Comparative analysis of the impact of influenza protein PB1-F2 on H5N8 virus pathogenesis in avian and human cell lines

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Introduction

Over the past years, highly pathogenic avian influenza viruses (HPAIV) from the H5Nx subtype have been responsible for recurrent epizootic diseases in domestic and wild birds, posing not only a serious threat to animals but also to public health. From October 2016 to December 2017, HPAIV H5N8 outbreaks in wild birds and poultry farms occurred in 30 European countries, resulting in massive economic losses, with no human cases reported. France was the most affected country, with 485 outbreaks reported in farms, mainly in the southwestern part of the country.

The viral protein PB1-F2 (polymerase basic 1-frame 2) is a small non-structural protein with a strong polymorphism in sequence and length (between 8 to 101 amino acids)[1]. This protein is encoded by a +1 open reading frame (ORF) overlapping the PB1 ORF. PB1-F2 is considered as a virulence factor but its role during the influenza A virus (IAV) infection is controversial. While some studies suggest that PB1-F2 contributes to viral pathogenesis in mammalian host by inhibiting type I interferon induction[2-4], investigations conduct by our group and others indicate that the expression of a functional PB1-F2 (>79 aa) during the infection leads to an exacerbated, and deleterious, inflammatory response in mammalian host[5-8]. Surprisingly, in avian host, the expression of a functional PB1-F2 reduces the inflammatory response, favors viral spreading and improves the survival of infected animals, indicating a dual role of PB1-F2 depending on the host type[9-11]. These observations were corroborated by the analysis of more than 7500 PB1-F2 sequences that highlighted that 8.6% and 38.8% of avian and human strains, respectively, encode a non-functional protein[1]. The same phenomenon also occurs in pig, among which 52.7% of isolated strains encode a truncated PB1-F2[1], suggesting that PB1-F2 is advantageous for IAV propagation in avian host but deleterious in mammalian host

Interestingly, the sequence analysis of an H5N8 strain isolated in the southwest of France (A/duck/France/161108h/2016 (H5N8) revealed that the virus encodes a truncated 11 aa-long form of the PB1-F2 protein. Taking advantage of a reverse genetic system developed for the A/duck/France/161108h/2016 (H5N8) virus, we produced i) a mutant virus that have a restored expression of a full-length PB1-F2, and ii) a reassortant virus with a PB1 gene from the A/domestic goose/Poland/33/2016 (H5N8) which encodes a full-length PB1-F2. These viruses allow us to evaluate the impact of a functional PB1-F2 protein on H5N8 virus pathogenesis in avian and human cell lines.

