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Viral interference between H9N2-low pathogenic avian influenza virus and avian infectious bronchitis virus vaccine strain H120 *in vivo*

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ABSTRACT

The interaction between a low pathogenic avian influenza virus (A/CK/TUN/145/2012), a H9N2 Tunisian isolate, and a vaccine strain (H120) of avian infectious bronchitis, administered simultaneously or sequentially three days apart to chicks during 20 days, was evaluated using ELISA antibody levels, quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analyses and histopathology examination. First, the in vivo replication interference of avian influenza virus (AIV) and infectious bronchitis virus (IBV) was evaluated using qRT-PCR to detect accurately either AIV or IBV genomes or viral copy numbers during dual infections. Second, we have determined the amount of specific antibodies in sera of chick's infected with AIV alone, IBV alone, mixed AIV + IBV, IBV then AIV or AIV IBV 3 days later using an ELISA test. Finally, histopathological analyses of internal organs from inoculated chicks were realized. Quantitative results of AIV and IBV co-infection showed that interferences between the two viruses yielded decreased viral growth. However, in the case of super-infection, the second virus, either AIV or IBV, induced a decrease in the growth of the first inoculated virus. According to our results, vaccine application was safe and do not interfere with AIV H9N2 infection, and does not enhance such infection. In conclusion, co-infection of chicks with AIV and IBV, simultaneously or sequentially, affected the clinical signs, the virus replication dynamics as well as the internal organ integrity. The results proposed that infection with heterologous virus may result in temporary competition for cell receptors or competent cells for replication, most likely interferon-mediated.

1. Introduction

Avian influenza virus (AIV) and infectious bronchitis virus (IBV) are respiratory diseases of poultry, caused by type A Orthomyxovirus and Gammacoronavirus, respectively, with several traits in common. They are considered the most economically important respiratory viral diseases, and they threaten the poultry industry worldwide [1]. Mixed infection or co-infection with AIV and IBV has been described as a natural infection in different countries in Asia and the Middle-East [2,3]. More, the IBV vaccine is used extensively in chicken farms in many countries worldwide where both IBV and low pathogenic AIV H9N2 are endemic [3,4]. The H120 strain of IBV was one of the earliest live attenuated IBV vaccines to be developed and has continued to be use in most parts of the world. As with any live-attenuated IBV vaccine, the H120 vaccine strains must replicate in the respiratory tract if they are to stimulate protective immunity. It follows, therefore, that some damage to the epithelial layer of the respiratory tract will occur and a fine balance must be achieved between a strain that is so over-attenuated that it is unable to replicate sufficiently to stimulate immunity and one that is insufficiently attenuated so that serious damage may occur. The H120 virus meets these criteria well and is ideally suited for use in young, susceptible chicks [5].

Both viruses replicate in epithelial cells of the respiratory and the intestinal tracts, where trypsin-like enzymes allow virus entry. They likely compete to infect target cells and replicate. It has been shown that many viruses require the presence of well defined cell surface

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proteins for cell entry. The cell surface receptor for AIV or IBV on the cell surface is the N-acetyl neuraminic acid (α2,3-sialic acid-galactose) [6]. Such cell receptor also serves as a receptor protein determinant for primary attachment of group 3 corona viruses [7], allowing fusion events and entry of both viruses. Huang et al. have described as it is unknown whether co-infections with AIV and IBV exacerbate clinical signs of the disease in infected birds or produce viral interference, masking infections by either virus [8], even though it is reported that co-infection of IBV with AIV increased not only the severity of AIV-H9N2 clinical sings and gross lesions, but also the mortality rate with extended viral shedding period of AIV [9–11].

In Tunisia, AIV type A-H9N2 and IBV (H120 vaccine strain) are frequently isolated from broiler poultry farms, affecting their productions. Hatchery vaccination is actually opening the door to a real control of broiler diseases. Indeed, infection of animals with AIV and IBV, following simultaneous, super-infection or vaccination, may lead to severe complications having bad effect on the animal health and the farmer expenses. Mixed infection of poultry with AIV and IBV induced complex clinical picture making identification and diagnosis of either one or both viruses' difficult [12,22]. Still, questions remain regarding potential interactions between these viruses in **co-**infected bird [8]. So, it is worthy to better understand the in vivo interactions between these viruses for an efficacious disease control by adapting the vaccination programs (Kelli et al., 2010).

