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Knowns and Unknowns of Influenza B Viruses

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ABSTRACT (Words: 103)

Influenza B (FluB) viruses circulate annually along with influenza A (FluA) strains during seasonal epidemics. FluB can dominate influenza seasons and cause severe disease, particularly in children and adolescents. Research has revealed interesting aspects of FluB and highlighted the importance of these viruses in clinical settings. Yet, many important questions remain unanswered. In this review, the clinical relevance of FluB is emphasized, unique features are highlighted and gaps in knowledge pinpointed. Multiple aspects of FluB epidemiology, evolution, virology and immunology are discussed. Future research into FluB is needed to understand how we can prevent severe disease in high-risk groups, especially children and elderly.

In 1940, an outbreak of acute respiratory illness in children with clinical presentations identical to the contemporary influenza A epidemics led to the isolation of a new virus that was serologically distinct from previous influenza A isolates. This novel influenza virus was named Influenza B [1].

It is now well established that influenza A (FluA) and Influenza B (FluB) viruses co-circulate annually within the human population and cause respiratory illness with varying levels of severity. Unlike FluA, FluB does not have an established animal reservoir and thus does not pose the same pandemic risk as FluA [2]. Consequently, FluB has received considerably less attention. Nonetheless, FluB presents a substantial challenge during annual epidemics. Although current knowledge of FluB viruses highlights their unique features, a plethora of questions on the virology, immunology, epidemiology and evolution of FluB remains to be elucidated.

FluB viruses belong to the family of Orthomyxoviruses and have a segmented negative-sense single-stranded RNA genome. FluB has 8 segments and encodes for 3 polymerase proteins (PA, PB1 and PB2), the nucleoprotein (NP), the non-structural protein (NS1), the nuclear export protein (NEP or sometimes called BNS2), the matrix protein (BM1), the BM2 ion channel and three surface glycoproteins (HA, NA and NB) [3] (Fig 1A-B). FluA and FluB proteins generally show substantial differences in length, amino acid composition and function [2]. FluB viruses are currently divided into two genetically and antigenically distinct lineages, B/Victoria/2/1987-like and B/Yamagata/16/1988-like viruses [4-6].

Epidemiology and Clinical Presentation of FluB

FluB viruses from one or both lineages co-circulate annually with the seasonal H1N1 and H3N2, with FluB being the main cause of the seasonal epidemic every 2-4 years [7,8]. Multiple lines of evidence suggest that FluB can cause substantial disease [9-11]. In an analysis of influenza-related mortality between 1976 and 1999 in the US, highest morbidity was associated with H3N2, followed by FluB and then by H1N1 [12]. This highlights that FluB infections can cause a significant degree of morbidity, although the analysis was conducted in 2009 and the above hierarchy might have changed with the establishment of the H1N1pdm strain in seasonal epidemics. Regardless, various reports highlight a strong presence of FluB in seasonal epidemics across the world. An epidemiological analysis of influenza-attributable mortality in the US between 1997 and 2009, reported that 29% of total influenza associated deaths were attributable to a FluB infection. Importantly, in 4 out of 12 seasons FluB infection was the predominant cause of seasonal influenza associated mortality [13]. In the 2008 season in Australia, 54% of influenza notifications were due to FluB infections, and the majority of these infections were in children [14]. In England, in the first half of the 2012/2013 season, FluB was the predominant cause of influenza infections, with the highest activity occurring in children aged 5-14 [15]. Additionally, in Thailand between 2010 and 2014 the majority of FluB infections occurred in children aged 5-19 [16]. These are only a few studies indicating that FluB can cause severe disease, especially in the young. The factors determining the predominance of FluB over FluA in a given season are largely unknown, but most likely depend on specific viral characteristics and interactions from all three circulating viruses.

The symptoms of FluB infection include a sore throat, coughing, nasal discharge and fever [17]. Overall, disease caused by FluB is indistinguishable from that of FluA [18], although complications can differ. Complications of FluB can include neurological and muscular manifestations [19,20], cardiologic complications [21-23] and secondary bacterial infections [22,24,25]. Paddock *et al* (2012) observed that secondary bacterial pneumonia was more frequently observed in adult fatal cases of FluB than pediatric fatal cases, and the reverse was observed for cardiac complications. The same study also determined that in fatal cases of influenza, the time from disease onset to death was shorter for FluB than that determined for the 1918, 1957, 1968, 2009 pandemic FluA or seasonal H3N2 [22]. It is however important to assess the validity of this observation in other FluB cohorts. It also appears that lineage does not impact disease outcome [26]. As mentioned above, although FluB can infect adults and the elderly, disease manifestations are more prevalent in children [27-29]. Moreover, FluB viruses of the Victoria lineage are more prevalent in younger populations than Yamagata viruses [6,30,31]. It has been proposed that this could be explained by higher expression of α-2,3 linked sialic acid glycans in children than in adults and the differential α-2,3 and α-2,6 sialic acid glycan binding preferences of viruses from the two lineages [6,32]. Overall, FluB can cause significant disease, particularly in children. The viral and host determinants of FluB pathogenicity and the reasons of increased susceptibility of young children remain to be elucidated.

Evolution and Phylodynamics of FluB

In the early decades of the 20th century FluB viruses belonged to a single lineage. Two antigenically distinct lineages emerged prior to 1980 [4-6] and now co-circulate globally, with one lineage usually predominating in any given year, although equal circulation of the two lineages in certain areas has been reported. For instance in the 2011-2012 season in Canada, 24% of all influenza cases were caused by B/Victoria-like viruses and 27% were of B/Yamagata-like origin [33]. In general, after the appearance of the two lineages, Victoria-like viruses dominated in the late 1980s, while viruses of the Yamagata lineage prevailed in the 1990s, with the exception of an outbreak of Victoria-like viruses in Asia in 1996/1997. The Victoria lineage re-emerged globally in 2001, and the two lineages have co-circulated ever since [34]. Interestingly, while new H3N2 viruses seem to originate from East and Southeast Asia in every season, FluB strains can persist during multiple seasons and re-seed subsequent epidemics [35,36]. This appears to be linked to less frequent global movements of FluB compared to H3N2 FluA [35].

