Review

H5N1 Avian Influenza: Global Circulation and Response Strategies

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Abstract: The highly pathogenic avian influenza virus H5N1 has garnered global attention due to its high pathogenicity and mortality rates. In recent years, the outbreak of the H5N1 subtype 2.3.4.4b in various mammals has raised concerns about its pandemic potential. This paper reviews the global prevalence and impact of H5N1 virus, explores the current status and challenges in the existing technological platforms for H5N1 vaccine and diagnostic development, and further evaluates the effectiveness and application prospects of current H5N1 therapeutics. This article aims to provide a robust reference to guide the global preparedness for future pandemic potential of avian influenza H5N1.

Keywords: H5N1, circulation, vaccine development, diagnostics, treatment

1. Introduction

In March 2024, the H5N1 virus was detected in dairy cattle in the United States (U.S.) for the first time [1]. By 4 October 2024, the U.S. health officials had confirmed 255 infected cattle herds in 14 states [2]. The spreading of the H5N1 virus among cattle, poultry, and cats, coupled with human infection cases, demonstrates the enhanced binding affinity of H5N1 virus for human receptors and its transmissibility in mammals. This significantly increases the potential of H5N1 for sustained human transmission, posing a huge threat to global health [3]. H5N1 is one of avian influenza viruses (AIVs) that primarily infects birds, causing avian influenza (AI) [4]. AIVs can be classified into 16 HA (H1-H16) and 9 NA (N1–N9) subtypes based on the antigenicity of hemagglutinin (HA) and neuraminidase (NA) proteins on the surface of influenza virus [5]. They can also be categorized into highly pathogenic avian influenza (HPAI) viruses (HPAIVs) and low pathogenic avian influenza viruses (LPAIVs) according to their pathogenicity for chickens. Among various HPAIV strains, H5N1 is considered the most virulent, having high mortality rates in both chickens and humans [6]. Wild waterfowl are the primary natural hosts for most influenza A virus subtypes, and multiple AIVs can infect free-ranging poultry through migratory wild birds, facilitating the viral spread and evolution. It has been demonstrated that the global transmission of the H5N1 virus is linked to the movements of migratory birds, especially wild waterfowl [7]. Since 1959, numerous HPAI H5 outbreaks in various countries have resulted in substantial losses in the poultry industry. Recent human and several mammalian H5N1 infections have further drawn increasing global attention. Here, we aim to review the global prevalence of H5N1 infection (see Table 1) and summarize the current measures for prevention and control of H5N1 infection, hoping to provide a useful reference for future scientific research and public health decision-making to contain a potential H5N1 pandemic.

1.1. Global Circulation of H5N1 Virus

The outbreak of the H5N1 virus has not only devastated animal populations—including poultry, wild birds, and mammals—but also threatens human health. From 2005 to 2024, HPAI led to the death and mass culling of 557 million poultry globally, with an unprecedented 141 million in 2022 alone. This year, 85 countries and regions worldwide were affected by HPAI [8]. The HPAI H5N1 epidemic has frequently erupted among birds and poultry

and has also been reported to infect mammals and humans in recent years [9]. According to the World Health Organization (WHO), from 2003 to August 2024, 902 human H5N1 cases, with 463 deaths, were reported across 24 countries [10]. There is rising concern that the continuous evolution of the H5N1 virus might eventually lead to a new pandemic capable of stable human-to-human transmission.

1.1.1. Initial Emergence and Early Outbreaks of H5N1 (1959–1997)

In 1959, highly pathogenic avian influenza H5N1 was first recorded in Scottish poultry [11]. In 1991, an H5N1 virus outbreak occurred among turkeys in the United Kingdom [12]. In 1996, the H5N1 avian influenza virus A/Goose/Guangdong/1/96 (Gs/Gd) was isolated from a goose in Guangdong Province [13]. In 1997, Hong Kong reported the first fatal H5N1 avian influenza virus transmitted from birds to humans, causing 18 infections and 6 deaths [14], proving that H5N1 can cross species barriers to infect humans for the first time. This Hong Kong outbreak was controlled by culling over 1.5 million poultry, eliminating the infection source. However, the Gs/Gd lineage of H5N1 and its derivatives have continued to spread among Asian poultry and wild birds [15].

1.1.2. Global Spread and Human Infections of H5N1 (2003–2005)

In late 2002, Hong Kong experienced a severe H5N1 avian influenza outbreak [16], followed by human infections in February 2003 [17]. By the end of 2003 and early 2004, human H5N1 cases were reported in China, Vietnam, and Thailand [18–20]. As the virus continued to spread, H5N1 avian influenza outbreaks were subsequently reported in several Asian countries, such as Vietnam, Thailand, Indonesia, China, Japan, South Korea, Cambodia, and Laos. These outbreaks caused millions of poultry deaths across East Asia, with several countries documenting human H5N1 cases [21]. The virus's spread via migratory waterfowl transformed H5N1 into a global public health issue. In 2005, an H5N1 avian influenza outbreak among migratory waterfowl at Qinghai Lake in China (Clade 2.2) killed about 6000 birds [22], marking the first observation of H5N1's sustained spread among migratory birds.

1.1.3. H5N1 Cross-continental Transmission and Emergence of the Derivative H5Nx (2005–2019)

Since 2005, H5N1 virus has spread rapidly across Central Asia, the Middle East, Europe and Africa via migratory bird flyways, causing outbreaks in poultry across more than 30 countries [23]. In 2006, H5N1 virus spread across Asia, into North Africa and Europe [24]. During the 2010s, multiple HPAI H5 viruses emerged, featuring new NA subtypes such as H5N2, H5N3, H5N6, H5N8 and H5N9, following reassortment (gene swapping) with viruses circulating among wild and/or domestic birds [25]. By 2011, the United Nations Food and Agriculture Organization (FAO) identified six countries as enzootic for HPAI H5N1 in poultry: Bangladesh, China, Egypt, India, Indonesia, and Vietnam. Sporadic outbreaks of HPAI H5N1 virus also occurred among poultry in non-enzootic countries. In January 2014, the first human infection with Asian lineage HPAI H5N1 virus in the Americas was reported in Canada in a traveler returning from China. In January 2015, an HPAI H5N1 virus was identified in a U.S. wild bird in 2014. This virus resulted from reassortment of the Asian lineage H5 viruses with an N1 NA from North American wild birds [25].

