Viral interference between low pathogenic avian influenza H9N2 and avian infectious bronchitis viruses *in vitro* and *in ovo*

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A B S T R A C T

Background: Low pathogenic avian influenza (LPAI) H9N2 and infectious bronchitis virus (IBV) are important pathogens of poultry, causing important economic losses for the sector. Replication interference between these two viruses was described using cell cultures (CC) and embryonated chicken eggs (ECE). Chicken embryo lung (CEL) and ECE were simultaneously or sequentially infected with IBV vaccine strain (H120) and LPAIV-H9N2 (A/Ck/TUN/145/2012) to evaluate viral interaction*in vitro* and *in ovo,* respectively. Real-time RT-PCR was developed to specifically quantify both AIV and IBV genomes as well as viral gene copy numbers during mixed infections. The amount of IL-1 beta, in supernatants of co-infected cell cultures, was determined using an ELISA assay.
Results: 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.
Conclusion: It appears that either AIV or IBV has a negative impact on the other virus growth when they are inoculated simultaneously or sequentially. The ELISA results showed that higher level of secreted IL-1beta varies, depending on the viral interference conditions between both viruses, during mixed infections.

1. Background

LPAIV and IBV are leading causes of economic losses in the poultry industry, due to increased mortality, impaired growth, and reduced egg and meat productions. They affect the respiratory and the reproductive tracts as well as the renal system of chickens, causing respiratory signs, reduction in weight-gain and usually life-long decrease of egg laying performances (Cavanagh, 2003; Liu and Kong, 2004). Farmers are always facing major problems related to co-infections with major respiratory pathogens, mainly avian influenza H9N2 and avian infectious bronchitis or Newcastle disease viruses (Wafa et al., 2011; Hager et al., 2012; Shengqiang et al., 2012; Fernandez et al., 2014). Studying the influence of infection by one virus on another virus is important to understand the interactions between respiratory viruses and the impact of the control measures applied such as vaccination. Multiple infections with AIV and IBV were rarely studied. However, none of these studies have quantitatively evaluated the degree of *in vitro* and *in ovo* interferences between these two viruses (Seifi et al., 2012). Owing to the

important position of both viruses, it is of theoretical and practical importance to continue discussions about viral interferences by evaluating AIV and IBV replication, using the newly developed multiplex real-time RT-PCR (rRT-PCR) (Nacira and Rim, 2018).

Both AIV and IBV are RNA viruses of birds, belonging to the *Orthomyxoviridae* and the Coronaviridae families, respectively, and having several clinical features in common (Carstens, 2009). The AIV genome has eight linear segments of negative-sense ssRNA. The IBV genome has a non-segmented positive-sense ssRNA. The AIV type A viruses are classified as low pathogenic (LP) and high pathogenic (HP) viruses, based on their virulence for chickens and the presence of multiple basic amino acids at the cleavage site of the hemagglutinin (HA), the precursor protein (Capua and Alexander, 2009; Boursnell et al., 1987). The receptor of AIV on the cell surface is the *N*-acetyl neuraminic acid (α2, 3-sialic acid-galactose) (Fernandez et al., 2014). On the other hand, the Coronaviruses have evolved with various initiation of infection strategies for both the attachment stage and the subsequent membrane fusion. They have even required the presence of

defined cell surface proteins for infection; the N-acetyl neuraminic acid (α2,3-sialic acid) being the receptor protein determinant for primary attachment for group 3 Coronavirus to which belongs the IBV (Delmas et al., 1992; Wickramasinghe et al., 2011; Winter et al., 2006). Alternatively, IBV may bind to only very specific subset of α2,3-sialylated glycans, present only on chicken cell surfaces (Wickramasinghe et al., 2011).

Initiation of super-infection inhibition was described for many bacteria, plants and animal viruses (Christen et al., 1990; Cockley et al., 1988). Although AIV and IBV are the causative agents of two major avian diseases, unfortunately, little is known about their interactions when co-infecting chicken hosts. In fact, there is a lack in evaluating the growth of individual viruses and the determination of the exact number of progeny viruses produced, during co-infections; the only reported study on AIV and IBV co-infection being performed without any quantitative evaluation of the degree of such interference (Groupé and Pugh, 1952). Owing the importance of such viruses and their impact on the poultry industry, new quantitative techniques such as rRT-PCR were developed, establishing *in vitro* and *in ovo* model system in which many different viruses and their interactions could be studied (Slomka et al., 2013).

Cytokines are expressed rapidly after viral infection, as they represent a first line of defense initiated by the innate immune response (Saacs et al., 1957). It was thus shown that immediately after chicken infection with AIV, broad range of effector molecules do express pro-inflammatory cytokines including IL-1β, which is the most important one expressed along with IL-6 and type I-IFN (Saacs et al., 1957). A general antiviral response is generated through the activation of a broad range of effectors molecules, including myxovirus resistance gene I, RNA-activated protein kinase and 2′,5′-oligoadenylate synthetases (Daviet et al., 2009; Garcia-Sastre, 2001). IL-1β expression is induced by toll like receptors that warrant its presence early after viral infection of chickens (Jianlin et al., 2016; Kim et al., 2015).