Evolution of FluB involves a constant turnover of antigenically-distinct viruses, in a process similar to the antigenic drift of FluA, as well as reassortment. The evolution rate of FluB is slower than that of FluA, as calculated by the rate of nucleotide substitutions for each segment. ($0.14x10^{-3}$–$3.32x10^{-3}$ substitutions/site/year for FluB, $2.68x10^{-3}$ – $12.5x10^{-3}$ substitutions/site/year for FluA H3N2) [34,37]. HA exhibits the highest rate of adaptive evolution, suggesting a degree of positive selection, depicting the effect of immune evolutionary pressure [5,34] Additionally, the NA and NS1 segments exhibit high levels of adaptive evolution, possibly due to immune

pressure [34]. In addition to these changes, reassortment also contributes to FluB genetic diversity. Reassortment can occur between viruses of the same lineage or viruses of the two distinct lineages [5,6,34,38]. Inter-lineage reassortment, in which Victoria viruses acquire genes from the Yamagata lineage have been more frequent than the reverse [6]. Interestingly, different segments can exhibit different patterns during reassortment events. Two bioinformatics studies revealed that only the PB1, PB2 and HA segments have maintained separate Victoria or Yamagata lineages. In contrast, the NS1/NS2 segment of current strains is derived from the Victoria lineage, while the rest of the segments (PA, NP, NA/NB, M1/BM2) come from the Yamagata lineage, suggesting substantial exchange of genetic information between the two lineages [6,38]. The observation that novel reassortant viruses can dominate the viral population implies that reassortment can generate novel strains with increased fitness.

Intriguingly, the two lineages exhibit different phylodynamics. Distinct clades of Victoria viruses generally circulate for 1-3 years before being replaced by new clades, while multiple clades of Yamagata viruses can co-circulate for longer periods of time. Additionally, Victoria viruses exhibit great genetic diversity between seasons and a stronger antigenic drift compared to Yamagata viruses. Furthermore, viruses of the Victoria lineage contain more amino acid substitutions in the HA and these are located near the receptor binding site of the HA, while changes in Yamagata viruses are less frequent and tend to appear in sites more distant to the receptor binding site. Overall, the Yamagata viruses are more conserved and show weaker antigenic drift and selection than the faster evolving Victoria viruses [6].

In addition to these evolutionary forces, interactions seem to occur between FluA and FluB. For instance, FluA (H1N1pdm09) infection can prevent FluB infection within a short interval of time but the opposite is not true, in the ferret model [39]. Additionally, analysis of FluA and FluB viruses over 30 years revealed that in certain years, strong genetic bottlenecks have been imposed on FluB viruses and these were associated with high prevalence of FluA. Such bottlenecks have led to changes in the dominant FluB antigenic cluster[34].

In summary, FluB evolution is a complex function of interactions between FluB viruses of the different lineages, interactions between co-circulating FluB and FluA viruses as well as the selective pressures from the host's immune mechanisms.

Virology of FluB

The Haemagglutinin (HA)

The HA of FluB mediates attachment and entry of the virus into cells via interaction with sialic acids. FluB HA binds to sialic acids with lower affinity than FluA [40,41]. Also, the FluB HA can interact with both α-2,6- and α-2,3-linked sialic acids. Despite their low sequence homology (~20-30%), the overall structures of FluB and FluA HAs are similar. Similarly to FluA, the FluB HA has an elongated membrane-proximal domain and a globular membrane-distal domain. The functional form of the FluB HA is a homotrimer. The Receptor Binding Site (RBS) of FluB HA has some significant differences to that of FluA HA. One important difference is located at amino acid position 95

188 (FluB HA numbering), which in FluB is a phenylalanine, while in FluA is a
189 highly conserved tyrosine [40,42]. The Phe95 residue has been shown to be
190 involved in the ability of FluB HA to interact with both human-like and avian-
191 like receptor analogues and with the lower affinity of FluB HA. Replacement of
192 Phe95 with a tyrosine residue increased receptor binding to levels
193 comparable to FluA and reduced binding to avian-like receptor analogues
194 [43]. The role of the HA protein in determining pathogenicity of FluA is well
195 established [44], however this has not been studied for FluB.
196
197 **The Neuraminidase (NA)**
198 The overall structure of FluB NA is similar to that of FluA NA, despite
199 only 30% sequence homology. The active form of the FluB NA is a tetramer
200 and each monomer contains two glycosylation sites (Asn283 and Asn143)
201 and a calcium-ion binding site. Asn283 is specific for FluB, while Asn143 is
202 conserved among Influenza viruses [45]. Nineteen highly conserved residues
203 form the NA active site of influenza A and B viruses. Residues in the catalytic
204 site (R118, D151, R152, R224, E276, R292, R371, and Y406) directly interact
205 with sialic acids, while framework residues structurally support the enzymatic
206 active site (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294,
207 and E425). Neuraminidase inhibitors (NAIs) licensed in the clinic for treatment
208 of influenza infection appear to be less effective against FluB [46].
209
210 **The NB Protein**
211 The NB protein is uniquely encoded by FluB in segment 6, overlapping
212 with the NA coding sequence and is not present in FluA. The start codon of

NB is located 4 nucleotides upstream of the NA start codon [47]. NB is an integral membrane glycoprotein of 100 amino acids that is incorporated into the virion [48]. The N-terminus contains two N-glycosylation sites, which are further modified by the addition of multiple N-acetyl-glucosamine residues [49]. Palmitoylation of C-terminal residues is important for trafficking of NB to the cell surface [50]. NB is dispensable for virus rescue and *in vitro* replication [51] (unpublished observations Elderfield, Koutsakos & Barclay). Hatta and Kawaoka observed attenuation in absence of NB in BALB/c mice [51], however, this could be due to an altered NA activity as the mutations introduced have been previously reported to affect the NA expression [47]. In contrast, our data suggest that a NB-knockout virus with unaffected NA activity replicates equivalently to the WT virus in C57B/6 mice. This mutant virus showed no fitness cost in ferrets and also transmitted to a new host, although overall transmission in that study was low (unpublished observations Elderfield, Koutsakos & Barclay). This apparent redundancy of NB is surprising and could be due to a host-specific role of NB. Additionally, the *in vivo* role of NB has only been assessed in the early stages of infection (up to day 4). It is possible that NB plays a role in the later stages of FluB infection.