1.1.4. Emergence of the New H5N1 Variant (2020–2024)

In October 2020, a new H5N1 virus, generated by reassortment between Clade 2.3.4.4b HPAI H5N8 and the local LPAIVs from the Eurasian gene pool, emerged in the Netherlands [26]. This novel H5N1, together with its further reassortant variants, spread among poultry and wild birds in various Western, Northern, and Eastern European countries, reaching some African nations by August 2021 [27]. From September 2021, it triggered a significant avian influenza outbreak across Europe, later spreading to multiple Asian and North American countries [28,29]. This new H5N1 virus exhibited a broader transmission range, higher genetic reassortment potential, and an enhanced ability to cross species barriers, effectively and sustainably infecting birds, poultry, and mammals [30]. In October 2022, an H5N1 outbreak among farmed minks in Spain resulted in over 50,000 minks being culled, demonstrating for the first time that the H5N1 strain can stably spread among mammals [31]. From January to April 2023, over 5000 sea lions died in Peru's marine reserves [32]. On 25 March 2024, the U.S. detected HPAI H5N1 in dairy cattle, reporting for the first time the transmission of H5N1 from cattle to humans [33], causing concern in the scientific community.

Table 1. History of Global Circulation of H5N1 Virus and Derivatives of A/Goose/Guangdong/1/96 (Gs/Gd, H5N1) Lineage.

Year	Key Events	Geographic Distribution	Clade *	Confirmed Human Cases	
				Cases	Deaths
1959	The first documented occurrence of the H5N1 virus in avian species [11].	Europe	/	0	0
1991	An outbreak in turkeys in the United Kingdom [12].	Europe	/	0	0
1996–1997	Initial isolation of the H5N1 virus and the first reported human infection [13,14].	Asia	0	18	6
2001–2002	Detection of H5N1 viruses in multiple live bird markets in 2001 and a significant avian influenza outbreak in Hong Kong in 2002 [34].	Asia	1	0	0
2003–2005	Re-emergence and continuous evolution of H5N1 in China, extensive outbreaks across Asia, and later spreading to the Middle East, Europe, and Africa [35].	Asia, Europe, the Middle East, Africa	1, 2.2, 2.3, 2.3.4	148	79
2006–2009	Expansion of the virus to Africa, with sporadic reoccurrences in Asia, Africa, and the Middle East [36].	Asia, Europe, the Middle East, Africa	2.2.1 2.2.1.1 2.2.1.1a	320	203
2010–2014	Spread from East Asia to North America, West Asia, and Europe, leading to globally dominant outbreaks and notable mortality cluster near Cambodia and Vietnam [37].	Asia, North America, Europe	2.2.1.1a 2.2.1.2	233	125
2015–2019	Emergence of H5N6 and H5N8 strains globally, replacing original H5N1 strains in some regions. Significant rise in H5N1 cases in Egypt [38–40].	Asia, Africa, Europe, the Middle East, North America	2.3 2.3.2.1c 2.2.1.2 2.3.4.4	160	48
2020–2021	Identification of new H5N1 strain, clade 2.3.4.4b, with wild bird-adapted N1 NA gene, originating in the Netherlands and transmitting across continents [28].	Asia, Africa, Europe, and the Middle East, America, Antarctica	2.3.4.4b	3	1
2022–2024	Sporadic human infections and detections in mammals with the emergent H5N1 strain [31–33].	Europe, America	2.3.4.4b	32	7

* /: Virus belonging to the Eurasian gene pool (non-Gs/Gd lineage). The other clades are all from the Gs/Gd lineage.

1.2. Clade Classification and Evolution of H5N1 Virus

The H5N1 virus continuously mutates and evolves, resulting in antigenic diversity in the HA protein and forming various H5N1 clades. In 2006, Professor Guan Yi's team first classified these clades through a phylogenetic analysis of all gene segments of the H5N1 virus, confirming that long-term circulation in different Asian regions led to the establishment of regional clades. Their research showed that the H5N1 virus continues to spread from its established source in South China to other regions via poultry trade and bird migration. Establishing regional sub-lineages of the virus allows tracking of its origin and transmission mechanisms, providing direct information for controlling poultry infection sources [41]. In 2008, the H5N1 Evolution Working Group, consisting of WHO, the World Organization for Animal Health (WOAH), and the Food and Agriculture Organization of the United Nations (FAO), developed a naming system based on the phylogenetic characteristics and sequence similarity of the HA gene, identifying 10 different virus clades (numbered 0–9) [42,43]. Over time, possibly due to proactive control strategies (including culling and vaccination), most H5N1 clades have gradually disappeared in the wild, with only clades like 2.2, 2.3.2, and 2.3.4.4 remaining among wild birds [44–46].