Since the introduction of H9N2 in Tunisia, in 2011, poultry farms became exposed to AIV or IBV (vaccines or wild strains), alone or mixed, leading to more severe disease (Wafa et al., 2011). In fact, chickens vaccinated with IBV-H120 strain or infected with wild IBV might also become infected with circulating H9N2 influenza strains. Laboratory diagnostic tests have revealed co-circulation of LPAI H9N2 and IBV in Tunisian poultry farms. Thus, it becomes important to study interactions between these two viruses during dual viral infection.

Understanding the interaction between AIV and IBV is important for the diagnosis of clinically suspected diseases as well as the implementation of appropriate control measures. Actually, several countries are reporting important losses in poultry farms related to natural viral co-infection, essentially with AIV and IBV (Nacira et al., 2016). In Tunisia, farmers are continually facing difficulties with effective control of these infections even in presence of recommended vaccination program. In fact, routinely vaccinated animals against IBV could be naturally infected with AIV; similarly, animals vaccinated against AIV could also be naturally infected with IBV. Understanding viral interaction between two viruses is needed to improve vaccination program and control multiple viral infection.

In the early 1940s, Henle and colleagues discovered that inactivated influenza virus particles were capable of interfering with the replication of a live vaccine virus added later. This finding opened the way to the study of a live virus vaccine inoculation either mediated either by interferon system or through competition for receptors on the cell surfaces that contain sialic acid (Fernandez et al.; 2014; Delmas et al., 1992).

For this, the aim of the study was to obtain information on viral interference between LPAI-H9N2 (strain A/Ck/TUN/145/12) and IBV-H120 during mixed and sequential infections as compared to results of single viral infections *in vitro* and *in ovo*. Experimental infections of cell cultures or ECE with either IBV vaccine strain in presence or absence of AIV-H9N2 virus and *vice versa*, added at different times were

monitored, for better understanding of dual infection and implementation of more efficient control measures (Huang et al., 2017).

2. Read methods

2.1. Virus strains

The (A/Chicken/TUN/145/12) strain, a field isolate of H9N2 and the vaccine strain H120 (Massachusetts type) of avian infectious bronchitis were used. Virus stocks were prepared after virus inoculation into the allantoic cavity of embryonated specific pathogen free (SPF) eggs. Following incubation at 37.5 °C for 48–72 h, allantoic fluids were collected, clarified by low speed centrifugation and titrated for virus contents. The viral titers, expressed as fifty-percent egg infectious dose (EID$_{50}$), were determined before freezing at -80 °C until use.

For virus titration, serial dilutions of each virus suspension were inoculated into cell culture plates and their fifty-percent Tissue 37.5°C with 5% CO_2 and use for virus Culture Infectious Dose (TCID$_{50}$) titers determined using the method of Reed and Muench (1938).

2.2. Cell cultures

Vero cell line (ATCC (CCL-81)), grown in Minimum Essential Media (MEM) containing 10% fetal calf serum (FCS), MDCK (ATCC/CRL-2935) and primary Chick Embryo Lung (CEL) cells, grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, were used to determine which cell type would allow better replication of both viruses, and could be used to study the *in vitro* viral interference.

The CEL cultures were prepared from 18-day-old ECE. Thus, the lungs of the embryos were collected, washed with PBS, sliced, digested with trypsin/EDTA, and then DMEM with 10% FCS was added. After clarification at 1000 g for 10 min, the decanted cells were resuspended in medium and filtered through sterile gauze. The cell suspension was counted and distributed into tissue culture flasks before their incubation at 37.5 °C with 5% CO_2 and use for virus growth.

2.3. Specific-pathogen-free (SPF) chicken eggs

SPF ECE (LohmannLtz-Germany) were incubated for 9–10 days at 37.5 °C and 80% relative humidity. They were candled before their use for isolation; amplification and titration of IBV and AIV, through inoculation into the allantoic sac and incubation for 48–72 h.Embryonated SPF eggs were also used to study virus interference during single, mixed and super-infections *in ovo*.

2.4. Virus replication cycles

The three types of cultured cells (Vero, CEL and MDCK) were used to estimate virus yields during single infection with either AIV or IBV. For this, ten-fold dilutions of either AIV or IBV were added to cultured cells and incubated at 37 °C with 5% CO_2 for 72 h. The cell layers (cells and supernatants) were then scraped off at 6 h, 12 h, 24 h, 48 h and 72 h after viral infection. At each time, a sample of the collected mixture was stored frozen until tested for total virus content. Each virus suspension was then centrifuged at 1500 rpm for 10 min to separate the cells from the culture media, kept separately frozen at -80 °C until analyses for intra and extracellular virus yields. For this, frozen viral suspensions underwent 2 cycles of freeze thawing followed by clarification, and collected materials titrated for virus contents in the appropriate cell cultures.

2.5. Viral interference in vitro

For *in vitro* viral interference studies, stock viruses of either AI or IB were thawed, clarified and then diluted in DMEM to obtain viral suspensions containing 10^2 TCID$_{50}$/50 µl.