The objectives of this study was to evaluate the effect of multi-infections of chicks with IBV vaccine strain and field AIV isolate (A/Chicken/TUN/145/12 H9N2) by inoculating them simultaneously or sequentially and determining the induced immune response (ELISA test), the virus pathogenicity (clinical signs and lesions, histopathology), their body distributions and titers by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). And to study consequences of vaccination on AIV challenge.

2. Material and methods

2.1. Viruses

The AIV (A/CK/TUN/145/12-(H9N2)) strain was isolated and characterized in 2012 [13] and the IBV-H120 (Massachusetts) vaccine strain was provided from Pasteur Institute of Tunisia. Both viruses were grown in 10-day-old embryonated chicken eggs (ECE) at 37 °C for 72 h, as described by Senne [14]. Allantoic fluids were collected, titrated and then the 50% egg infective dose (EID_{50}) calculated, according to the Reed-Muench method (1938).

2.2. Birds

One-day-old conventional broiler chicks were purchased from a local hatchery. The immune status of birds contains IBV and H9N2 maternally-derived antibodies. They were then housed in three self-contained isolation units which are ventilated under negative pressure with inlet and exhaust HEPA-filtered air and maintained under continuous lighting; feed and water were given adlibitum. All experiments were conducted in accordance with ethical guidelines of the Tunisian Council on Animal Care and approved the ethics committee of the Institute Pasteur of Tunis under the reference 2017/05/I/LEMVIPT/V1.

2.3. Experimental design

A total of 150 broiler chicks was divided into one control group and five virus infected groups containing each 25 birds. The birds were inoculated via the intraocular and intranasal routes; with 0.1 ml of 10^3EID_{50} of each virus. We used low inputs of pathogenicity strains in order to follow easily viral dominance than when we used higher virus inputs of high pathogenicity because it will damage all the experiment system.

The viruses were administered either alone, mixed or consecutively, 3 days apart, as previous reported and showing that the maximum of virus replication in the organism is reached three days after infection [15,16].

Clinical signs were noticed and chick body weights along with blood sample and tracheal (TC) and cloacal (CL) swabs collections from five chicks of each group, at 1, 3, 7, 11 and 20 days post inoculation (dpi), were realized. Thereafter, the chicks were ethically culled and internal organs placed in 10% neutral buffered formalin for microscopic lesion examinations. The virus contents in collected swabs and organs were also determined [17,18].

2.4. Viral RNA extraction and viral gene copy numbers quantification

TC and CL swabs were collected, placed in 2 ml MEM containing final concentrations of gentamycin (200 µg/ml), penicillin G (2000units/ml), and amphotericin B (4 µg/ml) and maintained frozen at −80 °C, before being thawed and centrifuged at 3000 rpm for 15 min. Viral RNA was then extracted using 200 µl of virus suspensions with Trizol, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and precipitated with absolute ethanol. The final pellets were suspended in 20 µl RNase-free water and stored at −20 °C. Standard precautions to avoid contamination were taken.

The number of viral copies present in each sample was computed using a newly developed one-step multiplex qRT-PCR assay in our laboratory [19]. The reaction mixture contained 2 µl of RNA samples, 0.6 µl of each primer at a concentration of 10 pmol/µl and 7.5 µl of enzyme with buffer Agpath (Applied Biosystem, USA) in a final volume of 15 µl (Table 1). The qRT-PCR assay was done in an EscoSpectrum 96 Real Time Thermal Cyclers with the following cycling conditions: 45 °C for 10 min, initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 45 s with a single fluorescence acquisition step at the end of the annealing step. For quantitative purposes, positive Ct values (Ct ≤ optimal cutoff point) for each analyzed sample were determined from the corresponding standard curves derived from AIV and IBV standard

Table 1
Number of chicks positive for IBV and AIV in tracheal (TC) and cloacal (CL) swabs during single and dual infections tested by qRT-PCR.