The spread of the H5N1 virus shows temporal and spatial specificity [47]. Clade 0, the earliest H5N1 clade identified, includes viruses isolated in Guangdong, China, in 1996, primarily circulating in China (including Hong Kong) in the early years [13]. Clade 1 viruses are entrenched in the Mekong Delta and widely distributed in Southeast Asia, such as Vietnam, Laos, and Cambodia [48], in 2003. Clade 2 viruses have generated new subclades over time, representing extensive geographical spread and complex evolutionary processes. Clade 2.2 viruses, with a widespread transmission history, caused an H5N1 outbreak among migratory birds in Qinghai Lake, China, in 2005, spreading to over 30 nations in Europe, the Middle East, and Africa [49]. Clade 2.3 is the most complex subclade, further divided into subclades like 2.3.2 and 2.3.4, widely distributed in China and Southeast Asian countries. Clade 2.3.2.1 viruses show particular transmission patterns, found among wild birds in China, Mongolia, and Russia. The detection of clade 2.3.2 viruses among Qinghai Lake migratory birds in May–June 2009 expanded the phylogenetic analysis [50], classifying Qinghai Lake isolates into clade 2.3.2.1 [51]. This subclade further evolved into lineages 2.3.2.1a, 2.3.2.1b, and 2.3.2.1c, circulating among poultry in various Chinese provinces [52]. In 2008, the subtypes H5N2, H5N5, and H5N8 with Gs/GD lineage H5 Clade 2.3.4 genetic backbone were identified, later evolving into various subclades, including 2.3.4.4 [53]. Clade 2.3.4.4, a highly pathogenic lineage, consists of eight subclades (2.3.4.4a–2.3.4.4h), causing severe outbreaks among several countries' poultry, becoming a major global concern. HPAI H5Nx viruses in this clade, paired with different NA subtypes, began widespread transmission from late 2014, spreading from East Asia to North America, West Asia, and Europe, dominating outbreaks globally [54–56]. In 2020, an H5N1 virus carrying the clade 2.3.4.4b HA gene appeared in the Netherlands, spreading from Eurasia and Africa to North America by 2021, and further reaching South America and Antarctica [29,57,58], affecting various wild birds and poultry [59,60]. Notably, outbreaks caused by clade 2.3.4.4b reassortants among mammals have drawn global attention to H5N1 viruses due to their potential risk to human health [61,62].

1.3. Current Prevention and Control Strategies on H5N1 Virus

Inter-species transmission or genetic exchange among avian, swine, and human influenza viruses has led to four influenza pandemics [63]. Currently, the H5N1 virus persists globally, enhancing its interspecies transmission capability, with certain strains proven to breach species barriers and infect humans. Transmission through contact with infected poultry or their secretions and excretions can cause mild to severe, potentially lethal illnesses, threatening human health [64,65]. The first report of H5N1 infecting dairy cattle and subsequent human cases in the U.S. in 2024 underscores the rising public health risk posed by the H5N1 virus. Close monitoring and improved preparedness are essential to achieve early warning or prompt detection of potentially pandemic H5N1 influenza viruses, along with planning and implementing appropriate control measures. Global influenza surveillance and diagnosis, culling of infected animals and other non-pharmaceutical measures to prevent transmission, mass vaccination programs, and rational antiviral drug use are key strategies for influenza prevention and harm reduction. This review summarizes the current research status of vaccines, diagnostic reagents, and therapeutic drugs for H5N1 virus control, providing reference data to guide further studies.

2. Status and Challenges of H5N1 Vaccine Development

Vaccines are the most effective method for preventing infectious diseases. In response to the global spread of the H5 subtype avian influenza virus, H5Nx vaccines (including H5N1, H5N6 and H5N8) are continuously developed using various technological platforms to facilitate pandemic prevention and control. However, several

challenges persist. This section reviews the advantages and disadvantages of various H5 vaccines (see Table 2) to aid future development.

2.1. Inactivated Vaccines

The inactivated H5N1 vaccine is produced by rendering the virus non-replicative. Following vaccination, the vaccine elicits an immune response without causing disease, inducing a strong systemic response that provides protection against the virus through the production of neutralizing antibodies. Due to their high safety and relatively low production costs, inactivated vaccines account for the largest portion of the global influenza vaccine market. Inactivated vaccines include whole-virus inactivated vaccines, split-virus inactivated vaccines, and subunit vaccines. Most poultry influenza vaccines are traditional inactivated oil-emulsion whole-virus influenza vaccines, with less than 5% being live vector vaccines [66]. To combat the H5N1 avian influenza outbreak in Indonesia, chickens were vaccinated with an inactivated vaccine derived from the highly pathogenic vaccine seed virus A/chicken/Indonesia(Legok)/03(H5N1) [5]. To address vaccine failure caused by viral mutations, reverse genetic systems are used to construct vaccine seed virus platforms by combining the HA and NA genes of H5 viruses with the internal framework genes of the high-growth A/Puerto Rico/8/1934 (H1N1) (PR8) strain, allowing for timely vaccine updates [67]. Currently, H5-re13 and H5-re14 vaccines can control H5 viruses carrying the local 2.3.4.4h HA lineage and the globally spreading 2.3.4.4b HA lineage [68,69]. These vaccines are prepared as inactivated adjuvanted vaccines and can induce a strong systemic immune response. The U.S. Food and Drug Administration (FDA) has approved three H5N1 avian influenza vaccines for humans: two split-virus vaccines (Sanofi Pasteur's Influenza A (H5N1) Virus Monovalent Vaccine, and GlaxoSmithKline's Arepanrix H5N1) and one subunit vaccine (CSL Limited's Aflunov) [70–72]. In July 2022, Park J et al. [73] developed an inactivated whole-virus avian influenza vaccine consisting of H1N9, H3N8, H5N1, and H7N3 subtypes, which provided protection to animal models such as mice and ferrets against various influenza A viruses. This vaccine has been approved by the FDA and is currently undergoing human clinical trials.

Current commercial vaccines are whole-virus inactivated vaccines produced using embryonated chicken eggs (ECEs), which have several drawbacks. Inactivated vaccine production requires at least six months [74], and the virus cultured in chicken embryos might introduce glycosylation not present in wild-type strains, reducing the vaccine's protective efficacy against circulating strains [75]. HPAIV can be lethal to chicken embryos, making high-titer cultures challenging [74]. Most H5N1 inactivated vaccines have poor immunogenicity, requiring at least two doses to elicit a durable immune response [74]. Inactivated split-virus and subunit H5N1 vaccines without adjuvants achieve only 58% neutralizing antibody titers in recipients, and antibody titers significantly drop six months after the second dose [76]. Therefore, new vaccine formulations are needed to address these issues. The influenza vaccines QIVc [77] and Vepcel® [78], developed using mammalian cell lines, have demonstrated excellent tolerance and immunogenicity in clinical trials and have received approval from the European Union. These vaccines are pivotal in the active immunization against the influenza A subtype H5N1. Cell-based vaccines effectively mitigate the risk of adaptive mutations associated with chicken embryo production and are independent of the chicken embryo supply. This independence allows for the rapid and efficient scaling of production in the event of a large-scale influenza pandemic, ensuring timely and adequate public protection. Furthermore, cell-based inactivated vaccines enhance the potential for future vaccine research and application, offering novel insights and directions for the advancement of global public health.

