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Unlocking influenza B: exploring molecular biology and reverse genetics for epidemic control and vaccine innovation

Ghayyas Ud Din[1,2], Chunchen Wu[3], Zahra Tariq[4], Kinza Hasham[4], Muhammad Nabeel Amjad[1,2], Bei Shen[1], Lihuan Yue[1], Muhammad Asif Raza[1,2], Muhammad Awais Ashraf[1,2], Lingdie Chen[1,2] and Yihong Hu[1,2*]

Abstract

Influenza is a highly contagious acute viral illness that affects the respiratory system, posing a significant global public health concern. Influenza B virus (IBV) causes annual seasonal epidemics. The exploration of molecular biology and reverse genetics of IBV is pivotal for understanding its replication, pathogenesis, and evolution. Reverse genetics empowers us to purposefully alter the viral genome, engineer precise genetic modifications, and unveil the secrets of virulence and resistance mechanisms. It helps us in quickly analyzing new virus strains by viral genome manipulation and the development of innovative influenza vaccines. Reverse genetics has been employed to create mutant or reassortant influenza viruses for evaluating their virulence, pathogenicity, host range, and transmissibility. Without this technique, these tasks would be difficult or impossible, making it crucial for preparing for epidemics and protecting public health. Here, we bring together the latest information on how we can manipulate the genes of the influenza B virus using reverse genetics methods, most importantly helper virus-independent techniques.

Keywords Influenza, Virus, IBV, Reverse genetics, Disease control

Background

Influenza Viruses are segmented, single-stranded RNA genome viruses that belong to the family Orthomyxoviridae. Depending on the host types, genetic properties, severity of infection, and vaccine coverages, these viruses could be divided into four types: A, B, C, and D (Table 1) [1]. Influenza, a highly contagious acute viral illness affecting the respiratory system, poses a significant global public health concern. It spreads from person to person, indiscriminately impacting individuals of all ages but more prevalent in children [2]. The trend of disease severity is high in children as compared to adults and individuals with underlying other chronic diseases (liver, immunosuppression, lungs, cardiac, kidney, and neurological disorders) [3]. Children under five years of age are at greater risk of getting sick with the influenza, and the

*Correspondence:
Yihong Hu
yhhu@ips.ac.cn
[1]CAS Key Laboratory of Molecular Virology & Immunology, Institutional Center for Shared Technologies and Facilities, Pathogen Discovery and Big Data Platform, Shanghai Institute of Immunity and Infection, Chinese Academy of Sciences, No. 320 Yueyang Road, Shanghai 200031, China
[2]University of Chinese Academy of Sciences, Beijing, China
[3]Department of Laboratory Medicine, Maternal and Child Health Hospital of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430070, People's Republic of China
[4]Sundas Molecular Analysis Center, Sundas Foundation, Gujranwala, Punjab, Pakistan

Table 1 Comparison of different type of influenza virus

Feature	Influenza A	Influenza B	Influenza C	Influenza D
Hosts	Humans, Birds, Animals	Humans only	Humans and Pigs	Cattle
Severity	Can cause severe pandemic/epidemic	Generally milder than A /epidemic	Mild respiratory illness	Mild respiratory illness
Genetic Segments	8	8	7	7
Surface Glycoproteins	Hemagglutinin (HA), Neuraminidase (NA)	Hemagglutinin (HA), Neuraminidase (NA)	Hemagglutinin esterase-fusion (HEF)	Hemagglutinin esterase-fusion (HEF)
Subtypes	Multiple subtypes H1 to H18, N1 to N11	Lineages (Victoria, Yamagata)	Not classified into in to subtype	D/C, D/M, D/H
Antigenic Drift	Commonly undergoes antigenic drift	Commonly undergoes antigenic drift	Limited antigenic drift	Limited antigenic drift
Antigenic Shift	Can undergo antigenic shift leading to pandemic	No antigenic shift	No antigenic shift	Limited impact
Vaccine Coverage	Seasonal flu vaccines target A subtypes	Seasonal flu vaccines target B lineages	No specific vaccine	No specific vaccine

annual incidence is up to 30% [1, 4]. According to estimates from the WHO and CDC, up to 650,000 people worldwide die from these illnesses annually [5].

The development of reverse genetics and the molecular engineering of recombinant viruses, which have revolutionized the field of virology, have made the study of genetic modifications in virus genomes possible [6, 7]. Exploring the structure and function of a virus, known as reverse genetics or viral rescue, is accomplished by creating an infectious clone of the virus and modifying it in-vitro at the DNA level. Infectious molecular clones include infectious cDNA and infectious in-vitro transcripts. Reverse transcription-polymerase chain reaction (RT-PCR) is used to amplify the cDNA fragment of the RNA virus genome. The restriction enzyme sites are then utilized to clone it into an appropriate vector to produce a full-length cDNA clone of the genome [7, 8].