Virus detection in TC and CL	Inoculated groups		Days post inoculation (dpi) and number of positive chick CL + TC		
TC	Day 1	Day 3	1	3	7 11 20
TC	LPAIV	-	0/5[a]	0/5	0/5 0/5 0/5
CL	IBV	-	0/5	5/5	5/5 5/5 5/5
	LPAIV + IBV	-	0/5	0/5	0/5 1/5 3/5
	LPAIV	IBV	0/5	5/5	5/5 5/5 5/5
	IBV	LPAIV	0/5	0/5	0/5 0/5 0/5
	LPAIV	-	0/5	0/5	0/5 0/5 0/5
	IBV	-	0/5	5/5	5/5 5/5 5/5
	LPAIV + IBV	-	0/5	0/5	0/5 1/5 3/5
	LPAIV	IBV	0/5	5/5	5/5 5/5 5/5
	IBV	LPAIV	0/5	0/5	0/5 0/5 0/5
	LPAIV	-	0/5	5/5	5/5 5/5 5/5
	IBV	-	0/5	0/5	0/5 0/5 0/5
	LPAIV + IBV	-	0/5	0/5	0/5 1/5 3/5
	LPAIV	IBV	0/5	0/5	0/5 0/5 0/5**
	IBV	LPAIV	0/5	5/5	5/5 5/5 5/5
	LPAIV	-	0/5	5/5	5/5 5/5 5/5
	IBV	-	0/5	0/5	0/5 0/5 0/5
	LPAIV + IBV	-	0/5	0/5	0/5 0/5 0/5
	LPAIV	IBV	0/5	0/5	0/5 0/5 0/5
	IBV	LPAIV	0/5	5/5	5/5* 5/55/5

[a] Number of positive birds/total number of birds sampled at each time point.
* Significant difference for number of positive chickens by qRT-PCR, as compared to single virus infected groups (* $P < 0.05$; ** $P < 0.01$).

plasmid templates. More details about the cloning, extraction of recombinant plasmid and the construction of standard curves are fully provided by Laamiri et al. [19].

2.5. Serology

ELISA antibody tests were realized to measure specific antibody titers in the sera collected from five chicks of each experimental group at 1, 3, 7, 11 and 20dpi using ID screen ELISA Kit, for AIV and IBV (ID vet–France). Antibody titers were calculated according to the kit's instructions.

2.6. Histopathological examination

Various internal organs (trachea, lung, liver and intestine) were collected from 5 sacrificed chicks of each group, at 1, 3, 7, 11, and 20dpi, and conserved in 10% formaldehyde solution. They were cut into small pieces and prepared for histopathological analyses using classical steps of molding in paraffin, embedding and sectioning the organs into very thin sections using a microtome. The slices were layered on a glass slide for staining with a mixture of hematoxylin and eosin (H&E), and then analyzed under a light microscope with 100X objective.

2.7. Statistical analysis

Data were analyzed using Prism v.5.01 software (GraphPad Software Inc. La Jolla, CA, USA) and values are expressed as the mean ± standard error of the mean (SEM). One-way ANOVA with Tukey post-test was used to analyze body weights. The number of birds shedding of viruses was tested for statistical significance using Fishers exact test. Two-way ANOVA with Bonferroni multiple comparison analysis was used to evaluate virus titers in swabs and organs. For statistical purposes, all qRT-PCR negative TC and CL swabs were given a numeric value less than 10 copies/ml. Statistical significance was set at $p < 0.05$ unless otherwise stated, using Student's test.

3. Results

3.1. Clinical signs and macroscopic lesions

None of the chicks infected with either AIV or IBV alone or both viruses, showed any clear clinical signs. Besides, significant changes in chick body weights of virus-infected chicks were noted only at 20dpi for all infected groups except co infected ones, in comparison with the control non-infected animal group (Fig. 1). Chicks inoculated with AIV showed pneumonia, enlarged spleen, serous effusion and congested kidney and spleen. These lesions appeared 7dpi in all infected chicks. Similarly, the most noticed lesions were observed at 11dpi in chicks

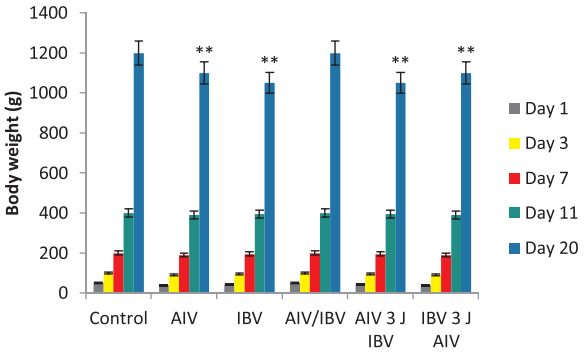

Fig. 1. Body weights of check at various times pi in virus infected and control groups. Bars represent the standard deviations for two experiments carried out on three check.