2.2. Live Attenuated Influenza Vaccines (LAIVs)

Live attenuated influenza vaccines primarily replicate in the upper respiratory tract, inducing mucosal IgA, serum IgG, and cell-mediated immune responses, mimicking natural infection, and representing a potential option for pandemic influenza vaccines. With the WHO recognition of LAIVs, they have become an attractive candidate for pandemic vaccines.

The early LAIV is based on cold-adapted influenza virus. In 1999, Li et al. [79] constructed an H5N1 vaccine by recombining the genes of A/Ann Arbor/6/60 with the HA and NA genes of H5N1. The recombinant strain showed temperature-sensitive and cold-adapted phenotypes, exhibiting good safety and immunogenicity in ferrets. This recombinant cold-adapted vaccine strain is a potential candidate of human influenza LAIV. In 2017, Lee YH et al. [80] used a recombinant cold-adapted H5N1 vaccine for intranasal immunization of mice. A single immunization induced hemagglutination inhibition (HI) and neutralizing antibody titers as well as respiratory tract IgA antibodies, protecting mice against the lethal H5N1 virus challenge. The study indicated the vaccine's stability and immunogenicity, suggesting it as a reserve pandemic preventive measure, replacing current egg-based production.

Alternatively, a live attenuated influenza vaccine named DelNS1-H5N1 constructed by deleting the NS1 gene of H5N1, showed a replication-deficient phenotype and reduced the possibility of genetic reassortment between the vaccine and circulating viruses. Phase I clinical trial results indicated that a single intranasal dose safely induced significant vaccine-specific antibody levels, and a second dose resulted in 41.7% local IgA responses specific to the vaccine. This provided proof of concept for DelNS1-H5N1's efficacy and laid the foundation for future clinical studies [81].

A replication-deficient LAIV reduces the possibility of reassortment while retaining the benefits of the LAIV approach. Researchers have constructed a replication-deficient recombinant influenza virus, WM01ma-HA (H5), expressing the hemagglutinin (HA) of H9N2 and H5N1 subtypes. Using the HA open reading frame (ORF) sequence of H5 to design a chimeric gene segment, with NA packaging signals from the mouse-adapted strain H9N2 (WM01ma), the HA ORF sequence of H5 was inserted in between. Due to the lack of NA protein expression from the ORF of the structural protein NA, this engineered H9N2 influenza virus WM01ma-HA (H5) showed restricted replication in vitro and in vivo. Intranasal vaccination of mice with WM01ma-HA (H5) stimulated a robust immune response, providing complete protection against H5N1 subtype influenza virus infection [82].

Live attenuated vaccines can induce strong humoral and cellular immune responses, providing long-lasting broad protection, thus reducing the need for frequent vaccinations. However, despite being attenuated, there is a risk of reversion to pathogenicity, especially in immunocompromised individuals. As a live virus, there is also a potential reassortment risk with circulating viruses.

2.3. Recombinant Protein Vaccines

Four types of recombinant protein vaccine platform have been used to design H5N1 vaccine.

Recombinant HA Protein Vaccines: In a clinical study, recombinant H5 HA protein expressed in baculovirus (rH5) induced neutralizing antibodies in adults at a rate comparable to egg-derived H5N1 subunit vaccines [83]. Although baculovirus-expressed H5 HA can induce functional antibodies in individuals not previously exposed to H5 viruses, its immunogenicity is low, requiring high doses for adequate response. Recombinant protein vaccines, which do not involve live viruses, offer higher safety. They are flexible in production and can quickly adjust vaccine components to adapt to viral changes, making them applicable to multiple viral proteins. However, their immunogenicity is lower, limited to adults aged 18–49, and they require frequent HA updates to match circulating strains' antigenicity.

(i) Virus-Like Particle (VLP) Vaccines: VLP vaccines are derived from self-assembling viral structural proteins into well-organized, non-infectious particles. VLPs mimic live viruses and can induce strong humoral and cellular immune responses [84]. For poultry, a single immunization with H5N1 VLP vaccines provided protection equivalent to commercial inactivated vaccines [85,86]. Plant-based influenza quadrivalent virus-like particle vaccines (QVLP) demonstrated greater efficacy than traditional quadrivalent inactivated influenza vaccines in human trials [87]. QVLP has reached phase III clinical trials, confirming its immunogenicity and safety [88], but it is not yet commercialized. Additionally, VLP vaccines based on different H5N1 clades have shown good immunogenicity in animal models [89–91]. However, the VLP production process is complex and costly, requiring further optimization for large-scale production.

(ii) Antigen-Presenting Induced Vaccines: During viral infection, CD11c+ dendritic cells (DCs) convert viral antigens into peptides via the major histocompatibility complex (MHC) and present them to T cells in secondary lymphoid organs, thereby significantly promoting adaptive antiviral immune responses. T cell responses to influenza viruses can be enhanced by inducing antigen-presenting cells. Studies indicate that the presence of α-Gal epitopes (Galα1-3Galβ1-4GlcNAc-R) on HA enhances the uptake of influenza virus vaccines by antigen-presenting cells [92]. Post-immunization in mouse models has shown induction of virus-specific $CD8^+$ and $CD4^+$ T cells and higher antibody titers, providing increased protection levels. This suggests that expressing α-Gal epitopes on recombinant envelope proteins might serve as an effective alternative to inactivated virus vaccines.

(iii) Epitope Peptide Vaccines: Next-generation vaccine strategies aim to induce immunity against a wide array of viral variants, thereby avoiding the need for frequent vaccine updates. By computationally identifying T cell and B cell epitopes of target proteins and utilizing synthetic peptides, "epitope-focused vaccines" can be developed [93]. FLU-v, a peptide-based, broad-spectrum influenza candidate vaccine, comprises short peptides derived from conserved regions of influenza virus proteins M1, M2, NP-A, and NP-B, inducing both human antibody and cellular immune responses. FLU-v significantly increases the number of IFN-γ and granzyme B-secreting cells responsive to vaccine antigens [94]. Currently in phase III clinical trials, this vaccine holds potential as a universal influenza vaccine.