Reverse genetics techniques for generating recombinant viruses were first developed in 1999 for influenza A virus because of the pandemic potential of this virus [8, 9]. In 2002, almost three years later, two different groups separately announced that the influenza B virus could be successfully recovered entirely from plasmid DNA [10]. The capacity to alter a virus genome completely revolutionized the study of influenza, enabling the development of infectious, recombinant viruses that are genetically altered [11]. Studies have clarified information on virus virulence and pathogenicity, host range and transmissibility, packing signals, the function of viral proteins, and virus-host interaction [12, 13]. Reverse genetic technologies have also been used to make flu vaccines and recombinant flu viruses that carry foreign peptides and/or proteins [14, 15].

Currently, reverse genetics stands as a fundamental approach in molecular virology, extensively employed in influenza virus laboratories for generating mutant as well flu viruses that are both wild-type and reassortant generated from viral cDNA [15] (Fig. 1). This review explores reverse genetics methods for influenza B virus, driving advancements in basic and applied virology. These methods enable the study of extinct viruses, rapid characterization of new strains, and development of novel vaccines to overcome substantial challenges.

Structure and genome organization

Through molecular virology studies, researchers have gained valuable insights into the genomic organization and replication RNA segmented within the genome of influenza B viruses (IBVs) [7, 8]. When viewed through electron microscopy, both influenza A and B viruses look like circular or filamentous structures. The width of spherical forms is about 100 nanometers, and the length of filamentous forms can be more than 300 nanometers [9, 12]. The influenza B virus consists of eight genomic segments including nucleoprotein (NP), nuclear export protein (NEP), matrix protein (BM1), BM2 ion channel, and three surface glycoproteins (HA, NA, and NB), giving the virus its characteristic molecular properties (Table 2). Each of the encoded proteins are crucial in the life cycle of the virus and its interactions with the respective hosts [16, 17].

Although the Influenza A and B virus have similar genomes encoding homologous proteins, yet they are different in their virion structures, genomic organization, host range, and glycan binding specificities [15]. Variable lengths of the encoded proteins, and non-coding regions (NCRs) serving as promoters for replication and

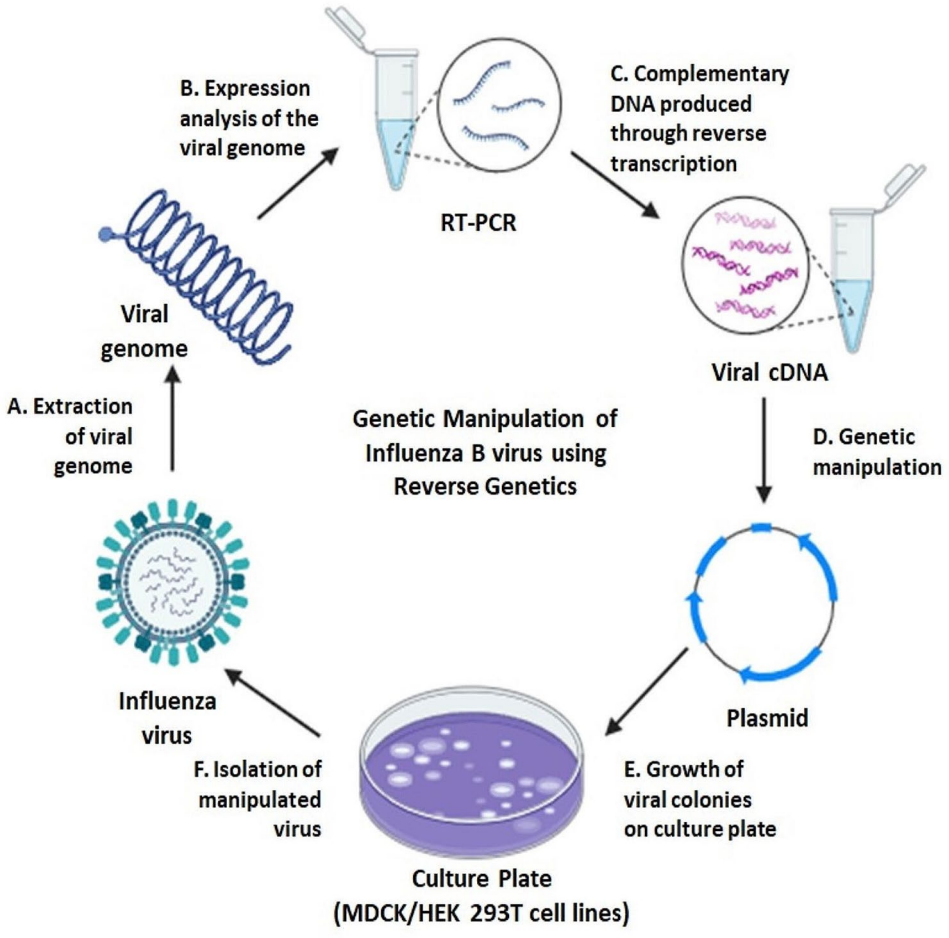

Fig. 1 Overview of genetic manipulation of influenza B virus using plasmid-based approach