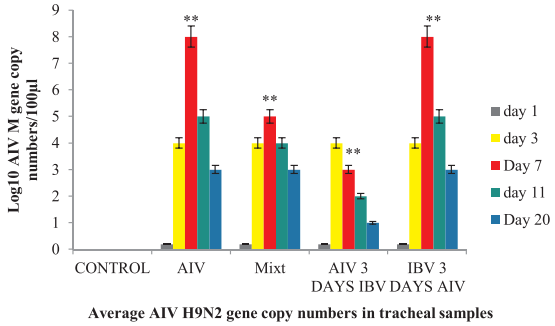

Fig. 2. Kinetics of AIV H9N2 excretion in TC check after single or dual infection with IBV as measured by qRT-PCR.

infected with IBV, showing congested trachea and kidneys, pneumonia and enlarged spleen.

Simultaneously AIV and IBV infected chicks showed less pronounced lesions as compared to super-infected groups, which animals showed pneumonia, congested spleen, liver discoloration and enlarged kidneys.

3.2. Single infections

Using qRT-PCR, the viral interference levels in various inoculated groups, as compared to the control group, was quantitatively evaluated. TC and CL viral shedding was examined and the results are shown in Table 1 and Figs. 2–5, representing the average values of three independent experiments with standard error bars. Chicks inoculated with only IBV or AIV excreted the virus from day 3 to 20dpi, as determined by virus detection in TC and CL swabs (Table 1). In co-infected groups, significant IBV virus excretion was noticed in both TC and CL swabs taken at 11 and 20dpi ($p < 0.01$). Super-infected groups showed that IBV virus is detected in TC and CL swabs from 3 to 20dpi in chicks infected with AIV followed by IBV inoculated 3 days later; no AIV shedding being noticed. Chicks infected with IBV then super-infected with AIV-3 days later, showed no IBV shedding either in TC or CL, during the experimental period (Figs. 2–5). When comparing the kinetics of AIV or IBV virus replication at different time points after chick inoculations, the patterns of TC and CL viral titers were different depending on the virus exposure, as shown for AIV or IBV viral gene copy numbers detected by qRT-PCR (Figs. 2–5). The results of single AIV infected groups showed classical viral curve with higher virus excretion, in either TC or CL swabs, at 7dpi. However, co-infected birds (AIV + IBV mixture) presented significant lower amounts of viral gene copy numbers at 7dpi, as compared to single AIV infected birds ($p < 0.05$) (Figs. 2 and 3).

3.3. Super infections

When AIV was first inoculated and followed by IBV administration,

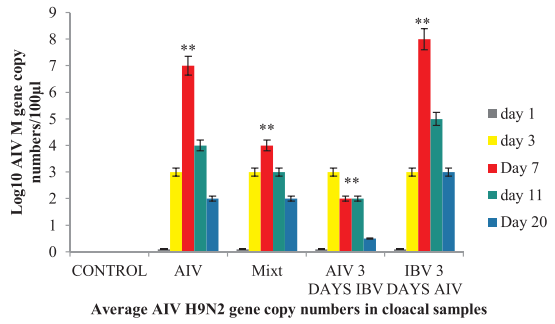

Fig. 3. Kinetics of AIV H9N2 excretion in CL check swabs after single or dual infection with IBV, as measured by qRT-PCR.

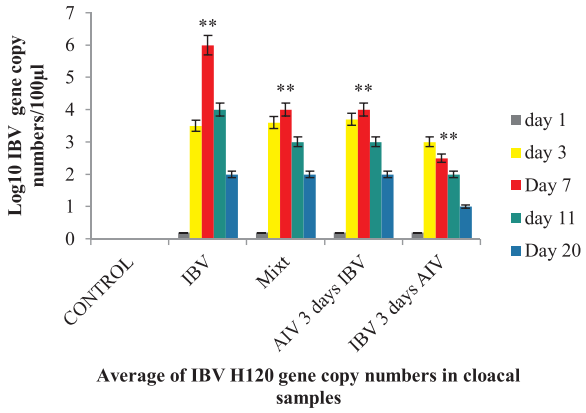

Fig. 4. Kinetics of IBV H120 excretion in CL check swabs after single or dual infection with AIV, as measured by qRT-PCR.