In various experimental settings, NB has displayed the ability to conduct ions. In *Xenopus* oocytes, NB could stimulate the conductance of Cl^- ions [52], while in *E.coli*-derived membranes NB was permeable to protons [53]. Additionally, purified protein and peptides corresponding to the transmembrane region of NB incorporated into artificial lipid bilayers could form cation-selective channels [54,55]. A review of these studies questioned the observed ion channel activity due to the limitations of the experimental

systems utilized, especially as previous observations showed that membrane proteins with no known ion channel activity were able to form multimers and conduct ions in similar experimental settings [56]. Subsequently, it was reported that a single amino acid substitution (S20A) in the NB transmembrane domain resulted in altered gating and proton permeability, supporting the idea of a NB ion channel. However, unlike the AM2, BM2, and CM2 ion channels of FluA, FluB and Influenza C viruses, NB has no pH-modulating activity in the *trans*-Golgi network (TGN), which the AM2, BM2 and CM2 ion channels do [57]. It is therefore unclear whether NB operates as an ion channel. Overall, more than 30 years after the discovery of NB, its role and function remain enigmatic. Understanding the role of NB could benefit the design of novel live-attenuated vaccines [51].

The Matrix Protein (BM1)

Very little is known about the M1 protein of FluB, which is encoded in segment 7. M1 plays an important role in FluB adaptation in mice and attenuation of the master donor vaccine strain (B/Ann Arbor/1/66) of the Live Attenuated Influenza Vaccine (LAIV) [58]. The functional role of M1 in the life cycle of FluB is not entirely known. M1 contains a Nuclear Localization Signal (NLS), two Nuclear Export Signals (NES) and can act as a shuttling protein between the nucleus and the cytoplasm in infected cells. All of these features were necessary for the function of M1, as they were required for the production of viable virus [59]. Thus, the exact role of BM1, and whether it is of a similar function to that of FluA M1, remains to be determined.

The BM2 Ion Channel

Like in FluA, segment 7 encodes for a second protein, namely BM2 [60]. The BM2 protein is a small hydrophobic, integral membrane protein [61] that is synthesized in the late stages of infection [62]. The protein is transported through the TGN to the cell surface [63] and is incorporated in the virions [62]. The N-terminus of the protein appears to be important for its transport within infected cells [63]. An interaction of BM2 with M1 has been proposed [64], supporting previous observations that BM2 is involved in the incorporation of the vRNP in the virions [65]. Additionally, BM2 is necessary for the production of viable virus [65-67] and decreased incorporation of BM2 in virions reduces infectivity, indicating a role for BM2 similar to that of AM2 [66]. Indeed, BM2 contains a HXXXW motif like AM2 and thus can conduct ions. This activity is similar to the one of AM2 in acidifying the endosome and modulating pH in the Golgi [68]. The crystal structure of BM2 reveals similarities to AM2 but also unique features. BM2 forms coiled-coil tetramers, with polar residues lining the pore that is formed, a feature important for the inactivity of adamantanes against BM2 [69]. Zhang *et al* , recently suggested a novel role for BM2 in inhibiting p53-mediated transcription and apoptosis [70].

A unique feature of BM2 is its expression mechanism. The BM2 coding sequence lies directly downstream of the M1 coding sequence and the two are translated from the same mRNA molecule. The translation termination codon of M1 overlaps with the start codon of BM2 in the following penta-nucleotide sequence: UA**AUG** (M1 stop-codon underlined, BM2 start codon in bold) [71]. The 45 nucleotides in the M1 coding sequence upstream of penta-

nucleotide appear essential for BM2 expression [72]. Additionally, the sequence upstream of the penta-nucleotide exhibits complementarity to the 18S component of the small ribosomal subunit [73]. An interaction between the mRNA and the rRNA as well as the involvement of the translation initiation factor eIF3 are important for BM2 expression [74]. This termination:reinitiation mechanism was the first one of its kind when initially identified, but since then similar mechanisms have been identified in other viruses, like caliciviruses [73].

The Nucleocapsid Protein (NP)

The NP of FluB (BNP) is encoded by segment 5. It differs greatly from the NP of FluA (ANP). The most striking differences are the lack of the two NLSs of ANP and the extended N-terminus of the protein in FluB. Specifically, BNP contains 50 amino acids in its N-terminus that are not found in ANP [75].

Structural studies revealed that BNP consists of a head, a body and a flexible tail loop. The groove between the head and the body contains an extended charged loop (residues 125-149), within which there are two lysine clusters critical for RNA binding. The flexible tail loop of BNP enables the assembly of homo-oligomers. Comparisons with the structure of ANP revealed an interesting similarity. In both proteins, a highly conserved salt bridge (R472-E395 in BNP, R416-E339 in ANP) seems to be involved in the stability of the loop and thus the assembly of homo-oligomers [76]. Pharmacological inhibition of this salt bridge in ANP can inhibit FluA [77], and thus might be a possible drug target for FluB.