The appropriate cell suspension, diluted in DMEM, was distributed in 96 well plates. Cell monolayers were infected with 50 μl/well of each virus dilution to observe viral interferences during single infection with AIV or IBV, dual infection with AIV and IBV simultaneously added or super-infection with AIV then IBV or IBV then AIV. The inoculated viruses were allowed to adsorb for 1 h at 37 °C before the cell layers were washed once with phosphate buffered saline (PBS) to reduce non-adsorbed viruses. A volume of 200 μl of DMEM, supplemented with 10% FCS and 1% antibiotic/antimycotic mixture was added and the infected cultures were incubated at 37 °C with 5% CO_2. Each day, the cell monolayers (cells and supernatants) were collected and frozen until tested for virus contents and viral RNA extraction (Hager et al., 2012).

For single infections, each virus was added to the corresponding wells. After 96 h incubation, the cultured cells were scraped off and the cell suspensions were collected, clarified twice by centrifugation (1000 × *g*), divided into aliquots and stored at −80 °C until tested.

For dual infection with AIV and IBV, the procedure was identical as for single infection except that both viruses were mixed before adding the mixture to the cell layers.

For super-infections, cells were first infected with either AIV or IBV and then super-infected 1 h, 4 h or 8 h later, with either IBV or AIV, respectively. The delay between the two viral infections was selected, based on previous *in vitro* studies assessing interference between super-infecting viruses (Banfield et al., 2003; Glazenburg et al., 1994; Schynts et al., 2003), allowing evaluation of the interference levels at the beginning, the middle and the end of the virus replication cycles.

2.6. Virus interference in ovo

For the *in ovo* viral interference studies, 200 μl of the virus suspension were inoculated into the allantoic cavity of SPF ECE of 10 days of age. The inoculated eggs were incubated for 72–96 h at 37 °C, as reported for *in ovo* interference (Yachida et al., 1986; Shengqiang et al., 2012; Nil et al., 2014).

For single infections, ECE were infected with 200 μl with either AIV or IBV viruses at an input of multiplicity of $10^3 EID_{50}$/egg; five ECE were inoculated with the corresponding virus suspensions for single or dual infections. After 3 days incubation, the allantoic liquids of infected eggs were harvested and stored until tested for virus contents. The embryos were also crushed and stored at −80 °C for further use.

The level of viral interferences were estimated by comparing AIV and IBV yields from mixed and super infected cultures with those of the corresponding single infected controls as measured by the multiplex rRT-PCR.

2.7. Multiplex rRT-PCR

Viral RNAs were extracted from 200 μl of collected cell supernatants and egg allantoic liquids, using Trizol reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instructions, avoiding cross-contaminations. The final pellets were suspended in 20 μl RNase-free water and stored at −20 °C.

A one-step multiplex rRT-PCR assay was developed in our laboratory by Nacira and Rim (2018) for simultaneous quantification of the two avian respiratory viruses: AIV-H9N2 (isolate strain A/chicken/Tunisia/145/2012) and IBV-H120 vaccine strain AIV and IBV, as reference strains (Nacira and Rim, 2018). Plasmid standard, holding viral specific sequences with binding sites for type-specific primers and probes, were prepared as follows. Using TOPO® TA Cloning® reagent kit (Life Technologies, Invitrogen), pCR®2.1-TOPO® plasmids were constructed by cloning amplicons generated by RT-PCR using the primers shown in Table 1 and T-A cloning strategy, according to the manufacturer's instructions. Besides, constructed plasmids were transferred by electroporation into electro-competent E.coliDH5α(F-φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk −, mk +) phoA supE44 thi-1 gyrA96 relA1 λ) for propagation. The plasmid DNAs were purified with PureLink® Quick Plasmid Miniprep purification Kit (Life Technologies (Invitrogen)), amplified and then sequenced with IDT M13 primers. The DNA concentration of each plasmid preparation was determined with the Thermo Scientific Nano-Drop 2000 spectrophotometer. The copy numbers of the extracted plasmids, equivalent to the number of viral genome copies, were calculated (Huang et al., 2009). Each standard plasmid was equally mixed and adjusted to a concentration of 10^8 copies/μl and the mixed plasmids were used to prepare ten-fold serial dilutions allowing construction of the duplex standard curves for analytical validation (Nacira and Rim, 2018).

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) in a final volume of 15 μl (Table 1). The rRT-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 (Nacira and Rim, 2018). 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 plasmid templates. Consequently, the number of viral copies present in each sample was computed.

2.8. Cytokine analyses

To determine cytokine secretion during viral infections, a commercial sandwich ELISA kit (Mybiosource-USA) was used to quantify chicken IL-1β, according to the manufacturer's instructions. The levels of IL-1β in supernatants of single, mixed or super infected cells were measured and analyzed.

2.9. Statistical analyses

The data and statistical analyses were performed using Prism 6 software (GraphPad, La Jolla, CA). Two groups' means were compared with a two-tailed Student *t*-test, whereas multiple comparisons were carried out by analysis of variance (one-way ANOVA method). The differences were considered statistically significant at *p* values < 0.05.

3. Results

3.1. In vitro virus detection and quantification during single infection

When testing the yields of either AIV or IBV in three different cell types (Vero, MDCK or CEL), it appeared that both *Vero* and CEL cell cultures supported higher viral growth than MDCK cells as compared to MDCK cells, in three replicate assays. Infected CEL cultures showed that, after an eclipse phase of 6 h, both virus titers increased progressively to reach the highest values of $1.69\ 10^8$ viral RNA/μl at 48 h post-infection p.i.; then they decreased progressively until 96 h p.i. Both viruses showed similar growth curves with no statistically significant differences (Fig. 1).

