes indicate the bootstrap confidence values (1000 replicates).

(C345T) and two non-synonymous ones (T353G and T380G), leading to two aa substitutions: Val118Gly and Met127Arg. As for the 2T/2017 strain, sequence alignment revealed two non-synonymous mutations (C345T and A347C) resulting in one aa substitution (Lys116Thr). Outside the HVR, each of the two strains harbored one non-synonymous mutation leading to one aa substitution in the S1 glycoprotein (Cys13Trp and Ala19Val for the 16T/17 and 2T/17 strain respectively). Among these nucleotide changes, only that at position 353 in the S1 gene sequence of the 16T/17 strain seemed to be an *in vivo* mutation since the chromatogram of this reisolated vaccine virus showed a single peak at this position (T353G). At the rest of the positions, chromatograms of the two studied strains displayed major and minor peaks, indicating *in vivo* selection of virus subpopulations. Fig. 2 demonstrates that nucleotides corresponding at minor peaks in all chro-

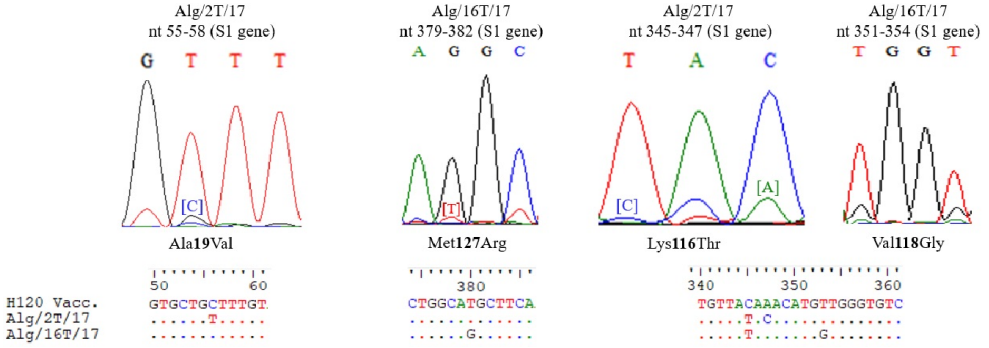

Fig. 2. Chromatograms of the two reisolated strains (S1 subunit of the spike glycoprotein gene). The last chromatogram on the right presents no minor peak at position 353, indicating that the nt substitution T353G was the result of a mutation. The rest of chromatograms present minor and major peaks. The nucleotides corresponding to the minor peaks (in square brackets) are exactly the same found in the genetic sequence of the vaccine strain H120 at the same positions, suggesting that predominant and minor genotypes were reversed in the chicken host system.

matograms were exactly the same found in the genetic sequence of the vaccine strain H120 at the same positions, meaning that predominant and minor genotypes were reversed in the chicken host system.

Several studies have reported the existence of coronaviral quasispecies (Nix *et al.*, 2000; Jackwood *et al.*, 2003; van Santen & Toro, 2008; Gallardo *et al.*, 2010), which is crucial for evolution, adaptation and persistence of the virus. Our results corroborate the experimental findings of McKinley *et al.* (2008) who demonstrated that genetic changes detected in reisolated vaccine viruses result from both point mutations and selection of intra-vaccine viral subpopulations. This phenomenon can be considered as a means of rapid adaptation of avian coronavirus vaccines not only to *in vivo* conditions but also to tissue microenvironments. Viral populations differing from the predominant vaccine population of attenuated vaccine strains have been shown to emerge after a single passage in chickens (van Santen & Toro, 2008). Gallardo *et al.* (2010) demonstrated that IBV vaccine strain undergoes rapid intraspatial variation during active immunisation under tissue selection pressure, leading to a selection of the most fit viral population among the various viral quasispecies present in the IBV vaccine, which allows the latter to survive in different tissue environments.

To induce broader protection, multiple IBV serotypes are combined and used for vaccination (Cook *et al.*, 1999). Viral subpopulations that emerge during the attenuation process in embryonated eggs would also give vaccines a greater ability to generate a broader immune response (Nix *et al.*, 2000) and higher mucosal and systemic humoral immunity (Ndegwa *et al.*, 2014), although this immune response is quite heterogeneous. If subpopulation-rich vaccines are still advantageous in terms of protection, they present the risk of reversion to virulence in the case of a positive selection of the more fit genotype/phenotype (Zhang *et al.*, 2018). Indeed, live attenuated vaccines producing significantly higher vaccine virus heterogeneity in vaccinated chickens are more likely to be selected and persist in the vaccinated host, offering genetic material for recombination or mutations with the

potential to result in increased virulence (Ndegwa *et al.*, 2014). Moreover, vaccines containing higher proportions of subpopulations are able to produce higher viral loads in the upper respiratory tract and hence severe vaccine reactions (Ndegwa *et al.*, 2012).

In conclusion, genetic variations in IBV vaccine strains, as well as in wild viruses, have to be monitored. This is crucial to appropriately control the disease and also to anticipate the reversion to virulence or the emergence of pathogenic strains resulting from recombination between wild viruses and selected IBV vaccine subpopulations.

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