312 The N-terminus of BNP has been of particular interest, as it is
313 considerably different from ANP. Wanitchang *et al* showed that the N-
314 terminus of BNP is required for nuclear localization and suggested that the
315 $K_{44}RTR_{47}$ motif in BNP might be acting as a NLS [78]. Reverse genetics were
316 later employed to generate FluB lacking the N-terminus of BNP, in order to
317 investigate the role of the N-terminus of BNP in the context of infection and
318 viral replication. Different mutations in the N-terminus affected the ability to
319 rescue virus or viral replication and transcription. Moreover, the entire N-
320 terminus appears to be essential for optimal localization in the nucleus during
321 infection and residues 51-81 were suggested to contain a bipartite NLS [79].
322 The NP protein of FluA is known to have multiple functions that are crucial for
323 effective virus replication, however, there are no similar studies for BNP.
324 Current knowledge suggests that BNP has features similar to ANP but also
325 possesses some unique domains, suggesting that at least some functions of
326 the two proteins are unique.
327
328 **The Polymerases (PA, PB1 & PB2)**
329 Although there is a vast amount of knowledge about the polymerases
330 of FluA, minimal data exist on the polymerase proteins of FluB, with the
331 general view being that they work similarly to those of FluA. However it is
332 pertinent that the FluB heterotrimeric polymerase complex structure was
333 solved before that of a human or avian FluA virus [80]. Indeed, similarities
334 exist, although recent studies suggest key differences. Localization studies
335 showed that all three FluB proteins have inherent nuclear import function and
336 encode NLSs. PB2 was found to predominantly localize in the nucleus, while

PB1 and PA were localized both in the nucleus and the cytoplasm. Pairwise interactions for all three proteins were also observed and localized in the nucleus. These observations are similar to what has been observed for FluA polymerase proteins [81].

A major difference between FluA and FluB polymerases is their cap-binding mechanism. Specifically FluB polymerases can bind to and cleave both m^7G-capped and unmethylated G-capped mRNAs. This is in contrast to FluA polymerases that exclusively bind to and cleave m^7G-capped mRNA [82]. Structural studies suggested that the overall structure and binding of the polymerases to the vRNA is similar between FluA and FluB [80], but also elucidate the details of the unique cap-binding mechanism of FluB [83]. The polymerases of FluA have central roles in host-adaptation and pathogenicity [44,84]. Conversely, the contributions of FluB polymerases to pathogenicity and host-restriction are unknown.

The Non-Structural 1 Protein (NS1)

The NS1 protein of FluB (NS1B) is a 281 amino acid long protein encoded by segment 8. NS1B is important for virus replication as knock-out viruses display impaired replicative capacity both *in vitro* [85] and *in vivo* [86]. It consists of an N-terminus (residues 1-90), a C-terminus (residues 120-281) and a linker domain (residues 91-119) [87]. NS1B localizes in the nucleus of infected cells in the early stages of infection, but mostly in the cytoplasm in the later stages. Residues 46-56 in the N-terminus function as a monopartite NLS and NS1B interacts with importin α3. Within the nucleus, NS1B localizes in nuclear speckles containing splicing factor SC35 [88]. Although NS1B can

bind RNA via its N terminus it cannot not inhibit export of host mRNA like NS1A does, as it does not interact with CPSF30 via its C terminus [89].

NS1B can antagonize multiple aspects of the interferon antiviral response [85,90]. Firstly, it can inhibit the IFN$\alpha\beta$ response by preventing activation and nuclear translocation of IRF3. Particularly, FluB lacking NS1B induces stronger IFNβ expression and activation of the IFNβ promoter than WT FluB [85]. Additionally, ectopic overexpression of NS1B can inhibit IFNβ promoter activity [90]. Interestingly, NS1B can complement the attenuated growth of ΔNS1A FluA [90]. All these studies implicate NS1B as a potent inhibitor of the IFN response. Both the N- and C- termini are involved in this function. The N-terminus of NS1B sequesters dsRNA to prevent activation of the antiviral PKR kinase, while the C-terminus is more involved in IFNβ promoter inhibition [91], although either termini could independently prevent activation of IRF-3 [90]. Interstingly, NS1B knock-out viruses are attenuated in IFN-deficient Vero cells [85], suggesting that NS1B has additional effects seperate of counteracting the IFN response.

NS1B also can directly bind to and block the interferon-stimulated Ubiquitin-like ISG15 protein, which is naturally conjugated to newly synthesized proteins [92]. By binding to ISG15, NS1B prevents activation of ISG15 by the UBE1L enzyme, and this activity is mediated by the N-terminus of the NS1B protein [87,93]. Published evidence suggests that NS1B re-localizes the bound ISG15 to the nuclear speckles [94]. NS1B specifically interacts with human and non-human primate ISG15, but not with other mammalian ISG15-like mouse or canine, implicating the ISG15-NS1B interaction in the host-restriction of FluB [94].

Overall, NS1B has a key role in innate immune response evasion, like NS1A, although the mechanism seems to be different for the two viruses. The unique targeting of ISG15 suggests that this interferon stimulated gene would otherwise play an important antiviral role against influenza B virus.

The Nuclear Export Protein (or Non-Structural 2) Protein (NEP)

The NEP protein of FluB interacts with the nuclear export machinery, contains a Nuclear Export Signal (NES) sequence in its N-terminus and possesses nuclear export activity [95]. Furthermore, it is produced in the late stages of infection, initially accumulating in the nucleus, then being transported to the cytoplasm and finally, in plasma membrane proximal regions, the sites of virion budding. NEP can also be detected in virions as part of the vRNP complex. Interestingly, in FluB NEP seems to interact directly with the vRNP and BM1 as opposed to FluA where M1 acts as a bridge between NEP and the vRNP [96]. Overall, FluB NEP appears to have a similar function to its FluA counterpart, although its precise function is yet to be determined.