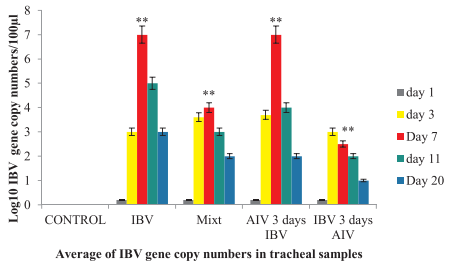

Fig. 5. Kinetics of IBV replication of in TC check swabs after inoculation alone or in a combination with AIV (A/Ck/TUN/145/12) as measured by the Real-time PCR.

no virus shedding was detected. However, chicks infected with IBV and inoculated 3 days later with AIV, excreted AIV in TC as well as CL, from 3 to 20dpi. During super-infection of AIV followed 3 days later by IBV, the AIV viral gene copy numbers detected by qRT-PCR in either CL or TC, decreased significantly ($p < 0.05$), as compared to single and mixed infections, starting on day 7dpi, indicating the inhibition of AIV growth (Figs. 2 and 3). However, when IBV was secondly inoculated 3 days after AIV infection, a clear growth of IBV was shown during the experimental period and no inhibition was noticed by the first day administration of AIV (Figs. 4 and 5). During super-infection of IBV by AIV 3 days later, the kinetics of IBV replication in either CL or TC, decreased significantly ($p < 0.05$), as compared to single and mixed infections, starting on day 7dpi, indicating the inhibition of IBV growth (Figs. 4 and 5).

On the contrary, when AIV was secondly inoculated 3 days after IBV infection, a clear growth of AIV was shown during the experimental period and no inhibition was noticed by the first administrated IBV (Figs. 2 and 3).

3.4. Co-infections

Similar results were observed during single IBV infected as well as co-infected (AIV + IBV mixture) birds as demonstrated for AIV infected groups, with significant lower amounts of viral gene copy numbers at 7dpi in either TC or CL swabs ($p < 0.05$) (Figs. 4 and 5).

3.5. Conclusion

Significant differences of AIV and IBV shedding, using the student's t-test, was noticed in TC and CL swabs taken at 3, 7, 11 and 20dpi, from co-infected chicks ($p < 0.05$), in comparison with single infected groups (Table 1).

3.6. Serology

The ELISA test was utilized to follow antibody levels against AIV and IBV in sera from non-infected as well as infected groups. No significant AIV and IBV antibodies were observed during the experimental period, in sera from all infected groups at 1-3dpi, as compared to the control non-infected group. However, a relatively low antibody levels against AIV or IBV were observed at 7dpi in single infected groups. During mixed infection, no significant levels of specific antibodies were detected against either AIV or IBV. In super-infected birds, low levels of specific antibodies against either AIV or IBV, were detected only in birds when the super-infecting virus is either AIV or IBV (Results not shown).

3.7. Histopathological examinations

Microscopic lesions of the respiratory tract of single and dual infected groups (Fig. 6, Fig. 7) showed congestion of pulmonary blood vessels and perivascular hemorrhage with pale bluish mucus and heterophile accumulation in lumen of secondary bronchioles (Fig. 6). Histopathological findings in the trachea demonstrated marked lesions, characterized by diffuse infiltration of sub epithelial and deep layer of mucosa with lymphocytes and histiocytes, resulting in an increase in the mucosathickness. There was also congestion of the blood vessels of the mucosa with marked lymphoid tissue hyperplasia of the lamina propria (Fig. 8). Collected control organs showed no changes. The jejunum of the intestine showed desquamation of intestinal villi (Fig. 7) and the liver showed congestion of portal veins with peri-portal aggregation of leukocytes, mainly lymphocytes and macrophages (Fig. 7).

4. Discussion

The aims of the study were to evaluate the biological interactions between AIV and IBV-H120 that might happen in co-infected chicks. And to study consequences of vaccination on AIV challenge. Infections with more than one virus, such as AIV and IBV, seem to frequently occur in poultry production; but, the effects of such co-infections or vaccination on several host responses such as viral shedding dynamic, antibody seroconversion and clinical signs are not fully known in chicks [15,20]. Besides, reported studies did not specify the various effects of dual viral infection on immune response, virus titers and genes copy number variations and histopathological changes during replication of both viruses. For this, we have deeply evaluated more than one aspects of viral interference between AIV and IBV, as compared to other reported studies [9,20], in terms of body weight, humoral response, macroscopic and microscopic lesions and viral replication using qRT-PCR.