I H5N8 outbreak in Europe is associated with a truncation of PB1-F2

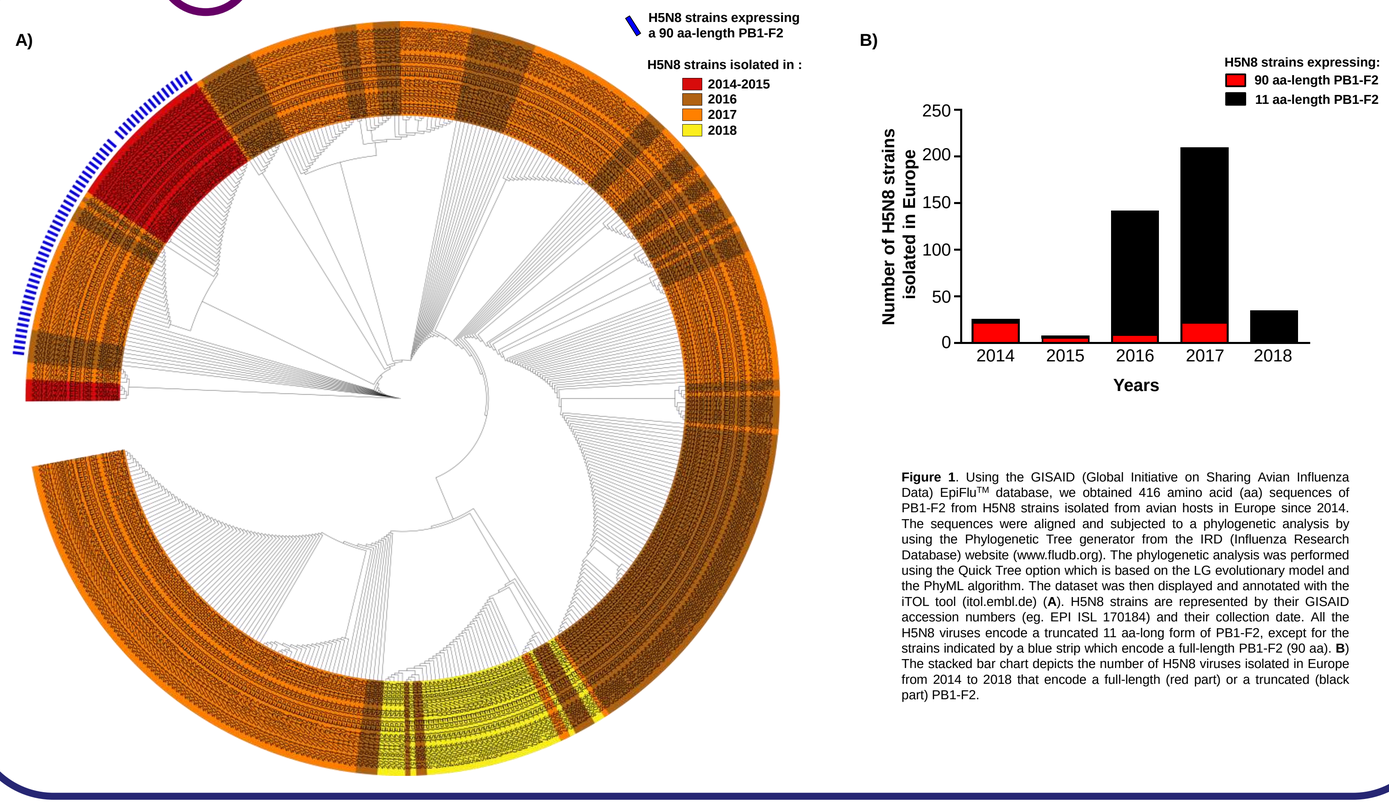

Figure 1. Using the GISAID (Global Initiative on Sharing Avian Influenza Data) EpiFlu™ database, we obtained 416 amino acid (aa) sequences of PB1-F2 from H5N8 strains isolated from avian hosts in Europe since 2014. The sequences were aligned and subjected to a phylogenetic analysis by using the Phylogenetic Tree generator from the IRD (Influenza Research Database) website (www.fludb.org). The phylogenetic analysis was performed using the Quick Tree option which is based on the LG evolutionary model and the PhyML algorithm. The dataset was then displayed and annotated with the iTOL tool (itol.embl.de) (**A**). H5N8 strains are represented by their GISAID accession numbers (eg. EPI ISL 170184) and their collection date. All the H5N8 viruses encode a truncated 11 aa-long form of PB1-F2, except for the strains indicated by a blue strip which encode a full-length PB1-F2 (90 aa). **B**) The stacked bar chart depicts the number of H5N8 viruses isolated in Europe from 2014 to 2018 that encode a full-length (red part) or a truncated (black part) PB1-F2.