Non-coding Sequences

The coding sequences of Flu viruses are flanked by stretches of non-coding sequences in both termini. These sequences form the promoter that drives viral replication and transcription and also contain packaging signals [3]. In the specific context of FluB, they have shown to be important for vRNA replication [97]. The sequences at the extreme of each terminus are highly conserved. Specifically, the first 9 nucleotides of the 3' vRNA end (5'-

412 AGCAGAAGC-3’ in cDNA) and the first 10 nucleotides of the 5’ vRNA end
413 (with the exception of position 6) (3’- TCATCxTTGT-5’ in cDNA) are highly
414 conserved among segments and strains (Fig 1C). The sequences in the two
415 termini show a high degree of complementarity and thus form secondary RNA
416 structures. Between these extreme terminal sequences and the coding
417 sequences, lie non-coding sequences that are conserved for each segment
418 but differ between segments [98]. Understanding the non-coding sequences
419 of influenza viruses has been pivotal in the generation of reverse genetics
420 systems[2,98-100] as well as the design of novel vaccination strategies [101].
421
422 **Immunology of FluB**
423 **Innate Immune Recognition and Response**
424 As with other aspects of FluB immunology, the innate immune
425 response has been largely unexplored. The recognition receptors for FluB
426 have not been identified but RIG-I seems to be essential in the response to
427 FluB. MDA-5 might play an additional, although not essential, role in initiating
428 an innate response against FluB [102]. PKR can also detect viral RNAs and
429 inhibit replication of FluB both in cell culture and in mice, as highlighted by the
430 inhibition of PKR by NS1B. Activation of PKR was temporally linked to the
431 appearance of viral RNPs in the cytoplasm and purified vRNPs could activate
432 PKR activity *in vitro* [103]. FluB induces IFN-dependent and independent
433 responses through activation of IRF3 [104]. The MAP Kinase and NFκB
434 pathways can be activated. Interestingly, the innate immune recognition of
435 FluB occurs earlier in infection of dendritic cells than FluA (peak IFN-λ mRNA
436 at 2-4 hours for FluB and at 8-16 hours for FluA) and is independent of viral

replication or transcription [105]. The mechanism behind this early recognition is not yet understood.

In response to *in vitro* FluB infection, epithelial and innate cells can produce type I and III interferons, specifically IFNα, IFNβ, IFNλ1, and other cytokines/chemokines, including CXCL10, CXCL8, CCL5, CCL2, IL1-β, IL-18 and IL-6, although the specific levels and roles remain to be investigated [105-107]. Similarly, although γδ-T cell hybridomas can respond to both FluA- and FluB-infected cells [108], the roles and functions of γδ-T cells or other innate immune cells in controlling FluB are unknown.

Antibody Responses

Similarly to FluA, the vast majority of antibody responses against FluB are directed at the HA. The four main antigenic sites are in the globular head of the HA1 and include the 120 loop (residues 116-137), the 150 loop (residues 141-150), the 160 loop (residues 162-167) and the 190 loop (residues 194-202) [42]. These sites of the HA are under positive selection, indicating the effect of the antibody response to the evolution of the virus [109]. Antibodies against the NA can also neutralize FluB and protect mice from sub-lethal infections. Interestingly, immunization with B/Yamagata/16/88 NA can confer protection against B/Yamagata/16/87 as well as B/Victoria/2/87 and B/Malaysia/2506/04, both of which belong to the Victoria lineage [110]. Although the two lineages are considered antigenically distinct, HA-directed antibody cross-reactivity between the two lineages has been observed in various contexts [111-115] potentially due to original antigenic sin[116].

461 Broadly neutralizing monoclonal antibodies have also been identified
462 for FluB, firstly in a seminal paper by Dreyfus et al [117]. Antibody CR8033
463 binds to the RBS region of the HA, while antibody CR8071 binds to the
464 vestigial esterase domain at the base of the HA head. Both can neutralize
465 viruses from the two FluB lineages *in vivo*, even at low doses. Interestingly,
466 they can inhibit the release of viral progeny rather than the entry of viruses
467 into cells. Another antibody, CR9114, binds to the HA stem, on an epitope
468 conserved across FluB and FluA viruses. Structural studies showed that this
469 antibody binds to the HA of H1, H3, H5, H7, H9 and FluB. CR9114 blocks the
470 pH-induced conformational changes in HA and subsequent membrane fusion.
471 CR9114 could protect mice against lethal infection. Interestingly, sequence
472 analysis of the V_H region of CR9114 showed a small number of somatic
473 mutations, suggesting that this broadly neutralizing antibody can be generated
474 from genomic sequences without extensive hypermutation [117]. Yasugi et al
475 [118] reported another broadly neutralizing antibody against FluB (5A7), which
476 targets the C-terminal region of HA1. This antibody showed therapeutic
477 efficacy even when administered after challenge. Broadly neutralizing
478 antibodies against the NA have also been reported. An antibody recognizing
479 residues 222-230 (N2 numbering), an epitope highly conserved across FluB
480 and FluA, can inhibit growth of FluB in cells but was not assessed *in vivo*
481 [119,120]. Vaccines that would prime antibody responses against such highly
482 conserved and cross-reactive epitopes would be of vital importance in
483 generating universal immunity against influenza viruses.
484
485 **T cells Responses**

The involvement of $CD8^+$ T cells in the immune response to FluA has been extensively studied in mice and human PBMCs [121-126]. However, little is known about T cell responses to FluB. Mice lacking any antibody responses are protected from lethal FluB challenge in a $CD8^+$-T cell-dependent manner. Primary infection can protect from secondary FluB infection and this is also dependent on $CD8^+$ as well as $CD4^+$ T cells [127]. Thus, similarly to FluA, T cells have an important role in promoting recovery from FluB infection. Three epitopes from NP of FluB have been identified for $CD8^+$ T cells. Robbins *et al* [128], identified the HLA-A*0201-restricted NP_{82-94} (MVVKLGEFYNQMM) epitope by its low similarity (leucine before a glycine followed by hydrophobic residues) to the FluA $A2^+$-$M1_{56-68}$ epitope. Two HLA-B8 restricted epitopes from the NP protein of FluB (NP_{30-38} RPIIRPAT and $NP_{263-271}$ ADRGLLRDI) were also identified later [129]. However, in a recent study, only the NP_{30-38} peptide could induce INFγ production from FluB polyclonal CTLs [130]. It has also been proposed that CTL responses against FluB are preferentially directed against HLA-B8 epitopes [131]. It was recently shown that FluB-specific polyclonal CTLs cross-react with viruses from the opposing lineage of FluB [130]. Some influenza A PB1 epitopes are conserved even in FluB PB1 sequence [132], but whether T cells against these epitopes are cross-reactive and cross-protective against both FluA and FluB remains to be determined experimentally. Although primary infection with FluB cannot protect from secondary infection with FluA and vice versa, in the BALB/c mouse model [133,134], immunization strategies that probe T-cell responses against conserved and cross-reactive epitopes could potentially confer universal heterologous immunity against Flu viruses. Thus, determining