To confirm the growth of both viruses in CEL cells, the kinetics of infectivity was studied and total, intra and extracellular virus yields were determined (Fig. 2). The average titers were calculated from three independent experiments.

It appeared that the kinetics of AIV and IBV growths are nearly similar and show a decrease in the viral titers 6 h after virus infections, followed by a rapid increase up to maximum titers of 10^8 and 10^8 $TCID_{50}$ at 36 h p.i., for IBV and AIV, respectively (Fig. 2). Cytopathic effects induced by either AIV or IBV started between 8–10 h p.i., respectively, followed by a complete cell degeneration between 48–72 h p.i. and a rapid decrease of virus titers.

Comparison of intra and extra cellular AIV and IBV titers showed an eclipse period of 6 h. This was followed by a gradual increase, reaching

Table 1
Primers and probes used in this work.

| Virus	Oligo	Primer/probe sequence (5'-3')	Genomic region	Amplicon Size (bp)	Target gene	Reference
AIV	Fr	AGATGAGTCTTCTAACCGAGGTCG	25–48	100	Matrix protein (M)	Spackman et al. (2002)
	Rv	TGCAAAAACATCTTCAAGTCTCTG	101–124			
	Probe	TCAGGCCCCCTCAAAGCCGA	64–83			
IBV	Fr	GCTTTTGAGCCTAGCGTT	391–408	143	5' untranslatedregion (UTR)	Callison et al. (2006)
	Rv	GCCATGTTGTCACTGTCTATTG	512-533			
	Probe	CACCACCAGAACCTGTCACCTC	473–494			
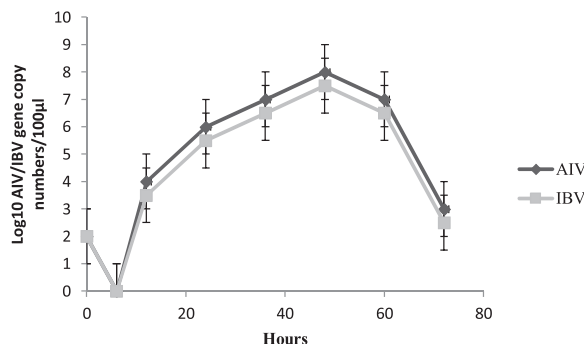
Fig. 1. Growth curves of AIV and IBV in CEL cultures, as determined by qRT-PCR. Symbols represent the mean titers and the standard deviations of triplicate tests.

maximum titers of $10^{4.5} TCID_{50}/100 \,\mu l$, in the intracellular (IC) viral suspension at 36 h, and $10^7 TCID_{50}/100 \,\mu l$ in the extracellular (EC) supernatants for either AIV or IBV, at 36 h p.i. The viral titers increased progressively to reach a pick at 36 h for IC and EC. Then after, the viral titers decreased rapidly (Fig. 2). The kinetics of infectivity of both viruses are similar with few differences not statistically significant ($p < 0.05$).

3.2. Gene copy quantification during in vitro single infection

Quantifying viral gene copy numbers by rRT-PCR confirmed the results obtained for the infection kinetics of CEL with either AIV or IBV. The rRT-PCR results of triplicate tests allowed detection of gene copies in the intra and extracellular virus suspensions collected from day 1 to day 4 p.i. Thus, it was shown that the majority of the produced viruses are cell-associated throughout the virus replication cycles and approximately 60–80% of the newly produced virions, during 12–24 h p.i., were cell-associated (Fig. 3A–C).

The numbers of gene copies of each virus, M gene for AIV and UTR

5' region for IBV, were lower in the intracellular than in the extracellular compartments. Besides, it appeared that the number of virus copies detected were higher than the virus titers obtained by micro plate-titration, indicatingthe higher sensitivity of the rRT-PCR technique (Fig. 3). Statistical significance of the results between the groups of AIV and IBV was determined using the student's *t*-test, as follows: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

3.3. Virus quantification during in vitro dual and super infection

The rRT-PCR was performed to quantify, over time, the number of gene copies in mixed and super-infected CEL cultures and better demonstrate virus interactions during co infection (Fig. 4), by comparing AIV or IBV yields from dual infected cells and ECE, evaluated by a multiplex rRT-PCR with those of the corresponding controls as measured independently by monoplex rRT-PCR. Such molecular analysis would demonstrate viral interference better than cell culture or ELISA titers by giving the exact gene copy numbers during mixed infections (Shengqiang et al., 2012). A standard curve was generated based on transcribed RNA analyzed in an EscoSpectrum 96 Real Time Thermal Cyclers.

Fig. 4 shows that the number of gene copies of AIV, during single infection, increased progressively from day 1 to 3 reaching 10^6 copies then decreased rapidly on the 4th day. However, when comparing these titers with those of AIV during mixed infection, it appeared that AIV or IBV growths were hindered by the presence of IBV or AIV, showing lower titers of 10^2 or 10^3 24 h p.i.; both viruses increased to reach 10^4 p.i., followed by a decrease, respectively. It is worth noting that the AIV titers appeared to be higher during mixed infection than those for IBV.