2.4. Recombinant Viral Vector Vaccines

Recombinant viral vectors serve as vaccine delivery platforms capable of inducing innate and adaptive immune responses without needing adjuvants. Various viral vectors have been employed to develop H5N1 influenza vaccines.

The recombinant fowlpox virus (FPV) vaccine for poultry was the earliest recombinant viral vector vaccine recognized in the United States, and it was among the first recombinant vaccines approved for use in poultry [95]. China developed an attenuated recombinant FPV vaccine expressing the HA and NA of HPAI H5N1 [96], with over 600 million doses used by 2009 [97]. Newcastle disease virus (NDV) is also an ideal vaccine vector for preventing HPAI infections in avian species [98]. Since 2006, China has used NDV vector-based H5 bivalent live vaccines in chickens [99], updating the vaccines by inserting different HA genes. Turkey herpesvirus (HVT) vectors have proven safe and effective against various avian viruses [100], with rHVT vaccines for H5 HPAI licensed in multiple countries. Duck enteritis virus (DEV) vector vaccines containing inserted H5 genes have demonstrated rapid and complete protection against H5N1 and DEV in ducks [101]. Zou et al. [102] constructed a DEV-based vaccine targeting the H5N1 avian influenza virus and duck Tembusu virus (DTMUV) using CRISPR/Cas9 technology and evaluated its efficacy. They demonstrated that the C-KCE-HA/PrM-E trivalent vaccine effectively prevents infections from H5N1, DTMUV, and DEV simultaneously. The CRISPR/Cas9 system offers increased convenience and efficiency compared to traditional DEV modification methods. Several adenovirus vectors have been used to develop influenza vaccines, demonstrating protection against influenza virus challenges in animal models, such as mice and ferrets, by expressing H5N1 antigens [103–109]. A HAd5 vector vaccine expressing A/VN/1203/04 (H5N1) HA (AdhVN1203/04.H5) demonstrated good tolerance and induced humoral immunity in healthy adults aged 19–49 [110]. Ad4-H5-Vtn, formulated as oral enteric capsules, showed good safety and immunogenicity in phase I clinical trials, inducing HA-specific T cell immunity [111], though with limited humoral immunity. A recombinant viral vector vaccine based on parainfluenza virus type 5, inserting the NP gene of HPAI H5N1, induced specific humoral and T cell immunity, providing protection against lethal H5N1 and heterotypic H1N1 virus challenges after a single dose [112]. These results suggest that PIV5 expressing H5N1 NP has potential as a universal influenza virus vaccine.

Nasal spray vaccines based on viral vector can build an immune barrier at the frontline of respiratory virus infection and offer user-friendly administration routes, showing significant application potential. Chen et al. [113] designed and constructed a dual-attenuated influenza vector COVID-19 vaccine, dNS1-RBD, validated for safety and broad efficacy in phase III clinical trials and approved for emergency use. This vaccine provided cross-protection against H1N1 and H5N1 influenza viruses. The protective immune mechanism of dNS1-RBD is attributed to innate immune responses of the nasal epithelium, local lung RBD-specific T cell responses, and RBD-specific IgA and IgG responses. This study suggests that intranasal administration of the dNS1-RBD candidate vaccine may be effective in combating influenza pandemics, addressing the limitations of current intramuscular injection vaccines. In response to the ongoing mutations of SARS-CoV-2, Chen et al. [114] developed an updated vaccine utilizing this viral vector. This updated vaccine demonstrates broadly protective efficacy against the Beta and XBB.1.9.2.1 variants. The swift replacement of antigen genes underscores the significant potential of the influenza virus vector in addressing both emerging and re-emerging infectious diseases and in mitigating potential future pandemics.

Recombinant viral vector-based nasal vaccines can induce multifaceted immune responses, providing broad and sustained immunity against various pathogens. However, pre-existing immunity to the vector virus can affect vaccine efficacy, and the safety of some viral vectors requires further evaluation due to potential immune responses or other concerns.

2.5. Nucleic Acid Vaccines

Nucleic acid vaccines have emerged in recent years as a promising type of vaccine. Among them, RNA vaccines and DNA vaccines are particularly notable for activating immune responses by encoding RNA or DNA sequences of antigens, demonstrating potential for rapid development and strong immunogenicity.

(i) mRNA Vaccines: The remarkable success of mRNA vaccines in addressing the COVID-19 pandemic has spurred research into their use for other infectious diseases, including influenza. In 2018, researchers at the University of Pennsylvania designed and evaluated an mRNA vaccine encoding the full-length HA of the H1N1 influenza virus, modified, purified, and delivered in lipid nanoparticles. Immunized mice achieved protection against heterologous H5N1 virus challenges, demonstrating the feasibility of mRNA platforms as broadly protective influenza virus vaccine candidates [115]. In May 2024, an mRNA vaccine targeting 2.3.4.4b H5 viruses, developed in lipid nanoparticles, induced high levels of neutralizing antibodies and HA-

specific $CD8^+$ T cell responses in mice, protecting ferrets from lethal H5N1 virus challenge [116]. However, this study has not validated the mRNA vaccine's protection against other H5N1 clades or its impact on pre-existing immunity. Given the continuous global spread of the 2.3.4.4b clade, this vaccine and platform offer rapid development and precise antigen matching, potentially serving as effective pandemic countermeasures. mRNA vaccines enable swift synthesis and production to address emerging virus strains and elicit strong immune responses. Nevertheless, they require stringent cold chain logistics and storage conditions, which increase costs, and there is currently limited long-term safety and efficacy data. The Biomedical Advanced Research and Development Authority (BARDA) is nearly finalizing an agreement with Moderna to fund human trials of its experimental mRNA avian influenza vaccine. The agreement includes provisions for stockpiling millions of doses if the trials prove successful. Furthermore, GlaxoSmithKline and CureVac have recently commenced H5N1 vaccine clinical trials, and additional companies are developing influenza vaccines that can be administered orally as capsules or through skin patches.