Table 2 The genomic segments organization of influenza B virus

Segment	Segment length in nucleotides	Encoded protein(s)	Protein length in amino acids	Protein function
1	2300–2400	Polymerase PB2	759–760	Polymerase subunit; mRNA cap recognition
2	2300–2400	Polymerase PB1	757–759	Polymerase subunit; RNA elongation, endonuclease activity
3	2150–2250	Polymerase PA	716–747	Polymerase subunit; protease activity
4	1700–1800	Hemagglutinin HA	550–583	Surface glycoprotein with antigen-binding, receptor-binding, and fusion activity.
5	1500–1600	Nucleoprotein NP	498–500	Responsible for viral transcription and replication.
6	1400–1500	Neuraminidase NA	467–470	Sialidase activity, viral release, surface glycoprotein
7	1000–1100	Matrix MP	252–255	Matrix protein; vRNP interaction, RNA nuclear export regulation, viral budding
8	900–1000	Non-structural NS	227–230	Interferon antagonist protein; regulation of host gene expression nuclear export of RNA

transcription, different accessory proteins encoded from open reading frames (ORFs), and different internal proteins inducing antigenic differences are the significant distinguishing features [18, 19]. Translation strategies of several encoded proteins such as M2 and BM2 in both IAV and IBV have also been reported to be different [15]. Furthermore, presence of NB ion channel in IBV which is absent in IAV is another characterizing feature to differentiate both the viruses [15, 20].

The surface glycoproteins Hemagglutinin (HA) and Neuraminidase (NA) are encoded in both the virus types. However, the 18 HA (H1–H18) and 11 NA (N1–N11) subtypes classify IAVs based on different antigenic properties. Yet, currently only two subtypes H1N1 and H3N2 are circulating in humans [20]. Contrastingly, reassortant

influenza B viruses, featuring a hemagglutinin (HA) similar to B/Shandong/7/97 from the B/Victoria/2/87 lineage and a neuraminidase (NA) closely related to B/Sichuan/379/99 from the B/Yamagata/16/88 lineage, have been found globally, becoming the most common strain causing influenza B epidemics [17, 21, 22]. The main viral proteins, including HA, NA, NP, and polymerase proteins, have been fully characterized [1, 7]. This indicates the important roles of genomic organization, replication, transcription, and translation in the characterization of IBV (Fig. 2).

Life cycle of influenza B virus

During the viral infection, the initial stage involves the binding of the viral HA protein to a cellular receptor which is a sialylated glycoprotein with either α-2,3 or α-2,6 bonds. Upon this binding event, the process of receptor-mediated endocytosis is triggered, resulting in the containment of virus particles within an endosome [23]. Acidification of the endosome induces a structural alteration in the HA protein of the influenza virus, facilitating the fusion of the viral and endosome membranes [20]. The M-2 (IAV) and BM2 (IBV) ion protein channels play crucial roles in releasing viral ribonucleoprotein (vRNP) complexes into the cytoplasm of the host cell. The transportation of viral ribonucleoproteins from the cytoplasm to the nucleus is aided by the export of nuclear protein as well as matrix 1 protein. This translocation is essential for initiating viral genome replication and gene transcription [20, 24]. The packaging of viral RNA into new virion is regulated by RNA-RNA interactions that take place between vRNA packing signals located at the terminal ends of each vRNA segment [25]. The final phase involves the enzymatic function of NA, which eradicates the receptors and liberates newly generated viral particles from the exterior of infected cells (Fig. 3) [23].

Viruses have developed different strategies to take over the cellular immune response in the hosts. This is because, as soon as the viral infection begins, the immune cells particularly the type I interferons try to overcome the viral attack by activating the immune response [26]. Therefore, the viral cells adopt different splicing strategies to produce multiple primary and functional transcripts. For example, the nonstructural gene produces nonstructural protein 1 (NS1) as a primary transcript which plays important roles in the replication cycles of influenza virus but also produces NEP by the alternate splicing of NS mRNA [27]. The NS1 inhibits the IFN-1 response induction and innate immune response activation [28]. Therefore, the ongoing fight between the host and the virus, particularly involving type I interferon, is crucial in controlling viral infection [20].