When the cells were first infected with AIV then super-infected 1 h later with IBV (Fig. 4A), a very significant inhibition of AIV growth ($p < 0.01$) was seen from day 2 up to day 4-p.i. Interestingly, when AIV infection was preceded by IBV, AIV yield increased slowly from day 2 up to day 4 but the observed titers were lower as compared to AIV single infection (Fig. 4A). The observed results were statistically significant between the experimental groups **: $p < 0.01$, as determined
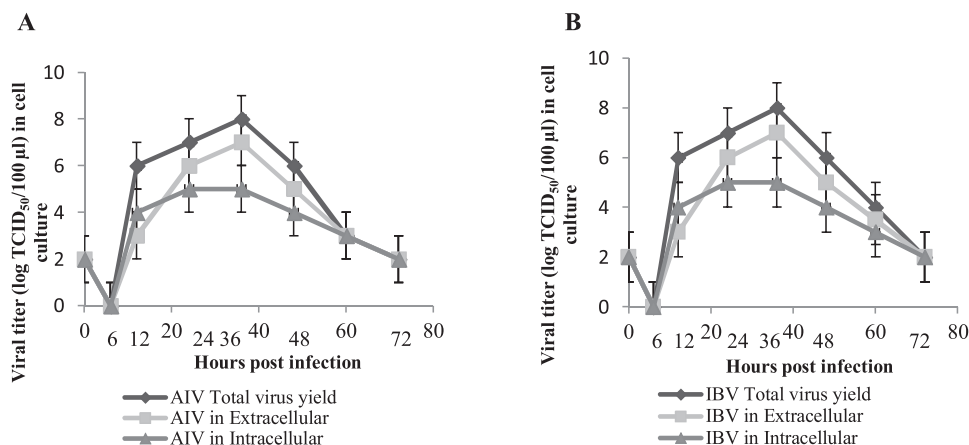
A

B

Fig. 2. Single-step growth curves in CEL infected with AIV (H9N2) strain (A) or IBV (H120 strain) (B) showing total virus yield and extracellular and intracellular virus yields, as measured by qRT-PCR.
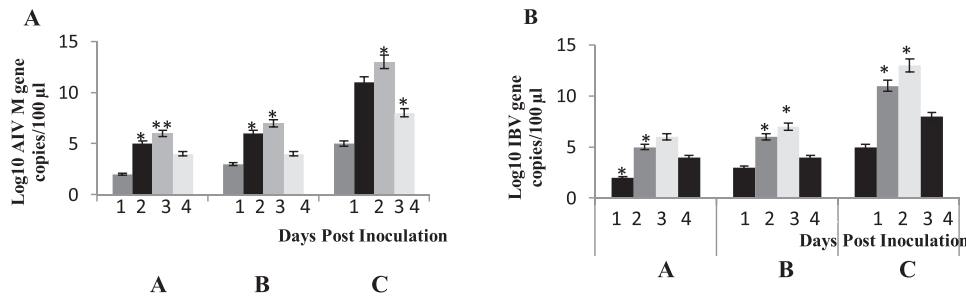
Fig. 3. Total, intra and extracellular gene copies of AIV or IBV during single infection of CEL cultures following 4 day incubation, as measured by q-PCR. A: Extracellular; B: Intracellular; C: Total Data are presented as the mean ± SD (* p ≤ 0.05; ** p ≤ 0.01 (significant increase)).

by the student's *t*-test (Fig. 4A).

As for AIV virus growth, Fig. 4B shows that during single infection, IBV growth increases progressively up to day 4 p.i. However, during mixed infection (AIV-IBV mixture), similar IBV growth was observed but the titers detected were statistically lower as compared to IBV single infection. When IBV infection was followed 1 h later by AIV, a clear inhibition of IBV replication was noticed (Fig. 4B). However, when IBV infection was preceded 1 h by AIV infection, its growth was hindered during the first 3 days p.i., followed by an increase on the 4th day to reach about 10^5 gene copies. The observed results were statistically significant between the experimental groups ($p <$ 0.01), as determined by the student's *t*-test (Fig. 4B).

Thus, the yields of AIV and IBV during mixed infection with H9N2 and H120 were significantly lower than those obtained during single infections. When both AIV (H9N2) and IBV (H120) were added together, signs of interference between the two infecting viruses were observed with an interfering effect highlighted by an inhibition of the growth of both viruses (Fig. 4).

When AIV and IBV were inoculated at 1 h interval, interference phenomenon was different from simultaneous inoculation. In fact, there was a strong inhibition of the growth of AIV after IBV super-infection and *vice versa*, during the first three days. It was clearly shown that AIV was capable of interrupting multiplication of IBV and IBV was also capable of interrupting the multiplication of AIV replication. However, on the fourth day of infection, either virus was able to grow again, even in presence of the competing virus but to a lesser level as compared to single infections.

In cases of super infection after 4 h, gene copy numbers of both viruses were similar (Fig. 5A and B). However, super infection after 8 h later showed that IBV gene copy numbers were higher than those of AIV (Fig. 6A and B). Statistical significance between experimental groups was determined by the student's *t*-test, as follows: *: $p <$ 0.05, **: $p <$ 0.01, ***: $p <$ 0.001.