511 the cross-reactivity of T cells against FluA and FluB is a key step in
512 understanding universal anti-influenza T cell immunity.
513
514 **Flu B Vaccines**
515 Immunization against FluB is provided in the traditional Trivalent
516 Inactivated Vaccine (TIV). However, until recently the annual formulation of
517 this vaccine only contained a virus from either the Victoria or the Yamagata
518 lineage depending on the year (Table 1). The high levels of co-circulation of
519 the two lineages in the last decade results in frequent mismatch of predicted
520 and circulating strains and has necessitated the inclusion of viruses from both
521 lineages in the formulation of a quadrivalent vaccine [27]. The quadrivalent
522 vaccine is available as an inactivated intramuscular vaccine or as a LAIV in a
523 nasal spray.
524 High yields of virus are required during the production of inactivated
525 vaccines. For FluA, this is achieved by reassortment, resulting in the
526 combination of the antigenic surface proteins in the PR8 (A/Puerto Rico/8/34)
527 backbone, which grows to high titers during production in eggs. There is no
528 established 'high-yield' strain for FluB and the yield of FluB during annual
529 production varies greatly. Ways to generate high yields of FluB for vaccine
530 purposes as well as studies into the attenuation mechanisms of influenza B
531 LAIV have been reviewed [2]. Recently Le *et al* [135], generated reassortant
532 viruses with B/Lee/40 as a donor strain and found that the NP gene from the
533 B/Lee/40 strain was central for high-yield production. However, overall there is
534 lack of a 'high-yield' FluB strain.
535

Antiviral Strategies against FluB

Two classes of licensed anti-influenza drugs exist, adamantanes (eg. amantadine and rimantadine) and NAIs, like oseltamivir and zanamivir. Adamantanes block the AM2 ion channel activity but not of BM2. This is attributed to the polar amino aside residues in the BM2 ion channel cavity, as they reduce accessibility of the drug to the ion channel pore [69]. Consequently, these drugs are ineffective against FluB.

NAIs can inhibit FluB replication, although with variable efficiency. The inhibitory effect of NAIs against both FluA and FluB can be attributed to the high conservation of the catalytic site across both FluA and FluB. The efficacy of NAIs and the resistance mutations of FluBs were extensively reviewed recently by Burnham et al [46]. Overall, oseltamivir shows lower efficacy against FluB than FluA, while zaramivir shows comparable efficacy.

Mutations conferring resistance to NAIs have been reported in clinical isolates, surveillance isolates as well as reverse genetics studies. These mutations can be found either in the catalytic site of the enzyme (eg. R152K) or in framework residues (eg D198N, I222T). Studies on the fitness cost of NAI resistance mutations have shown that, contrary to most resistance mutations, the E119A and H274Y mutations have little impact on viral replication and fitness [136,137]. Interestingly, it appears that different mutations have different frequencies and effects in the NA gene from the two FluB lineages [138]. Studies in NAI resistance by FluB highlight the possibility of uncompromised NAI-resistant viruses emerging in human populations, suggesting the need for increased surveillance and potentially novel antiviral strategies.

Host Range of FluB

Influenza B viruses are restricted to human hosts [2]. In 1999, a FluB outbreak in seals was reported in the Netherelands. Serological and virological data indicated that a strain of FluB circulating in humans was introduced to the seal population in 1995 [139] and further evidence of seal FluB infections were reported later [140,141]. Sequence analysis of the B/Seal/1999 virus revealed high identity to a human FluB strain, suggesting no adaptation of the virus in seals. A serological survey in swine farms across the US revealed antibodies against FluB in pig serum samples but virus was only detected by RT-PCR of the NS segment in a limited number of nasal swabs. Unfortunately, virus could not be isolated. The partial sequences derived from the RT-PCR were of high homology to human isolates, suggesting no adaptation. In an experimental setting, FluB could replicate in the respiratory tracts of pigs and infected animals exhibited flu-like symptoms, lung lesions and seroconversion. FluB transmitted via direct contact from infected to sentinel animals, although transmission was limited [142]. Antibodies against FluB have also been detected in guinea pigs [143], and FluB replicates and transmits efficiently during experimental infection of guinea pigs [144]. In addition, FluB replicates in ferret airway epithelial cells as well as infected animals and transmits between ferrets, which are considered the gold standard animal model for human influenza infections [145](unpublished observations Elderfield, Koutsakos & Barclay). Overall, while endemic circulation of FluB is restricted to humans, there are indications of FluB cross overs from humans to other species. However, there is currently

no evidence of sustained animal reservoirs of FluB. As discussed above, this restriction of FluB in humans may, at least to an extent, be attributed to the human host specific interactions of NS1[5,87,94] and M1 [58] proteins.

Conclusions & Future Perspectives

Over the last decade, it has become apparent that FluB constitutes a substantial part of annual flu epidemics. The particular pathogenicity of FluB in children, the co-circulation of the two lineages, which results in frequent mismatches of vaccine and circulating strains, and the emergence of NAI-resistance mutations with no apparent fitness cost further highlight the importance of FluB as a human pathogen.

Although it has generally been assumed that FluA and FluB viruses are similar, studies from the last decade emphasize multiple levels of uniqueness in FluB virology, immunology and evolution (Table 2). Some FluB proteins, like NP, have unique domains, others, like NB, are uniquely encoded by FluB viruses and still others can work in unique ways, like the cap-snatching mechanism of the FluB polymerases.

The numerous gaps of knowledge regarding FluB mean that multiple avenues of research can prove fruitful in the future. A better understanding of the evolution and phylodynamics of FluB viruses, such as the interactions between the two lineages and the interactions between FluA and FluB, can aid surveillance and vaccine strain determination and may inform epidemiology [39]. A clearer picture of the molecular virology of FluB could provide a better understanding of FluB pathogenicity, adaptation and design of novel antiviral strategies. A more comprehensive understanding of FluB

immunology would be an important step towards more effective vaccines and possibly antibody- and/or T cell-mediated universal influenza immunity. Overall, FluB is a research field waiting to explode.