The results reported by Seifi et al. [20] have suggested that IBV infection increases the pathogenicity of H9N2-AIV in broiler chickens, based on serological analysis and clinical signs noticed during co-infection using high viral titers (10^6 EID_{50}) of H9N2 and IBV. However, our study showed that the majority of chicks simultaneously infected with AIV (A/CK/TUN/145/12)-H9N2 and IBV-H120, did not show clear clinical signs, indicating low viral growth. It is worth noting that the virus titers used in our study were relatively lower (10^3 EID_{50}), in regards to the exacerbated clinical signs reported [20].

It was interesting to note that the shedding patterns of both H9N2 and IBV were strongly influenced by each other. In fact, during co-infection, all chicks became infected with both viruses, as shown by virus serological and molecular results, but a significant reduction in virus replication was observed during the first 3 days, as compared to singly infected controls. A significant lower amounts of viral gene copy numbers were then noted at 7dpi up to 20dpi, in either TC or CL swabs ($p < 0.05$), collected from simultaneously (Figs. 2–5). It can be inferred that the transitory inhibition of viral replication may have an effect on the severity of the disease; the induced clinical signs were not

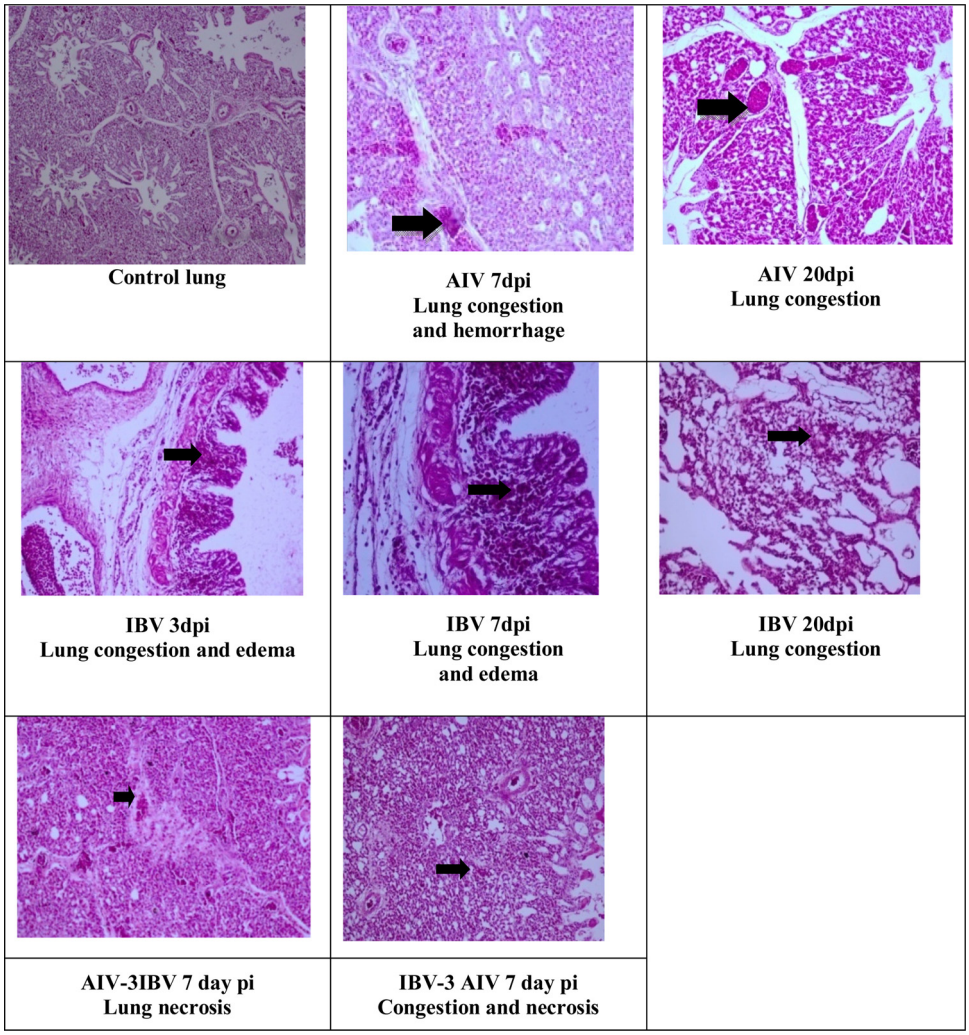

Fig. 6. Histopathological sections of the lungs in different cases of experience.

perceptible and only microscopic lesions were noticed. These findings are in concordance with other reported results [18,21,16]. Besides, lower specific antibody titers were produced since both viruses inhibited the growth of each other's, early after infection.