(ii) DNA Vaccines: Compared to traditional inactivated or attenuated vaccines, DNA vaccines offer prolonged expression of plasmid-encoded antigens and induce both humoral and cellular immunity. In 1999, Kodihalli et al. [117]demonstrated that a plasmid DNA vaccine encoding H5N1 HA, administered via gene gun to mice, elicited a low antibody response but provided protection against influenza virus, proving the feasibility of DNA vaccines for H5-related strains in humans. In 2007, the research team led by Chen Hualan demonstrated that a DNA vaccine expressing HA protected against the highly pathogenic H5 virus in chickens [118]. However, the high-dose intramuscular injection strategy posed practical application challenges. Given the low immunogenicity of DNA vaccines, combining them with nano-adjuvants presents promising prospects for improving transfection efficiency and immunogenicity. One study developed a co-delivery system using manganese-based liposomes as adjuvants for DNA vaccines. This system protected the DNA vaccine, enhanced phagocytosis, and promoted activation of antigen-presenting cells (APCs) and immune cells in draining lymph nodes, thus activating both humoral and cellular immunity in mice. This strategy opens new avenues for enhancing DNA vaccine immunogenicity using manganese nano-adjuvants [119].

Influenza viruses possess numerous subtypes, with continuous antigenic variation making epidemiological trends difficult to predict. Developing a universal influenza vaccine with broad-spectrum cross-protection remains the ideal strategy for controlling influenza. Universal influenza vaccines target conserved viral epitopes, aiming to overcome the challenges posed by the highly variable nature of influenza viruses. These vaccines offer broader protection against various influenza strains by inducing cross-protective and broadly neutralizing antibodies. The aforementioned vaccine platforms hold promise as universal vaccines, preparing for H5N1 and future influenza pandemics that may pose potential threats to humans.

Table 2. Current Status of H5N1 Vaccine Development using Different Vaccine Platforms.

Vaccine Platform	Vaccine Name	Species Targeted	Delivery Route	Production properties	Induced immune response	Safety Profiles (Adverse Events, AEs) [a]	Scalability	Status	References
Whole-virus vaccine	A/chicken/Indonesia (Legok)/03 (H5N1)	Chickens	Intramuscular	High yield; Directly prepared from allantoic fluid without a concentration process.	Induce 10 months of protective immune response in chickens; Effective against homologous H5N1 virus and early heterologous H5N1isolates.	Excellent	Limited scalability due to extended production cycle	Approved	[5,120]
	H5N1/PR8	Chickens Ducks geese	Intramuscular					Approved	[5,121]
	BPL-1537	Human	Intranasal or Intramuscular					In Phase 1	[122]
	IVACFLU-A/H5N1	Human	Intramuscular					In Phase 2/3	[123]
Split-virus vaccine	Sanofi Pasteur's Influenza A (H5N1) Virus 173 Monovalent Vaccine	Human	Intramuscular	Vaccine, Adjuvant, and the Mix-and-Match.	Less 6 months.	Well-tolerated	Limited scalability due to extended production cycle	In Phase 3 licensed in the US	[124,125]
	GlaxoSmithKline's Arepanrix H5N1	Human	Intramuscular					In Phase 4 licensed in the US	[125,126]
Subunit vaccine	CSL 174 Limited's Aflunov	Human	Intramuscular	Cell culture manufacturing methods.	Provide cross-protection against five genetic clades or subclades.	Well-tolerated; No vaccine-related serious adverse events occurred.	Scalable	In Phase 4 licensed in the US	[125,127]
Live, attenuated virus vaccine cold-adapted	FluGen	Human	Intranasal	Cell culture -based process; Cryopreserved, reconstituted, and scaled up at any time; Peak titer of the vaccine in MDCK cells was lower than eggs.	Single immunization induces neutralizing and IgA antibodies; Local and systemic antibody and T-cell responses; Protected against identical and drifted H5N1 viruses.	Well-tolerated	Scalable	In Phase 2	[80,128]
	A/17/Duck/Potsdam/86/92 (H5N2)	Human	Intranasal					In Phase 1/2	[129]
Recombinant HA vaccine	Multimeric-001 (M-001)	Human	Intramuscular	Produced in *E. coli* using standard fermentation and purification methods with ion exchange and hydrophobic columns.	Induces neutralizing antibody.	Well-tolerated; No significant adverse events were noted	Scalable	In Phase 3	[130]
	rHA-GLA/SE	Human	Intramuscular					In Phase 1/2	[131]
Virus-like particle vaccine	COBRA-VLP	Human	Intramuscular	Higher surge capacity, response speed, and simplicity in the plant-based technology.	Detectable cross-reactive neutralizing antibodies towards H5N1.	Well-tolerated; Typical mild and short duration adverse events; No serious AEs	Scalable	Preclinical	[132]
	H5VLP	Human	Intramuscular					In Phase 2	[133,134]
Recombinant viral vector vaccine	fowl pox virus (FPV)	Chickens, Ducks	Wing-web, Intramuscular, or Subcutaneous	Special production lines tailored for different viruses.	Specific antibody and robust cell-mediated immune responses.	Depends on the virus vector	Depends on the virus vector	Preclinical	[95,96]

	Newcastle disease virus (NDV) rLH5-1	Chickens	Oculonasal					Approved	[98,99]
	turkey herpes virus (HVT)	Chickens	Intramuscular, Subcutaneous					Preclinical	[100]
	duck enteritis virus (DEV)	Ducks, Broilers	Intramuscular					Preclinical	[101]
	infectious laryngotracheitis virus (ILTV)	Chickens	Ocular					Preclinical	[135]
	PIV5-NA	Human	Intranasal					Preclinical	[136]
	Wyeth/IL-15/5flu	Human	Subcutaneous					Preclinical	[137]
	VXA-A1.1 oral tablet	Human	Mucosal					In Phase 2	[138]
mRNA vaccine	H5-mRNA-LNP	Human	Intramuscular	Gene-synthesized and transcription in vitro.	Induces neutralizing antibodies and HA-specific $CD8^+$ T-cell responses.	No data available	Scalable	Preclinical	[116]
DNA vaccine	pCAGGoptiHA	Chickens	Intramuscular	DNA design and plasmid synthesis.	Cross protection; Higher effector memory $CD8^+$ T-cell responses.	No data available	Scalable	Finish Phase 3	[118]
	Micro-consensus DNA vaccine	Human	Intradermal/ Intramuscular electroporation					In Phase 1	[139]

a "vaccine AE" or an "AE following immunization" is defined as "any untoward medical occurrence which occurs during administration of a vaccine or follows immunization and which does not necessarily have a causal relationship with the use of the vaccine. The adverse event may be any unfavorable or unintended sign, an abnormal laboratory finding, a symptom or a disease" [140].