Executive Summary

Clinical Presentation and Epidemiology

- FluB strains dominate every 2-4 years.
- Clinical presentations are similar to FluA, although usually more severe in children and adolescents.

Evolution and Phylodynamics

- Two lineages of FluB co-circulate globally, although usually one predominates in any given year..
- The two lineages exhibit contrasting phylodynamics.
- Re-assortment between the same lineage or two lineages contributes to the evolution of FluB.

Virology

- FluB has 8 gene segments encoding for 11 proteins.
- These generally have low sequence similarity to FluA and can exhibit important functional differences.
- NB is uniquely encoded by FluB and its function is unknown.
- NS1 inhibits the IFN$\alpha\beta$ response as well as ISG15 in a species-specific manner.
- The exact contributions of FluB proteins to pathogenicity and host-adaptation are unclear.

Immunology

- Mainly unexplored.
- FluB stimulates IFN$\alpha\beta$ and cytokine antiviral responses, although with kinetics different to FluA.
- Antibody responses are directed against HA and universally neutralizing antibodies against HA have been identified.
- T cells confer protection against primary and secondary infection in a mouse model.
- Only a handful $CD8^+$ T cell epitopes have been identified and T cell cross-reactivity between two lineages has been described.
- Trivalent (two FluA and one FluB strain) and quadrivalent (two FluA and two FluB strains) vaccines are available and updated annually.

Antiviral Strategies

- Adamantanes are ineffective against FluB.
- Neuranimidase inhibitors work but not as efficiently as for FluA.
- Resistance mutations with no apparent fitness cost have been detected.

Host Range

- There is limited evidence of reverse zoonosis of FluB but no established animal reservoir.

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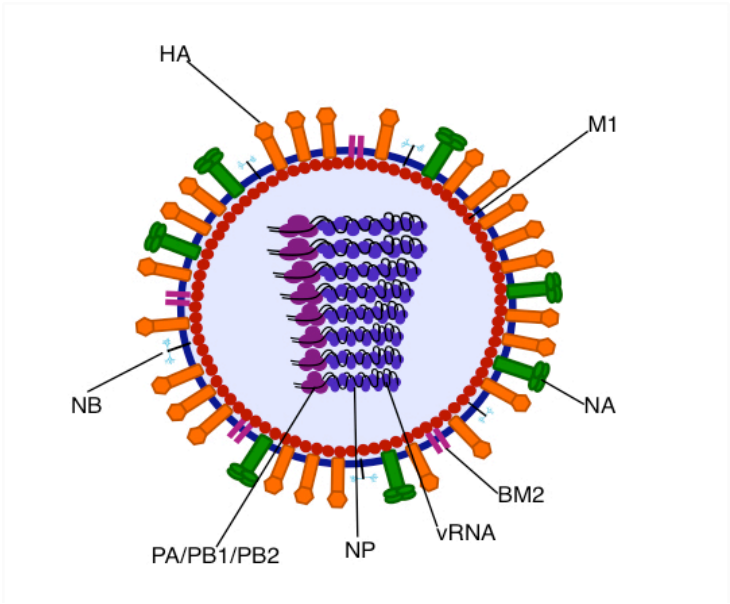
Figure Legends

Figure 1. Overview of Influenza B viruses. A) Schematic of FluB virus. B) Genome organization of FluB. C) Non-coding sequences of FluB.

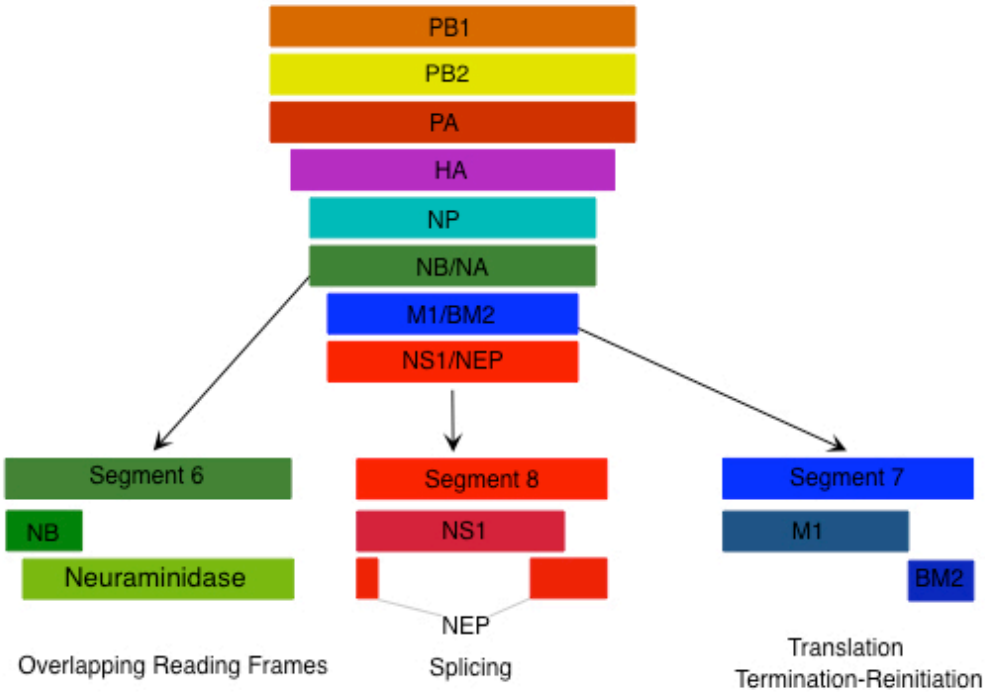
Table 1. Vaccines strains of FluB in the Northern and Southern Hemispheres. QV=Quadrivalent Vaccine, N/A= Not applicable.