Our results clearly showed that viral titers in TC swabs were distinctly higher than in CL sample. They are consistent with previous study showing that AIV viral shedding happens mainly by the TC route (Costa-Hurtado, 2014). In addition, virus shedding was lower in all groups simultaneously or sequentially treated, as compared to single infected animals. Previous in vitro or in ovo experiments have shown similar results, in which simultaneous infections with a mixture of AIV and IBV induced inhibition of the replication of both viruses during the first 3 days of infection. In fact, using cell cultures or chicken embryos, co-infections with AIV and (Newcastle disease virus) NDV mixture have shown interference between both viruses, suggesting inhibition of the growth of one virus by the other [22–24]. Such early inhibition may be related to competition for virus receptors on the cell surface, leading to an attachment interference phenomenon. Interference could also be due to intracellular interference related to competition for replication sites or essential factors for viral replication, virus-induced interferon interference or ARN or defective interfering particles [25].

Both inoculated viruses may also compete for sites or essential factors of virus replication, as they are both RNA viruses. It has been clearly established that the cell surface receptors for AIV are sialic acid containing glycol-conjugates [23], whereas the cellular receptors for IBV have been proposed as α2,3-sialylated glycans [26], both of which contain sialic acid [23]. These findings imply the existence of common receptor sites on the permissive host cells that are shared by AIV and IBV [27].

Historically, the interest for viral interference phenomena has led to the discovery of interferon in the early 1940s [28,29]; the interferon being secreted by cells following viral infection leading to inhibition of virus replication. Our results did not show an important secretion of interferon in cell cultures simultaneously infected with AIV-IBV mixture (data not shown), even though studies have reported secretion of cytokines in chickens infected with H9N2 [30]. It has also been reported that H120 and H9N2 viruses are weak interferon inducers, and the first inoculated virus could not interfere well with the second one by producing sufficient amount of interferon ([15,31]; 2015 [11];).

Thus, during in vivo super-infection, our results showed an important increase of the replication of the second inoculated virus that has the ability to out-compete and dominate the first virus replication, attaining almost its initial titer obtained during single infection. This result was in concordance with some studies that have been reported (Shengqiang et al., 2012), including the results in this study demonstrating the higher growth of the super-infecting virus. However, there are some reported studies that demonstrated contradictory findings in vitro, as it has been reported for the occurrence of AIV-NDV, Hepatitis B Virus-Hepatitis C virus and AIV-IBV interferences [32–34], showing that the pre-inoculated virus always induces a lower growth of the super-infecting virus [35].