II Restored H5N8 PB1-F2 colocalizes with mitochondria

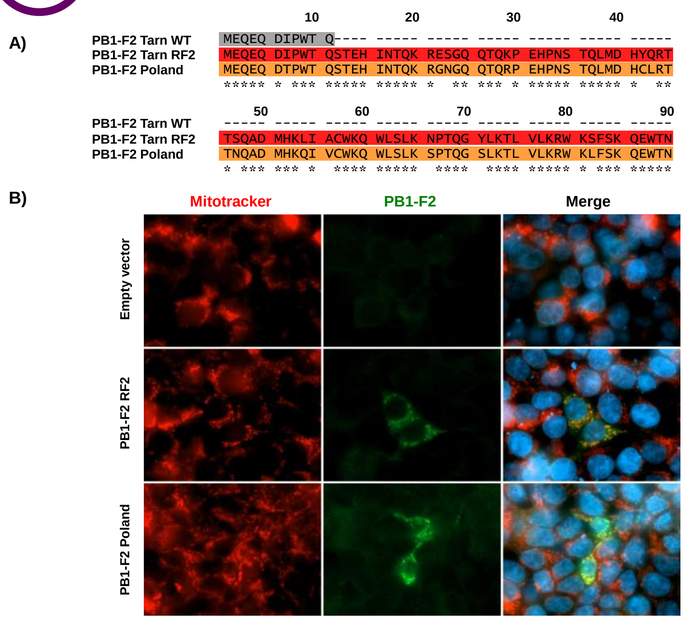

Figure 2. To evaluate the role of PB1-F2 during H5N8 infections, we developed a reverse genetic system for the A/duck/France/161108h/2016 (H5N8) (EPI ISL 240012) virus isolated in the south-west of France (in the Tarn department). Each viral segment was cloned into the bidirectional transcription plasmid pHW2000 (kindly provided by R. Webster , St Jude Children's Research Hospital, Menphis, USA). Since the A/duck/France/161108h/2016 (H5N8) virus encodes a truncated 11 aa-long form of PB1-F2, we generated a viral PB1 segment encoding a full-length PB1-F2 by mutating the premature stop codon (at position 12) into a serine. In addition, we also cloned a viral PB1 segment derived from a strain encoding a full-length PB1-F2 (A/domestic goose/Poland/33/2016 (H5N8), kindly provided by E. Swieton, National Veterinary Research Insitute, Poland) into the pHW2000 vector. Aligment of the three PB1-F2 is depicted in (**A**). PB1-F2 variants are abbreviated as follows: Tarn WT, A/duck/France/161108h/2016 (H5N8); Tarn RF2, A/duck/France/161108h/2016 (H5N8) with a restored expression of PB1-F2; Poland, A/domestic goose/Poland/33/2016 (H5N8). **B**) Human embryonic kidney 293T cells were transfected with an expressing vector encoding PB1-F2 Tarn RF2 or PB1-F2 Poland. Twenty four hours post-transfection, the cells were treated with the Mitotracker Red FM (Thermo Fisher) for 1h, fixed, immunolabeled with an anti-PB1-F2 antibody and analysed by wide-field microscopy,

III H5N8 PB1-F2 expression does not change viral growth

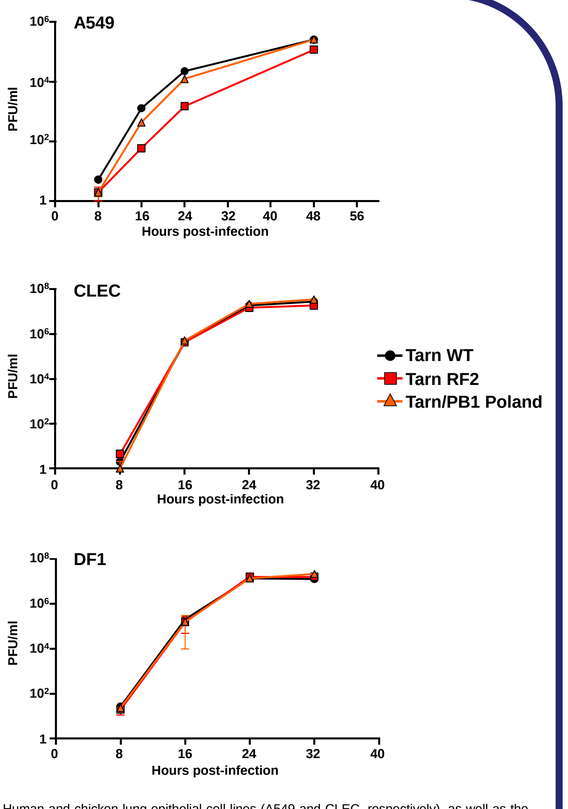

Figure 3. Human and chicken lung epithelial cell lines (A549 and CLEC, respectively), as well as the chicken fibroblast cell line DF1 were infected at a multiplicity of infection of 0,1 (A549) or 0,0001 (CLEC and DF1) with the A/duck/France/161108h/2016 (H5N8) virus (Tarn WT), the A/duck/France/161108h/2016 (H5N8) virus mutated to express a full-length PB1-F2 (Tarn RF2), or the reassortant A/duck/France/161108h/2016 (H5N8) virus containing a PB1 segment derived from A/domestic goose/Poland/33/2016 (H5N8) (Tarn/PB1 Poland). At the indicated time points, cell supernatants were collected to evaluate the number of infectious viral particles by plaque assay in MDCK cells.

IV H5N8 PB1-F2 exacerbates the inflammatory response in avian cells whereas it impairs this response in human cells