3. Status and Challenges of H5N1 Diagnostics Development

Timely and accurate diagnosis of H5N1 infection is crucial for reducing mortality in poultry and humans and for lowering the risk of a pandemic. Current H5N1 diagnostic methods include virus isolation and identification, molecular biology detection, immunodetection, and serological detection, each with its strengths and weaknesses (see Table 3). Various diagnostic methods with high specificity and sensitivity are being developed to improve the detection and monitoring of the H5N1 virus.

3.1. Virus Isolation and Identification for H5N1

Virus isolation and identification, the gold standard for avian influenza virus (AIV) detection, are performed by isolating and culturing AIV in chicken embryos or MDCK cells [141]. The cell isolation method involves inoculating samples into MDCK cells and observing cell cytopathic effects. For chicken embryo isolation, samples are inoculated into the amniotic and allantoic cavities of 9 to 11 day-old chicken embryos, and the culture fluid is subsequently analyzed through hemagglutination (HA) and hemagglutination inhibition (HI) tests. However, this method requires a long detection time (2–3 days) [142] and Level 3 Biosafety Laboratories (BSL-3) for handling HPAI H5N1 virus isolation. Additionally, stringent sample storage and transportation conditions make it unsuitable for field testing [143].

3.2. Nucleic Acid Molecular Detection for H5N1

Accurate and efficient nucleic acid molecular detection methods are vital for the diagnosis, monitoring, and prevention of the H5N1 virus. Three primary detection methods—RT-PCR, isothermal amplification, and NGS—have been developed for H5N1 diagnostics. Each method has unique application scenarios and advantages.

(i) Reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR are common molecular techniques for identifying specific gene sequences. RT-PCR is one of the earliest applied nucleic acid amplification technologies and has created a new method for detecting RNA viruses. RT-PCR converts RNA into cDNA through reverse transcriptase and then uses PCR to amplify cDNA and detect specific sequences. RT-PCR has higher sensitivity for detecting influenza A virus (>93%) compared to virus isolation culture (80%) and ELISA (62%) [144]. Initially used to detect H5 gene segments during the H5N1 outbreak in Hong Kong [115], RT-PCR has demonstrated rapid detection capabilities for clinical specimens. Wei et al. [145] utilized multiplex RT-PCR methods to subtype H5 and N1 AIV directly from samples. Payungporn et al. [146] developed a one-step multiplex RT-PCR method using primers targeting M, H5, and N1 genes, achieving good specificity. RT-qPCR is a further development of RT-PCR and offers real-time quantitative detection ability. By incorporating fluorescent labeling, it monitors the accumulation of products in real time during the amplification of complementary DNA. Quantitative analysis is conducted through changes in fluorescence intensity. RT-qPCR is capable of providing quantitative data for detection and can perform multiplex detection with high accuracy. Real-time fluorescence RT-PCR offers significant advantages in speed, viral load quantification, sensitivity, and specificity [147]. Chen et al. [148] established a real-time RT-PCR method capable of detecting H5 subtype influenza virus loads as low as 5×10^{-2} EID_{50}, compared to a minimum detection threshold of about 3 EID_{50} using WHO primers in conventional RT-PCR. Payungporn et al. [149] developed a one-step multiplex real-time RT-PCR using primers for M, H5, and N1 genes and TaqMan MGB probes labeled with various markers, capable of detecting three fluorescence signals with a sensitivity of 10^2–10^3 copies/μL. RT-PCR and RT-qPCR offer sensitive and specific diagnostic tools but have high diagnostic costs and stringent technical and laboratory requirements to avoid false positives or negatives. These molecular detection technologies require specific gene sequence information, such as HA, NA, and NP, which can complicate the detection of certain isolates or highly mutated H5N1 viruses.

(ii) Isothermal amplification technology is an innovative in vitro nucleic acid amplification method that operates at a constant temperature. By employing various enzymes with distinct activities and specific primers, it rapidly amplifies nucleic acids. Compared to RT-PCR, isothermal amplification is particularly advantageous for on-site clinical detection due to its simplicity and speed, eliminating the need for complex equipment. Techniques such as Nucleic Acid Sequence-Based Amplification (NASBA), Loop-mediated Isothermal Amplification (LAMP), and Recombinase Polymerase Amplification (RPA) are currently used for detecting the H5N1 virus. NASBA is a PCR-independent method allowing rapid amplification of specific nucleic acid regions, particularly suitable for RNA sequences. This technique employs RNAse H, T7 RNA polymerase, and avian myeloblastosis virus reverse transcriptase (AMV-RT) for amplification. In 2002, a research team in Hong Kong applied NASBA to detect H5 subtypes of avian influenza viruses, and identify multiple strains isolated from chicken embryos. Moore et al. [150] developed a commercial NucliSens EasyQ Influenza A H5 and N1 NASBA test. Studies

demonstrated NASBA's 100% sensitivity for 19 clinical samples and high sensitivity for H5N1 virus detection (0.01 $TCID_{50}$ and 10 copies/μL), showcasing its practical applicability in clinical research. Despite its high specificity, sensitivity, convenient operation, and time-saving, NASBA's complexity and reliance on three enzymes increase its costs. LAMP is another promising technique that uses DNA polymerase or RNA reverse transcriptase and multiple primers to enhance specificity [151,152]. RT-LAMP's high sensitivity, up to 100 times that of conventional one-step RT-PCR, makes it advantageous for AIV diagnosis [153–155]. However, LAMP primer design can be challenging due to the rapid mutation rates of viral genes. RPA was developed by Piepenburg et al. [156] in 2006, which offers a simple operation and high efficiency at low temperatures. Yehia et al. [157] established an H5 RT-RPA detection method with 100% sensitivity, capable of detecting a single RNA molecule within 7 min. Compared to other isothermal techniques, RPA offers simple operation, high efficiency, and low-temperature processing. However, RPA primer design remains a significant bottleneck.