Previous studies on in vitro and in ovo interference between AIV

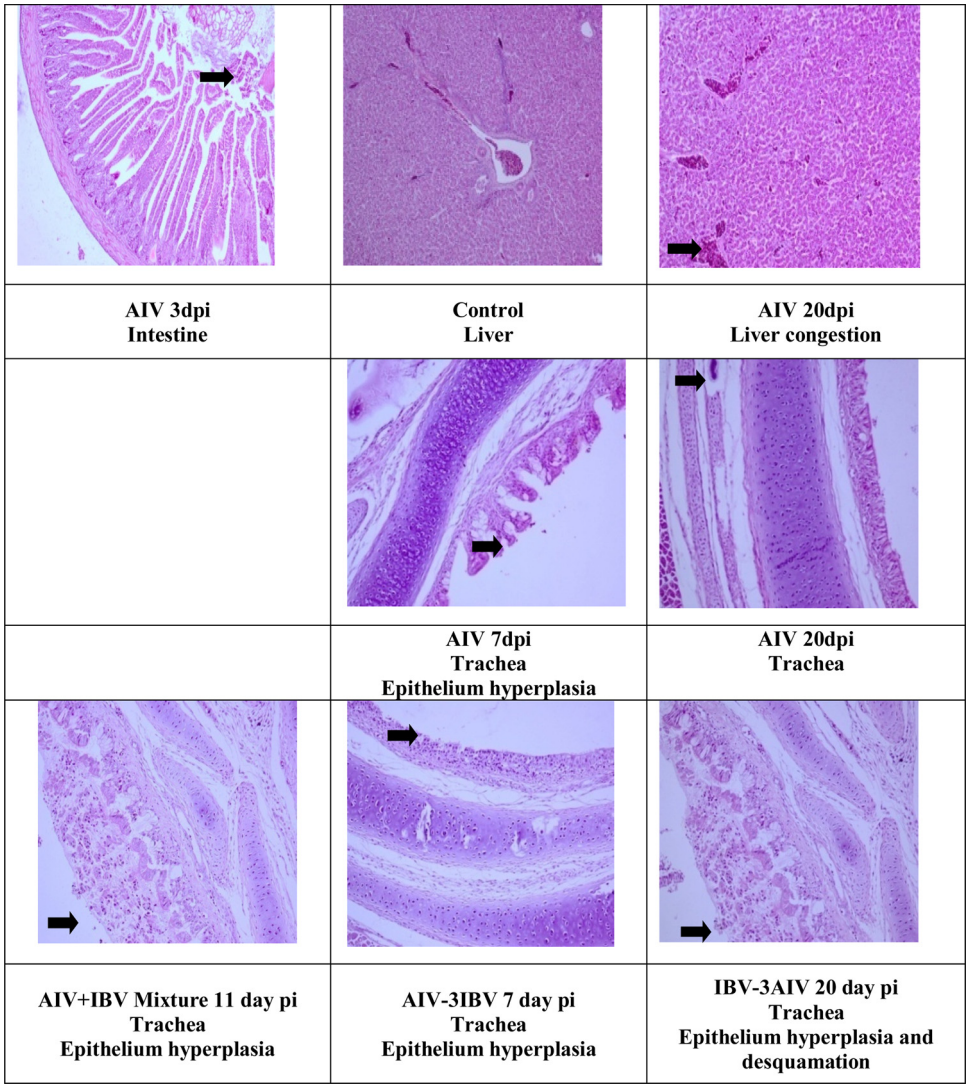

Fig. 7. Histopathological sections of the livers and the trachea in different cases of experience.

and IBV-H120 using, respectively, cultured chicken embryo lung cells and embryonated SPF eggs, have shown that the second inoculated virus slows down the process of viral replication of the first administered one [36]. At the same age, groups simultaneously infected had numerically lower antibody titers as compared to groups singly or sequentially infected. This was due to lower virus replication related to mutual inhibition during the first 3dpi and low virus titers challenging the immune system. Similarly, increased histopathological lesions in the trachea of singly or sequentially infected groups were observed. Viral infection has been reported to induce loss of TC cilia, hyperplasia, hyperemia, and [37,38]. Histopathological changes induced in the trachea, lungs, liver and intestine of PCR- positive chicks, during viral interference between AIV and IBV, were in accord with those previously reported [39–41].

In general, the effects of viral interference depend on their titers and their pathogenicity, the time of co-infection, the immune responses and the environmental factors leading to the adaptation of viruses to their host species. Identification and assessment of factors affecting viral interference and understanding those that cause delay in virus replication will help finding the path of pathogenicity and transmission of these viruses in chicks. Our results have shown an increase in virus replication in cases of super-infection, which may affect the severity of the disease. Determination of factors that may influence interference during co-infection or favor a delay of one virus growth at the expense of another virus will give new insights on the pathogenesis of these viruses, allowing a better design of diagnostic tools and improved vaccine and vaccination program for better controlling the diseases.

5. Conclusion

In summary, our study showed that simultaneous dual infection of one day old chick's leads to reduction of both virus growths. However, during super-infection, the second inoculated virus has a negative impact on the growth of the first virus inoculated and that the degree of interference depending upon the interval between interfering viruses. The results suggested that interference might interfere during the virus entry into the cells through a competition for shared virus sialic acid receptor that are essential for virus attachment. Interference may also happen when both viruses compete for sites or essential factors of viral replication (Shengqiang et al., 2012). These findings may have a strong influence on prevention and control strategies for the spread of economically important AIV and IBV diseases in the poultry industry. According to our results, vaccine application was safe and do not interfere with AIV H9N2 infection, and does not enhance such infection.

Ethics approval and consent to participate

We received the approval of the ethics committee on this work.

Reference: 2017/05/I/LEMVIPT/V1.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

RA analyzed and interpreted the qRT-PCR and ELISA data regarding H9N2 and H120 viruses and was a major contributor in writing the manuscript. NL performed the qRT-PCR examination, did statistical analyses of the work and contributed in writing the manuscript. SS did the experimental protocol of the chicks. RA did histopathological analysis. JN did organs examination. All authors read and approved the final manuscript. AG corrected the draft paper.

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