The results shown in Figs. 5 and 6 demonstrated that the second infecting virus inhibits the growth of the first virus, AIV or IBV, and also when this virus was added, 4 h or 8 h later, indicating mutual inhibition between both viruses. Besides, the second virus started slowly and reached a maximum titer at 24 h p.i., which was followed by a rapid

decrease at 48 h p.i.

3.4. Cytokine expression in CEL cultures

Using the known concentrations of IL-1β detected by ELISA, a calibration curve was plotted allowing determination of the kinetics of IL-1β secretion in infected CEL cultures. Thus, during single or dual infections, significant IL-1β expression was induced by either AIV and/or IBV. Statistical significance of the results between the groups (IL-1 beta secretion due to AIV and IBV in the different cases) was determined using a one way ANOVA test, as follows: **: $p <$ 0.01 (significant increase) (Fig. 7).

Fig. 7 shows that either AIV or IBV induces IL-1β; AIV being a much better IL-1β inducer. Thus, IL expression started on day 2 p.i. and then increased progressively up to 6 days p.i. However, IL-1β synthesis was totally inhibited during simultaneous infection with AIV and IBV mixture. However, during super-infection, IL-1β secretion seemed to be better when cells are infected with IBV 1 h before AIV infection ($p <$ 0.05) (Fig. 7).

3.5. Virus interference in ovo

It is worthy to obtain information on quantitative and temporal factors that may affect viral co-infection with AIV and IBV during chicken embryo growth. Harvested allantoic fluids of infected ECE were analyzed using rRT-PCR and dead and live embryos were examined for specific lesions; contaminated eggs being discarded (Table 1).The *in ovo* results of single, mixed and super infected, done in duplicate, are shown in Table 1 and Fig. 8.

The results demonstrated 100% embryo mortality in the different infected groups (single, mixed or super infected). The infected embryos showed specific lesions such as hemorrhagic head for AIV or curled embryos for IBV groups. Surprisingly, no lesions were detected in simultaneously infected group (Table 2).

It also appeared that yields of AIV and IBV viruses from dually infected ECE were significantly lower than those in singly infected eggs. When AIV and IBV were inoculated at different time intervals, the interference phenomenon was somewhat different from simultaneous
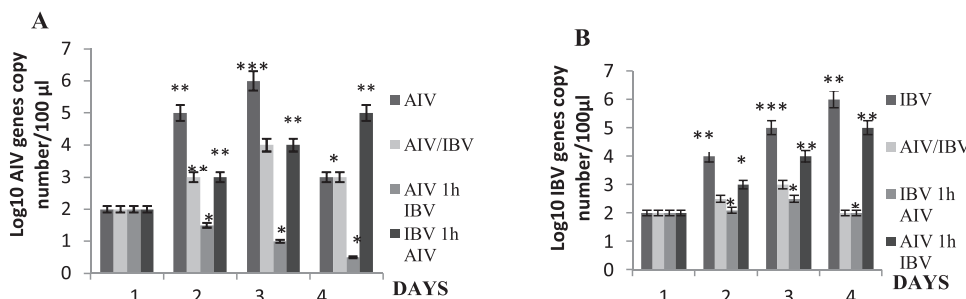

Fig. 4. Gene copy numbers of AIV (A) or IBV (B) in CEL cultures during single, dual and super infections, as measured by qRT-PCR.
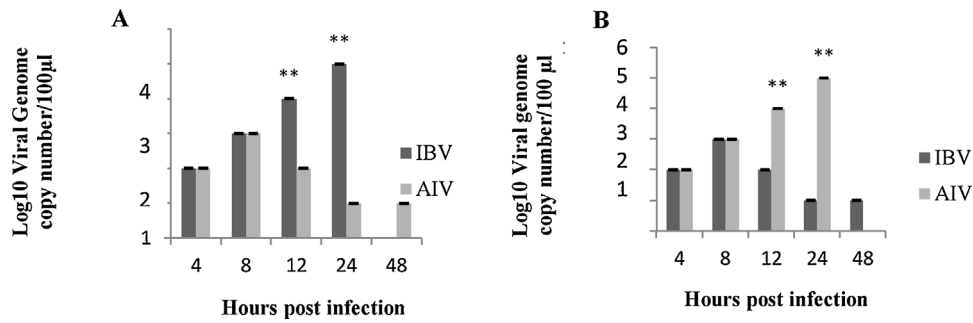
Fig. 5. Quantification of AIV and IBV viruses during *in vitro* super-infection. **A:** AIV-4 h IBV; **B:** IBV-4 h AIV.

inoculation. Thus, it was shown that AIV growth was hindered when IBV was added 4 h and 8 h later. Similar results were also obtained when IBV infection was followed by AIV super infection after 4 h or 8 h later. It is worth noting that higher growth inhibition of either AIV or IBV was observed when the second virus was added 8 h later. Statistical significance of the results between the groups (Indicated on figure) was determined using one way ANOVA test, as follows: **: $p < 0.01$ (significant increase). Bars represent the standard deviations for Log_{10} Gene copy numbers/100 μl for 7 cases of infection, co infection and super infection experiments (Fig. 8).