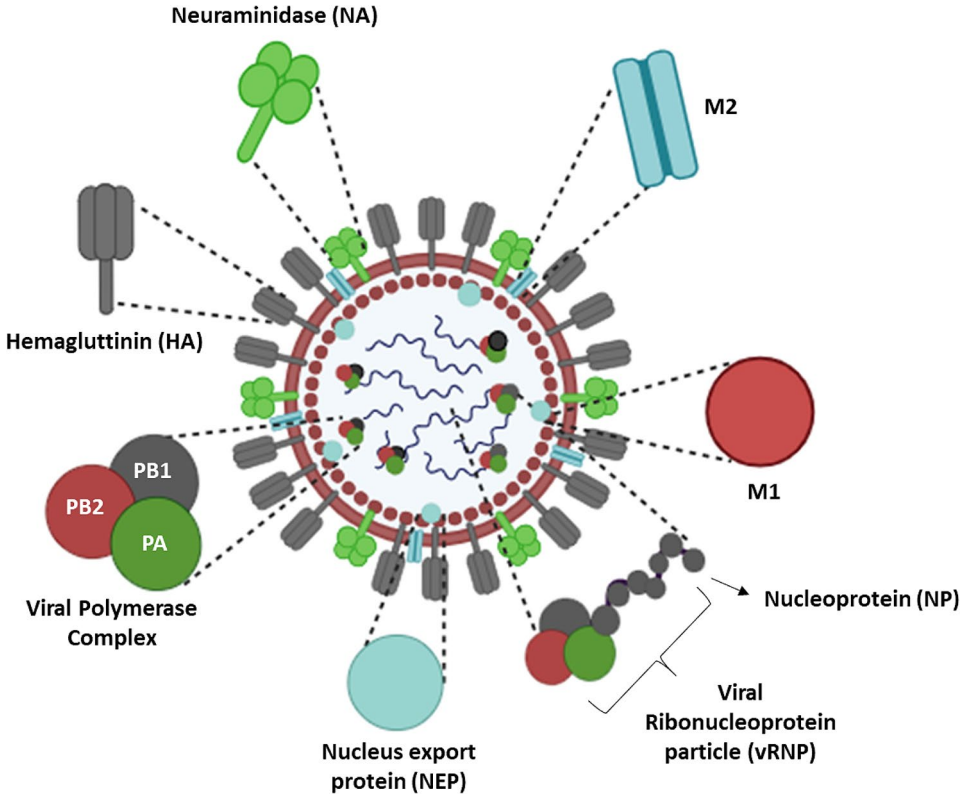

Fig. 2 Structural and genomic organization of influenza B virus

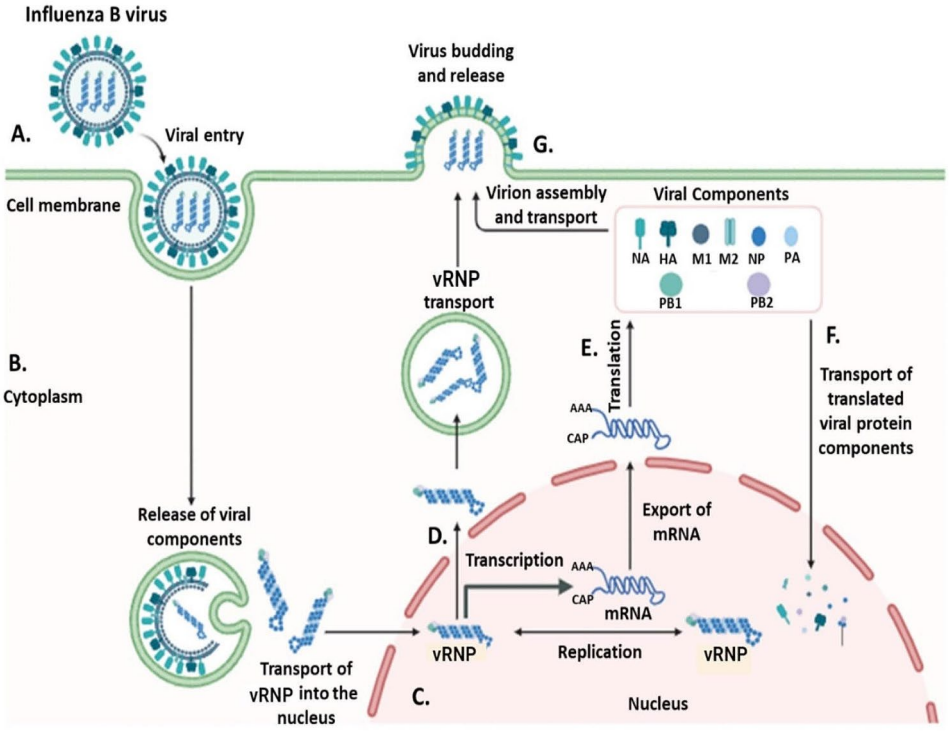

Fig. 3 Life Cycle of Influenza B virus: (**A**) Entry of Influenza B virus into the host cell by invading the cell membrane; (**B**) Release of the viral components into the cytoplasm followed by the transport of vRNP into the nucleus; (**C**) vRNP undergoes transcription for the production of viral mRNA and replication to produce cRNP. The mRNA is exported into the cytoplasm; (**D**) vRNP after being processed in the nucleus is transported in the cytoplasm from where it is budded into the cell membrane to be exported from the host cell; (**E**) Viral mRNA is translated to produce the viral protein components; (**F**) Viral protein components are transported into the nucleus to be assembled; (**G**) The viral components are assembled in the cytoplasm and released from the host cell through budding; viral nucleoprotein particle (vRNP); complementary nucleoprotein particle (cRNP); Neuraminidase (NA); Hemaglutinnin (HA); Nucleoprotein (NP)

Methods

Reverse genetics (RG) represents an indispensable tool for elucidating the intricate characteristics of viruses both in vivo and in vitro. Initially implemented with DNA viruses and later expanded to encompass RNA viruses, the pioneering success of RG manipulation was achieved in a positive-sense RNA virus, namely poliovirus [8, 25]. However, tackling negative-stranded RNA viruses posed daunting challenges, including the absence of genomic RNA, the stringent demands for exact genome length to enable replication and packaging, as well as the requirement for transient availability of viral RNA-dependent RNA polymerase [29]. Notwithstanding these formidable obstacles, the creation of a reporter IAV replicon was triumphantly accomplished using a helper virus [10]. A pivotal breakthrough emerged with the advent of plasmid-based systems, facilitating enhanced manipulation of negative-sense RNA viruses, marking a significant milestone in virology research [30, 31].