Table 2. Comparison of FluA and FluB.

A)



B)



C)

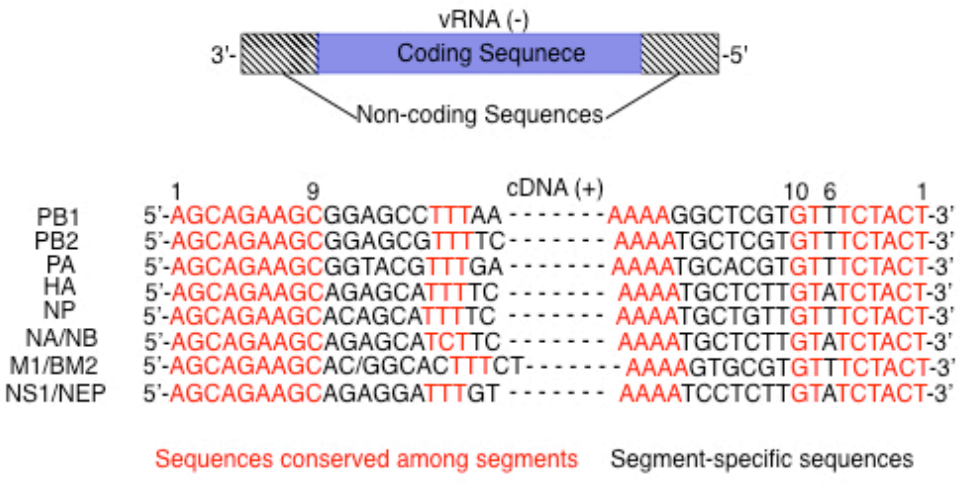


Figure 1 FluB Virus and Genome Organization

Northern Hemisphere

Flu Season	Strain	Lineage of TIV strain	To be included in QIV
1998-1999	B/Beijing/184/93	Yamagata	N/A
1999–2000	B/Beijing/184/93 or B/Shangdong/7/97	Yamagata	N/A
2000–2001	B/Beijing/184/93	Yamagata	N/A
2001–2002	B/Sichuan/379/99	Victoria	N/A
2002–2003	B/Hong Kong/330/2001	Victoria	N/A
2003-2004	B/Hong Kong/330/2001	Victoria	N/A
2004-2005	B/Shanghai/361/2002	Yamagata	N/A
2005-2006	B/Shanghai/361/2002	Yamagata	N/A
2006-2007	B/Malaysia/2506/2004	Victoria	N/A
2007-2008	B/Malaysia/2506/2004	Victoria	N/A
2008-2009	B/Florida/4/2006	Yamagata	N/A
2009-2010	B/Brisbane/60/2008	Victoria	N/A
2010-2011	B/Brisbane/60/2008	Victoria	N/A
2011-2012	B/Brisbane/60/2008	Victoria	N/A
2012-2013	B/Wisconsin/1/2010	Yamagata	B/Brisbane/60/2008
2013-2014	B/Massachusetts/2/2012	Yamagata	B/Brisbane/60/2008
2014-2015	B/Massachusetts/2/2012	Yamagata	B/Brisbane/60/2008
2015-2016	B/Phuket/3073/2013	Yamagata	B/Brisbane/60/2008

Southern Hemisphere

Flu Season	Strain	Lineage of TIV strain	To be included in QIV
1999	B/Beijing/184/93	Yamagata	N/A
2000	B/Beijing/184/93 or B/Shangdong/7/97	Yamagata	N/A
2001	B/Sichuan/379/99	Victoria	N/A
2002	B/Sichuan/379/99	Victoria	N/A
2003	B/Hong Kong/330/2001	Victoria	N/A
2004	B/Hong Kong/330/2001	Victoria	N/A
2005	B/Shanghai/361/2002	Yamagata	N/A
2006	B/Malaysia/2506/2004	Victoria	N/A
2007	B/Malaysia/2506/2004	Victoria	N/A
2008	B/Florida/4/2006	Yamagata	N/A
2009	B/Florida/4/2006	Yamagata	N/A
2010	B/Brisbane/60/2008	Victoria	N/A
2011	B/Brisbane/60/2008	Victoria	N/A
2012	B/Brisbane/60/2008	Victoria	N/A
2013	B/Wisconsin/1/2010	Yamagata	B/Brisbane/60/2008
2014	B/Massachusetts/2/2012	Yamagata	B/Brisbane/60/2008
2015	B/Phuket/3073/2013	Yamagata	B/Brisbane/60/2008

Table 1 FluB Vaccines Strains in Northern and Southern Hemispheres

FluA	FluB
Phylogenetics	
• Subtyped by HA and NA (e.g. H1N1, H3N2)	• Two lineages according to HA: Victoria and Yamagata
•	
Epidemiology	
• More severe in children and elderly	• More severe in children and adolescents
•	
Clinical Presentation	
• Symptoms: soar throat, coughing, fever, nasal discharge	• Symptoms: soar throat, coughing, fever, nasal discharge
• Avian strains lead to severe disease and high mortality rates	• Can cause sever complications in children
Virology	
• 8 segments, >13 known proteins	• 8 segments, 11 known proteins
• Unique proteins: eg. PB1-F2, PB1-N40, PA-X	• Unique proteins: NB
Immunology	
• Generally, strong IFN$\alpha\beta$ and cytokine response	• Strong IFN$\alpha\beta$ and cytokine response, details unclear
• HA is the major antibody target	• HA is the major antibody target
• M1 and NP are the major $CD8^+$ T cell antigens	• Major $CD8^+$ T cell antigens are unknown
• Two strains included in annual vaccine (one H3N2 and one H1N1)	• One or two strains in annual vaccine depending on formulation
Antivirals	
• Adamantanes used are effective	• Adamantanes are ineffective
• NAIs effective, but resistance detected	• NAIs effective, but resistance detected, in some cases without apparent fitness cost
Host Range	
• Multiple animal reservoirs and possible animal hosts	• No established animal reservoirs

Table 2. Comparison of FluA and FluB