4. Discussion

In this study, *in vitro* and *in ovo* dual infections, simultaneously and sequentially, using AIV (H9N2) and IBV (H120), were studied in CEL cultures and ECE, respectively. The primary CEL cells were chosen since they support the growth of AIV and IBV as well as the high Ct values obtained as compared to MDCK and CEL cells infected with both viruses, indicating good viral replications. Cells were inoculated with low virus titers to allow their replication without damaging the cell layers. In fact, when cells were infected with high virus titers, the infected cells were rapidly detached and the virus did not grow enough to follow the kinetics of virus replication and interactions.

We report here a study of virus interference between AIV and IBV in terms of viral replication *in vitro* and *in ovo,* using an in-house developed rRT-PCR. The results of total viral replication as well as intra and extracellular virus yields showed that when the value of the results of the infectivity titration ($TCID_{50}$) increases, the values of the results of the rRT-PCR technique also increase and *vice versa*. This suggests that the results of these two techniques are proportional.

The practical implication of this relationship is that the virus genomic titer in cells can serve as an indicator of the rRT-PCR infectious titer. Previous report showed that there was a statistically significant correlation between the genomic titer and the rRT-PCR infectious titer (Mahsouba et al., 2017).

Using rRT-PCR, our study showed that co-infection of CEL cultures with AIV and IBV caused reduction (50%) of virus growths, with a decrease in the levels of viral replication of both viruses, indicating a competition between simultaneously inoculated viruses. This result is
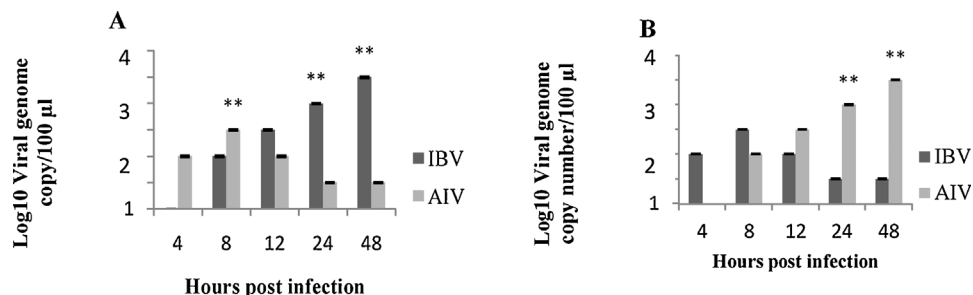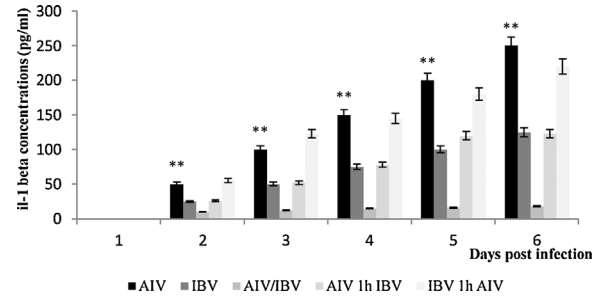

Fig. 7. IL-1βsecretions in supernatants of CEL cultures infected during single, dual, or super infection.
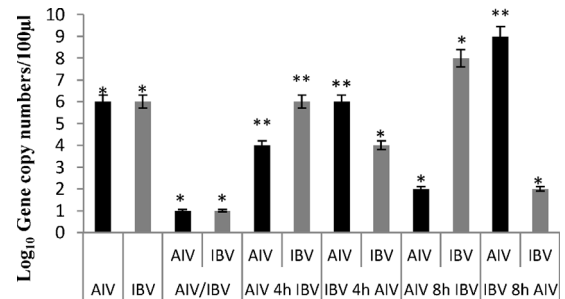

Fig. 8. Quantification of AIV or IBV in ECE in single infection, co-infection and super-infection at different time intervals (4 or 8 h), as measured by qRT- PCR.

quite different from other studies, in which they found a reduction in the replication of only one virus in case of simultaneous infection (Shengqiang et al., 2012). In fact, they have shown a slightly higher rate of replication for one virus and a relatively lower rate for the second virus (Shengqiang et al., 2012). Theoretically, when AIV and IBV are simultaneously added to cell cultures, it may be expected that interference would happen, probably in relation to direct competition for the same viral receptors on the cell surface (Ge and Wang, 2011). It has been clearly established that the cell surface receptors for AIV are sialic acid containing glycoconjugates (Ge and Wang, 2011), whereas the

Fig. 6. Quantification of AIV and IBV virus during *in vitro* super-infection. **A:** AIV-8 h IBV; **B:** IBV-8 h AIV.

Table 2
Mortalities and embryonic lesions in different groups of inoculated SPF eggs.