Recombinant virus generation techniques initially focused on DNA viruses, employing transfection with plasmids encoding the viral genome or heterologous recombination between plasmids and the viral genome with a helper virus [32]. Positive-sense RNA viral genomes, like poliovirus, were later manipulated through cell transfection with plasmid DNA or in vitro-transcribed RNA for recombinant virus production [29]. However, negative-sense RNA viruses, such as influenza, posed challenges due to their non-infectious nature without viral RdRps and vRNA [33]. The advent of reverse genetics and molecular engineering revolutionized influenza research, enabling exploration of viral replication, transcription, pathogenicity, host interactions, and vaccine development [32]. These technologies have also facilitated the creation of recombinant influenza viruses for vaccine vectors, expressing foreign proteins, or carrying reporter genes for easy infection tracking [29, 32, 34].

It's a new era for the study of influenza virus with powerful reverse genetics technology, and the comparison of different methods used for the reverse genetic of IBV is shown in Table 3. These discoveries have enabled the study and resuscitation of extinct influenza viruses, quick characterization of new viral strains, production of conventional influenza vaccines, and development of state-of-the-art influenza vaccines. Its application has yielded significant benefits, contributing to the development of inactivated or live-attenuated influenza vaccines and the exploration of anti-influenza treatments with elucidated

Table 3 Comparison of different methods used for the reverse genetic of influenza B virus

Method	Descriptions	Cell type	Applications	Advantages	Disadvantages
Helper Virus-Dependent Methods	Helper virus contains key viral proteins or activities required for the recombinant virus's packaging and replication. The viral regions of interest are transfected using plasmids and co-infected with a helper virus.	HEK 293T, MDCK	Generation of recombinant viruses, study of gene function, vaccine development	Streamlined process as a result of the helper virus	Possibility of helper virus and recombinant viral recombining
Helper virus-independent methods	Uses plasmids that, in the absence of a helper virus, encode the viral RNA segments and essential viral proteins. These plasmids can be transfected into host cells to produce recombinant viruses.	HEK 293T, MDCK	Development of recombinant viruses, investigation of gene function, and development of vaccines	No risk of helper virus contamination	Requires multiple plasmids and careful optimization of transfection conditions
Expression of viral RNA with the help of Promotors	Promotors used for the efficient recovery of influenza B virus strains.	HEK 293T, PER.C6, Vero, COS-1, MDCK, chicken embryo cells, Human, canine, quail cells	Facilitating efficient viral RNA production, overcoming species-specific limitations, reducing vector complexity	Species-specific efficiency, reduces number of vectors needed, efficient recovery of influenza B virus. High efficiency in virus recovery, suitable for vaccine production.	Limited to species-specific use, not broadly applicable. Requires expression of T7 RNA polymerase in cells, adding complexity. Limited cell line availability, requires specific cultivation conditions.
Plasmid-Based Reverse Genetics	Uses plasmids containing viral cDNA under the control of RNA polymerase I promoter for RNA transcription and RNA polymerase II promoter for protein expression. Uses a set of plasmids each encoding one of the eight viral RNA segments flanked by viral promoters, co-transfected into cells to reconstitute the virus.	HEK 293T, MDCK	Generation of recombinant viruses, study of gene function, vaccine development. Complete reconstitution of the virus from cloned cDNA, allowing for precise genetic manipulation.	High efficiency, precise control over viral genome, widely used and validated. Comprehensive system for whole-genome manipulation, well-established technique.	Requires multiple plasmids, complex cloning procedures, potential for recombination errors. Requires careful design of plasmids, potential for incomplete virus rescue, labor-intensive.
Reporter RNA and DNA Polymerase-Based Cloning	Incorporates reporter genes (e.g., luciferase, GFP) into the viral genome to monitor replication and transcription.	HEK 293T, MDCK	Monitoring viral replication, screening antiviral compounds, studying viral protein functions.	Allows real-time monitoring, quantitative analysis, high-throughput screening.	Reporter gene insertion might affect viral fitness, limited to studying specific aspects of viral biology.
Bacterial Artificial Chromosome (BAC)	Uses BACs to clone and manipulate large viral DNA fragments, allowing for easier genetic modifications.	HEK 293T, MDCK	Facilitates complex genetic manipulations, studying larger genomic regions, vaccine development.	Handles large DNA fragments, allows for complex and multiple genetic modifications.	Requires specialized techniques and equipment, potential for instability of large constructs.

antiviral mechanisms [35]. The ability to manipulate viral genomes through reverse genetics has brought a transformative impact on influenza research. Researchers now have the capacity to work with infectious, recombinant, and genetically modified viruses, allowing them to target and address specific research concerns with precision and depth [35–37].