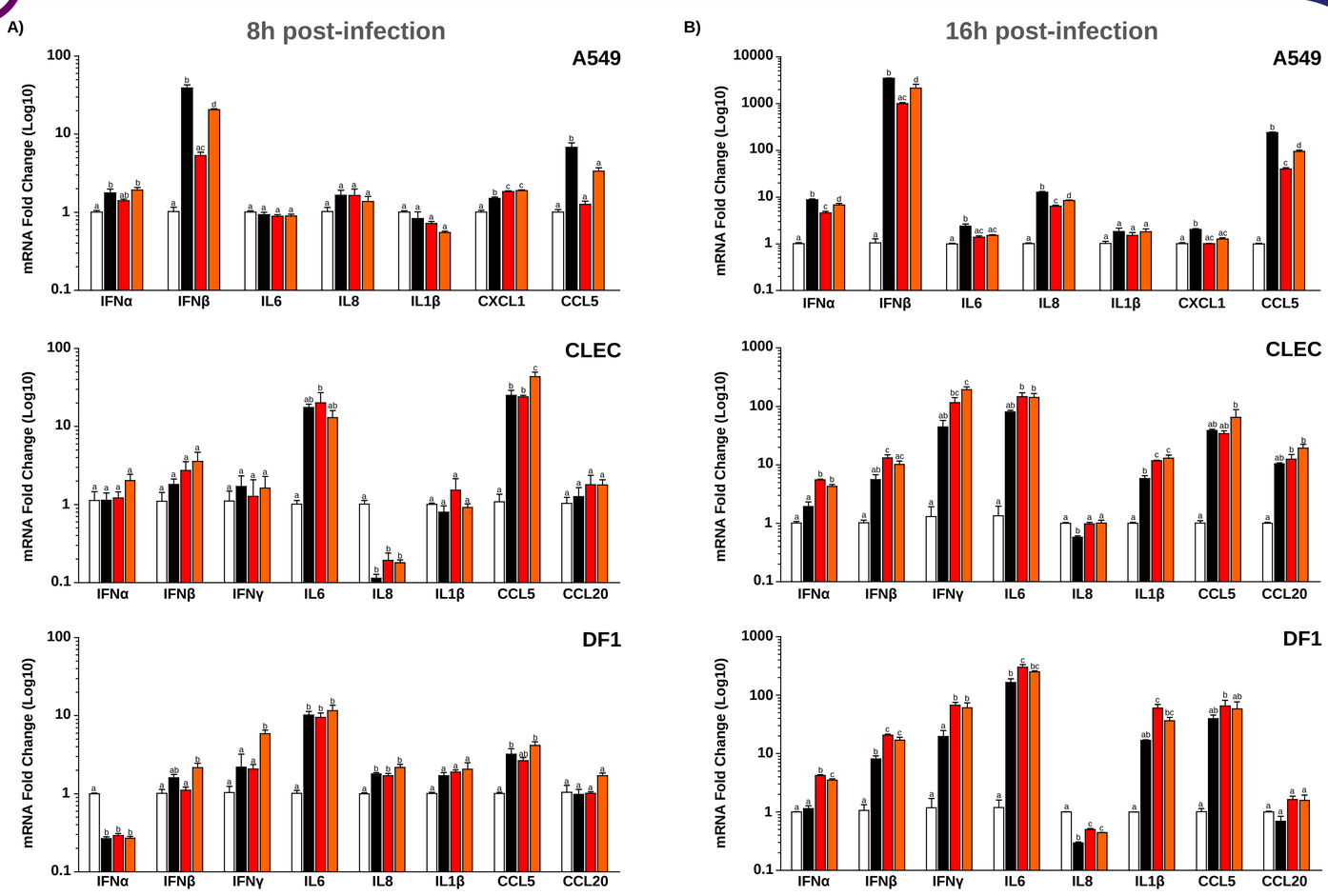

Figure 4. Human and chicken lung epithelial cell lines (A549 and CLEC, respectively), as well as the chicken fibroblast cell line DF1 were mock-infected of infected at a multiplicity of infection of 2 with the A/duck /France/161108h/2016 (H5N8) virus (Tarn WT), the A/duck/France/161108h/2016 (H5N8) virus mutated to express a full-length PB1-F2 (Tarn RF2), or the reassortant A/duck/France /161108h/2016 (H5N8) virus containing a PB1 segment derived from A/domestic goose /Poland/33/2016 (H5N8) (Tarn/PB1 Poland). Eight (A) or 16 hours (B) post-infection, the cells were lysed, and total RNA was extracted and reverse transcribed to quantify the expression of inflammatory genes by real-time qPCR. Gene expression was normalized to the expression level of GAPDH and presented as a fold increase relative to mock-infected cells. Data are means ±SEM of triplicates. Means with different letters are significantly different (one-way ANOVA test, $p < 0.05$).

Conclusions

Our first data shed in light that the H5N8 epizootic outbreak in Europe during 2016-2017 is concurrent with the loss of function of PB1-F2. Interestingly, our results revealed that restoration of a full-length PB1-F2 in an H5N8 virus increases the inflammatory response developed by infected avian cells. Even though the expression of PB1-F2 appears not to alter the viral growth of H5N8 viruses in these cells, we may assume that PB1-F2 could have a deleterious effect on H5N8 propagation in avian host, which may explain, in part, the loss of a functional activity of this protein. Conversely, our study also shows that restoring a functional PB1-F2 in the context of an H5N8 virus leads to a weaker inflammation in human cells. This feature may partly explain why no human cases were reported during the epizootic, even though a significant number of people were in contact with infected animals.
Surprisingly, data obtained in this work are very different from what we had previously reported in avian and mammalian models. To date, no answer can be given to this discrepancy. An approach using reassortant H5N8 viruses expressing PB1-F2 variants from different strains is under way to address this question.

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