Groups	Mortalities	Embryonic lesions
Controls (PBS injection)	No mortality	No lesions
AIV	100 %	Hemorrhagic head; Dwarfism ; No fluff
IBV	100 %	Appearance curled; No fluff; Hemorrhagea
Mixte (AIV and IBV)	100 %	No lesions
AIV 4 h IBV	100 %	Hemorrhagea + Dwarfism; No fluff
IBV 4 h AIV	100 %	Hemorrhagic head; Dwarfism; No fluff
AIV 8 h IBV	100 %	Appearance curled; No fluff; Hemorrhagea
IBV 8 h AIV	100 %	Hemorrhagea + Dwarfism ; No fluff

cellular receptors for IBV have been proposed as α2,3-sialylated glycans (Wickramasinghe et al., 2011), both of which contain sialic acid (Ge and Wang, 2011). These findings imply the existence of common receptor sites on the permissive host cells that are shared by AIV and IBV (Murphy et al., 1999). This would raise the possibility that when both viruses are simultaneously inoculated into cultured cells, they can compete for shared virus sialic acid receptors, essential for virus attachment, and viral interference could happen, as it was clearly shown in our study (Fig. 4).

Our results also showed that there is an increased replication of the super infecting virus following blockade of the pre infecting virus. This may be because H120 and (A/Ck/TUN/145/12) viruses are weak interferon inducers, and the first inoculated virus could not interfere well with the second one (Christopher et al., 1987; Mar et al., 2014; Mar et al.,2015). Both inoculated viruses may also compete for sites or essential factors of virus replication as they are both RNA viruses, or induce formation of defective interfering particles (Kimura et al., 1976; Ge et al., 2012).

It is well known that various viral super-infection interferences do occur at the cellular level, *in vitro*, as it has been reported for the occurrence of AIV-New Castle Disease Virus, Hepatitis B Virus-Hepatitis C virus and AIV-IBV interferences (Banfield et al., 2003; Christen et al., 1990; Cockley et al., 1988), showing that the pre-inoculated virus always induces a lower growth of the super-infecting virus (Sonnenfeld and Merigan, 1979). In fact, viral interference may be broadly classified in two categories relating to either attachment or intracellular virus interactions. In previous studies (Shengqiang et al., 2012), viral attachment was reported to be mediated through blockade or destruction of available cell surface receptor sites inhibiting the super infecting virus growth; however, some exceptions have been reported (Shengqiang et al., 2012), including the results in this study demonstrating the higher growth of the super infecting virus.

The results between the CEL cultures and the *in ovo* system are clearly different. Comparison of the qualitative results obtained from this study between the CEL and the in *ovo* system is that the second inoculated virus reduced the replication of the first inoculated one causing interference between these viruses. Quantitatively, there was a clear difference in gene copy numbers of both viruses during the different experiment cases in which a number of variables were measured. In fact, the effects of varying the time intervals between the two inoculations (1 h, 4 h and 8 h) could lead to variations in the rRT-PCR results.

It is also worth noting that pro-inflammatory cytokine such as IL-1β, IL-6 and IFN play crucial roles in avian respiratory diseases in coordinating and activating the adaptive immune response, which enables the host cells to combat the pathogens (Jang and Mo, 2013). Nil and colleagues reported that expression of pro-inflammatory cytokines (IL-1 beta and IL-6) was higher in IBV infected group than in control group, suggesting that these are involved in IBV progression as they are expressed in response to viral infection, and constituting another mode of resistance of the organism (Nil et al., 2014). IL-1β was reported to be the central cytokine that accompanies inflammation, as it is implicated in promoting pulmonary tissue pathology (Kim et al., 2015). The ELISA results showed important secretion of IL-1β in CEL cells following dual infection, which could explain the interference between AIV and IBV. Results from our experiments showed that infection with one virus induced the secretion of IL-1β that reduces the first virus growth, facilitating the infection and the dominance of the second virus during the 24 h p.i. of the mixed infections. This phenomenon associated with the changes in IL-1β expression suggested that expression of pro-inflammatory cytokine is related to viral replication as recently reported by Jianlin and colleagues (Jianlin et al., 2016). Taken together, these data indicated that the presence of high levels of pro-inflammatory cytokine might be correlated with high rate of replication of the second infecting virus.

Although the time interval between inoculations seemed to have the greatest effect on the growth of the second inoculated virus, super-infection also appeared to be influenced by the nature of the primary and the secondary virus strains (Dunn et al., 2010). To achieve exclusive or maximum growth during dual infection and follow the dominance of a one virus strain over another, lower virus inputs of low pathogenicity strains were used.

The *in ovo* interference of AIV (H9N2) and IBV (H120), and *vice versa*, was also studied during simultaneous and sequential inoculations, using the ECE system. Similar rRT-PCR results were obtained, confirming those observed during *in vitro* interaction studies. In regard to AIV replication, the pre-infection of CEL cultures or ECE with IBV was accompanied by a significant increase in the replication of the second inoculated virus ($p < 0.01$.

5. Conclusion

In summary, our study showed that simultaneous dual infection 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). Our results showed that both viruses are good IL-1 inducers with AIV showing stronger induction.

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.

Ethics approval and consent to participate

We received the approval of the ethics committee on this work. Reference: 2017/05/I/LEMVIPT/V0.

Competing interests

The authors declare that they have no competing interests.

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 culture and ECE data regarding H9N2 and H120 viruses and ELISA and was a major contributor in writing the manuscript. NL performed the rRT-PCR examination and did statistical analyses of the work. All authors read and approved the final manuscript. AG corrected the reduction of the study.

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