A major breakthrough occurred in 2002 when reverse genetics techniques successfully achieved the complete recovery of recombinant influenza B viruses from plasmid DNA [13]. This aided in the investigation of both the host and viral factors involved in influenza pathogenesis, transmissibility, host-range interactions and restrictions, and virulence [15]. The reverse genetics approaches allowed the researchers to determine the importance of the non-coding regions present in the genome of influenza B virus, generate novel vaccine strains, study the drug resistance mechanisms, and evaluate the function of viral proteins, which are analogous to influenza A virus proteins and uniquely present in influenza B viruses [10].

Helper virus-dependent methods

These were the first successful influenza virus reverse genetics methods. They relied on a helper virus and a selection system to obtain the desired recombinant/transfectant influenza virus [36]. During the era when

helper virus-dependent systems were the sole accessible RG systems for influenza virus, selection systems for only six out of the eight genomic RNA segments of influenza A virus were documented [37]. Consequently, the genetic manipulation of two RNA segments, the first PA and the second PB1, was rendered impossible [36]. Many selection procedures differed in their level of strictness and, consequently, in their effectiveness. The quantity of mutated or reassortant influenza B viruses produced using this system was significantly less than the quantity produced [10, 38]. These technologies represent the initial advancements in reverse genetics for influenza viruses. Although they have demonstrated effectiveness and great significance in influenza virus research, their drawbacks on the helper influenza virus and their reliance consequently require the implementation of a selection method to enable the separation of necessary transfectant or recombinant influenza B viruses [35, 37].

Helper virus-independent methods

Helper virus-independent reverse genetics methods for influenza B virus revolutionized the study and manipulation of viral genomes. In this approach, individual plasmids containing cloned cDNA of the eight influenza B viral RNA segments, driven by RNA polymerase I or II promoters, are co-transfected into permissive cells [38]. The transfected cells serve as a host for the transcription, replication, and translation of the viral RNA segments, ultimately leading to the reconstitution of infectious influenza B virus [13, 39]. Most approaches use plasmids or vectors to produce all viral genomic RNA segments and required 'helper' proteins in cells, removing the requirement for selection procedures or helper virus elimination. Recent research proposed an alternate technique based on isolated RNPs from influenza virus preparations, but it has yet to gain attraction in the literature [40].

Plasmid-only reverse genetics systems

Viral RNA segments and necessary viral proteins are typically expressed by transfecting cells with specific plasmids (plasmid-based RG systems) [41]. These systems can be categorized based on factors like promoter types, plasmid numbers/types, transcription control elements, and cell species. Plasmid-based RG systems have been established for influenza A, B, C, and D viruses, along with the tick-transmitted Orthomyxoviruses, and Thogoto virus [41–43]. Jackson et al. described the use of cassette vector, pPRGCAT, cloned with the segments of influenza B/Panama/45/90 and flanked by human polymerase I promoter at the 5′ terminus and the hepatitis delta virus (HDV) antigenomic ribozyme at the 3′ terminus, so that the transcription resulted in the synthesis of negative-sense RNAs with exact viral-like termini [10].

Nogales et al. [15] described the generation of recombinant influenza B virus using an ambisense bidirectional plasmid pDP-2002 containing two transcription units in opposite direction. The influenza B vRNAs are expressed using the human polymerase I (hPol-I) promoter and a murine Pol-I transcription terminator (TI) while the mRNAs are expressed using polymerase II-driven cytomegalovirus promoter (pCMV) and the bovine growth hormone polyadenylation signal (aBGH). The synthesis of negative-sense vRNA from the hPol-I cassette, and positive-sense mRNA from the Pol-II unit, from one viral cDNA template is allowed by the orientation of both the polymerase units. The cloning of influenza B/Brisbane/60/2008 was carried out by inserting the influenza B viral cDNAs between the polymerase I transcription/terminator cassette flanked by an RNA polymerase II-dependent (Pol-II) cytomegalovirus promoter (pCMV) and a polyadenylation site (aBGH) [15].

Expression of viral RNA with the help of promotors

The initial reverse genetics systems employed plasmids that were free of helper viruses and had only the necessary genetic material. These plasmids utilized the human Pol I promoter to enable the production of viral RNAs [44]. The 3′ terminus of the viral RNAs was produced through the action of the ribozyme or the (murine) Pol I terminator sequence. Pol I promoters are typically regarded as species-specific, meaning that they function exclusively in the species where the promoter sequence originated or in closely related species [45]. Canine and chicken Pol I promoters were also utilized in specific cell types. Murine Pol I promoters were not widely used due to limited transfectable cell lines [46].

To overcome species-specific limitations, a universal plasmid-based reverse genetics system employed the T7 promoter, relying on T7 RNA polymerase expression within transfected cells. This system worked in human, canine, and quail cells. An alternative patent suggests using Pol II promoters with self-cleaving ribozymes at both ends of viral RNAs for reverse genetics [46, 47]. Nogales et al. shared their methods, which described the use of bidirectional plasmid pDP-2002 containing two promoters, human polymerase I (hPol-I) promoter, and polymerase II-driven cytomegalovirus promoter (pCMV) for influenza B/Brisbane/60/2008. This plasmid allowed the synthesis of vRNA and mRNA from the same vector, thereby, reducing the number of vectors used to eight for the efficient recovery of influenza B virus [15].

Human 293T and PER.C6 cells, as well as monkey Vero and COS-1 cells, have been employed in reverse genetics studies related to influenza virus. The cells have been cultivated either individually or in conjunction with more vulnerable cells, such as MDCK or chicken embryo cells [27, 39]. The rescue of recombinant influenza B

viruses from the plasmid DNA have been reported using HEK293T and MDCK cell lines [15]. Another research has reported the efficient recovery of influenza B virus strains using human derived PER.C6 cell lines [45].

Reporter RNA and DNA polymerase-based cloning

Different techniques, such as using long-overhang primers and restriction enzymes, have been employed to create plasmids that produce RNA templates for influenza B virus reporters. Using these methods, the luciferase gene is added to target vectors, viral vectors are joined with UTRs, or a double-stranded DNA linker is used between terminator sequences and the Pol-I promoter [48]. It has been tried to create restriction enzyme-free methods for IBV reporter-based RNP activity assays using overlapping sequences and long overhang primers, but these methods cannot be standardized or established because no clear experimental protocols are currently present [49]. The difficulty is increased for influenza B viruses because their untranslated regions (UTRs) are greater in comparison to influenza A virus [48, 50].

Recent research shows a different way to make a firefly luciferase-based reporter plasmid for the influenza B/Brisbane/60/2008 virus that does not use restriction enzymes, specialized reagents or kits thus making the method fairly simple to be adopted. The cloning strategy developed by Kedia et al. utilized a single DNA polymerase, which was easily available due to its wide use in regular molecular biology work. The reporter RNA cassette with the reporter ORF which was flanked by the viral 5′- and 3′-UTR regions was generated by two consecutive PCR amplification reactions. It was then cloned into the selected vectors for the expression analysis to successfully establish a simple, adaptable, and user-friendly cloning of any other reporter RNA constructs [51].

Vaccine innovation and epidemic control

Epidemiologic studies have indicated the presence of selective pressure on influenza B viruses. This is certainly due to a phenomenon known as immunologic imprinting in which the individuals in the population exhibit the pre-existing immunity against the virus developed during the childhood by infection with the influenza strain circulating which then protect against unfamiliar HA or NA subtypes emerging from the same groups [52, 53]. The evolution of the influenza B virus is driven by the antigenic drift and reassortment mechanisms along with the mutations in the HA and NA genes allowing the evasion of pre-existing antibodies. Thus, to overcome the epidemic threats posed by rapidly evolving influenza B virus strains, it is crucial for the researchers to come up with vaccines developed from the updated strains [52, 54].

To mitigate the burden attributed to epidemics caused by influenza virus, a number of approaches including vaccines and antiviral drugs, are being developed. The high evolutionary rates of influenza B virus have constrained the production of a fully effective vaccine, making it difficult to prevent influenza completely. However, vaccination is deemed as an appropriate option to combat the viral attack [53]. The researchers have developed three types of vaccines (inactivated, live attenuated, and recombinant HA vaccines) with their advantages and disadvantages, respectively. The vaccine seed viruses for all these vaccines should be replaced periodically with respect to the antigenicity of the circulating viral strains which otherwise cause low vaccine efficacy. The epidemiologic information from individual countries, the genetic and antigenic characteristics of the circulating viruses are responsible for the selection of the correct influenza vaccine composition (Fig. 4) [55].

Previously, high uncertainty existed in the yield of influenza B virus for vaccine purposes based on the propensity of the selected antigenic variant to propagate in eggs. For example, with the change of recommended strain of influenza B virus to B/HK/330/2001, poor growth of the strain in the egg became apparent after a long time. Therefore, achieving the necessary antigen doses needed for the influenza B virus was difficult. Emergence of reverse genetics approaches eliminated the uncertainty of the reassortment process [52]. This was demonstrated by Hoffmann et al. (2002) who generated '6+2 reassortants' by reverse genetics with internal genes from B/Yamanishi/166/98 and HA and NA genes from B/Victoria/504/2000, B/Hawaii/ 10/2001 and B/Hong Kong/330/2001 influenza strains. The recombinant viruses grew along with the wild-type virus in eggs, with the enhanced growth exhibited by B/Victoria recombinant virus [16].

Researchers further developed tissue culture cell-based approaches for vaccine production which might be fast, adaptable, and pose minimal risk of biological contamination. Cell lines including 293T, MDCK, and PER.C6 have been used for the recovery of influenza B viruses using reverse genetics procedures. MDCK and PER.C6 cell lines have been licensed for influenza vaccine production which are being widely used to generate vaccines [45, 56, 57]. Initially, trivalent vaccines were formulated which contained two influenza A (H1N1 and H3N2) and one lineage of influenza B viruses. This was to develop protection against three different influenza viruses irrespective of two different lineages of circulating B viruses. To provide wider protection, the second lineage of the influenza B virus was included to produce a quadrivalent influenza vaccine. The current approved vaccines for influenza virus are quantitatively standardized with

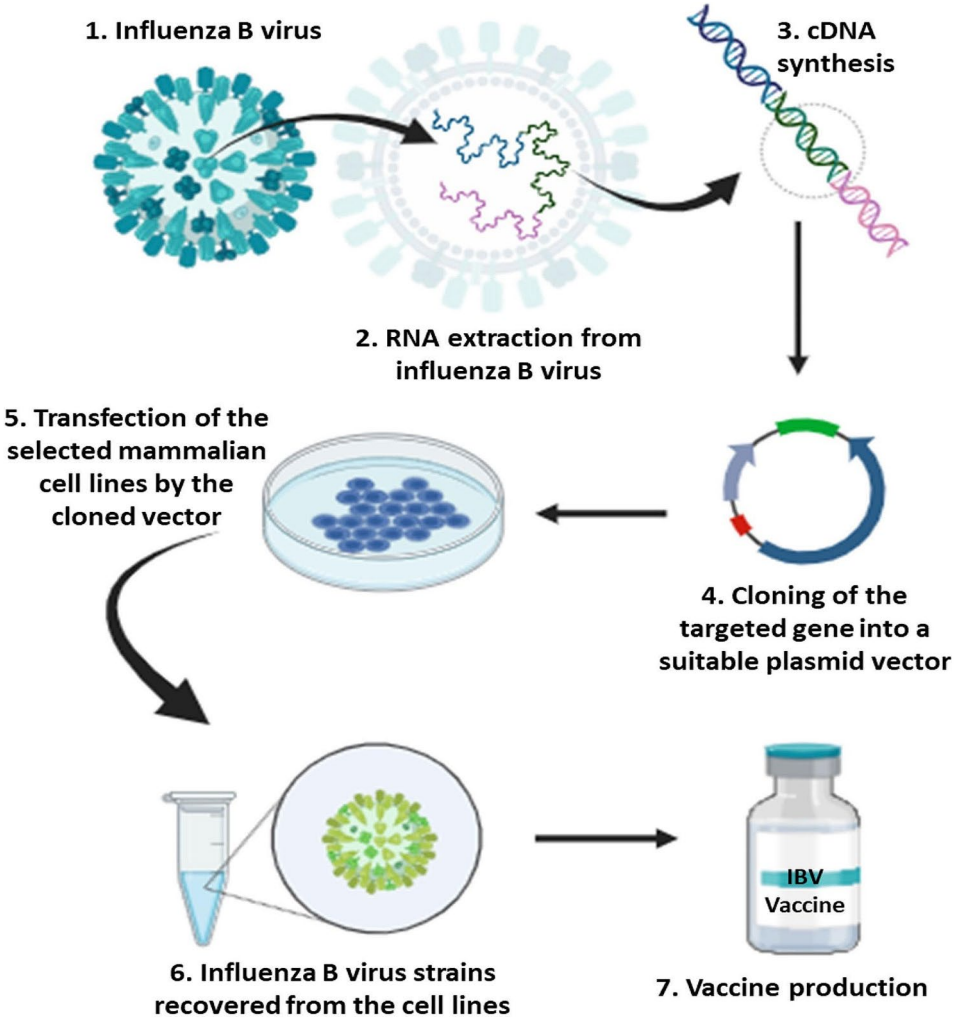

Fig. 4 IBV vaccine production: (**1**) Selection of IBV strain; (**2**) RNA extraction; (**3**) cDNA synthesis for gene manipulation; (**4**) Cloning of the targeted gene into a suitable plasmid vector; (**5**) Transfection of the selected mammalian cell lines by the cloned vector; (**6**) Recovery of the transfected viral strain from the cell lines; (**7**) Vaccine production

respect to the antigenicity or HA quantity but not by the presence of neutralizing antibodies (NA) [58].

Conclusion

Influenza B virus infections persist as a formidable threat to humanity. A relentless increase in incidence, notably during the 2019–2021 influenza season has been recorded. The challenge of antigenic mismatch and uncertainty in predicting prevalent IBV lineages poses a daunting obstacle in vaccine seed selection. Although quadrivalent vaccine formulations offer some relief, IBV vaccine efficacy remains insufficient due to increased antigenic drift and mutations in HA and NA genes. Present study highlights the transformative impact of reverse genetics on influenza B virus research, detailing its role in manipulating viral genomes. It categorizes methods, favoring plasmid-only systems for efficiency. In-depth analysis of reverse genetics and viral vector-based approaches has been conducted. Interestingly, a reporter plasmid free of restriction enzymes or complex reagents has been developed for influenza B virus. This standardized assay system proves valuable for studying viral and host factors, offering a high-throughput screening platform for antiviral drugs. Overall, it underscores the crucial role of reverse genetics in advancing influenza B virus research and potential interventions.

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Author contributions
G.U.D., C.W. and Y.H. Conceptualization; G.U.D., Z.T. and K.H. Writing-original draft preparation; Z.T. and K.H. Visualization; G.U.D. and M.A.R. Formal analysis; M.N.A. Software support; L.C. Methodology; G.U.D., C.W., Z.T., B.S., M.A.A., M.N.A., L.Y., L.C. and Y.H. Writing-review and editing; C.W. and Y.H. Supervision; C.W. and Y.H. Project administration; C.W. and Y.H. Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals. For consent to participate, it is not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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