(iii) Next-generation sequencing (NGS) has received extensive attention because it can perform rapid, accurate and high-throughput sequencing of the entire genome of viruses. NGS technology offers unparalleled advantages especially when it comes to the comprehensive analysis of genetic variations of viruses, the monitoring of virus evolution, and the identification of emerging virus strains. In 2018, researchers obtained the complete genome sequence of the HPAI H5N1 directly from the lung, trachea, colon tissue, and fecal samples of patients using NGS technology. This marked the first report of acquiring the complete H5N1 genome from human autopsy specimens [158]. In 2023, an HPAI H5N1 outbreak among cats in Poland further demonstrated the powerful application of NGS. The research team conducted whole-genome sequencing and phylogenetic analysis, identifying the virus as belonging to clade 2.3.4.4b with a CH genotype. The analysis revealed that the virus had partially adapted to mammalian hosts, suggesting a need for enhanced monitoring and detection of certain mammal species [159]. Despite its potential in pathogen detection, NGS's high costs and complex operation limit its applicability as a routine diagnostic method.

3.3. Antigen Detection for H5N1

Colloidal gold immunochromatography is a widely used sandwich immunoassay technology for detecting pathogens, DNA, small molecules, and proteins [160–163]. Cui et al. [164] developed a rapid immunogold test strip using monoclonal antibodies for HPAI H5 subtype-specific detection of the H5N1 virus. This method is rapid, sensitive, and cost-effective for field testing. However, traditional immunogold is mainly qualitative for antigen or antibody detection. Li et al. [165] modified colloidal gold immunochromatographic techniques to achieve rapid semi-quantitative detection of H5 AIV by setting multiple test lines, capable of detecting infection levels in different organs. Despite its speed and ease of use, colloidal gold immunochromatography has relatively low sensitivity, with specificity >90% and sensitivity around 60% [166]. Enhancing the efficiency of the immune amplification system is a critical factor in improving the performance of antigen detection. Fluorescent quantum dots and nanoparticles are considered promising substitutes for traditional markers. Akira Sakurai et al. [167] established fluorescence immunochromatography using conjugated microspheres for broad-spectrum H5 subtype influenza virus detection, achieving sensitivity 10–100 times higher than traditional immunochromatography. Atsuhiko Wada et al. [168] combined silver amplification technology with immunochromatography for H5 subtype virus detection, achieving 500-fold sensitivity improvement. Although fluorescence immunochromatography had high detection sensitivity, it is costly and requires special equipment investment for quantum dot labeling. To develop rapid diagnostic kits suitable for field use, Chen et al. [169] developed broad-spectrum H5-specific antibodies recognizing 10 major genetic groups and subgroups (clade 0–9). Using these antibodies, they established a dot enzyme-linked immunosorbent assay (Dot-ELISA) to detect H5N1 virus or isolate samples. This test employs horseradish peroxidase-labeled H5-specific antibodies to identify the H5N1 virus captured by fixed H5-specific antibodies. Compared to virus culture, the overall positive rate of this test is 96.7%, providing a simple and rapid method that does not require complex instruments. This method is suitable for field investigations of H5N1 avian influenza outbreaks and potentially for suspected cases of human H5N1 infections. They also applied this method for H1N1 virus detection, achieving high sensitivity and specificity, showing potential for rapid influenza virus detection and field application [170]. Enhancing the broad-spectrum and specificity of antibodies is a critical factor in improving antigen detection performance. As the H5N1 virus continues to evolve and mutate, screening for new broad-spectrum antibodies can improve the sensitivity and specificity of existing detection methods and increase the accuracy of virus antigen detection.

3.4. Serological Detection for H5N1

Serological detection primarily includes HI assays and enzyme-linked immunosorbent assays (ELISA). HI and microneutralization are sensitive serological methods considered the gold standard for detecting H5-specific antibodies in humans [171]. These methods are economical, rapid, and widely used [172], although cross-reactivity may occur among some subtypes. Various ELISA methods have been developed to detect AIV antibodies in poultry and other species, including humans [173]. Zhou et al. [174]developed a competitive ELISA (CELISA) using recombinant nucleoprotein and monoclonal antibodies for the serological diagnosis of various avian AIV strains. This method omits that use of multiple anti-immunoglobulin conjugates or live virus, thereby reducing potential exposure risks [175]. Wu et al. [176] expressed full-length influenza nucleoprotein in *E. coli* to detect influenza nucleoprotein antibodies in serum samples. However, H5N1 cross-reactive antibodies in serum may affect test accuracy [177]. Other studies have developed epitope-blocking ELISA for H5 virus HA [178] and highly conserved antigen epitope peptide-based detection methods for rapidly detecting H5N1 antibodies in human and animal serum [179]. Although ELISA is simple to perform with high specificity and sensitivity, it is limited by poor reproducibility and potential cross-reactivity.

3.5. Emerging Technologies for H5N1 Detection

Existing diagnostic methods face challenges in large-scale screening and resource-limited outbreak areas. New molecular diagnostic technologies such as nanopore sequencing, CRISPR-Cas systems, and immunoamplification technologies aim to improve detection efficiency and accuracy. Nanopore sequencing technology shows great potential in influenza virus detection, particularly for real-time monitoring, rapid diagnosis, and resistance gene detection, due to its high diagnostic sensitivity (100%) and rapid whole-genome sequencing capability [180,181]. CRISPR-Cas systems [182], combined with RT-RPA technology, have established new methods for detecting the H5 virus [183,184], achieving high specificity and efficiency suitable for clinical settings, emphasizing their importance as early detection and management tools for H5 subtype AIV outbreaks. Additionally, various biosensors have been developed for influenza virus detection, such as surface plasmon resonance [185], field-effect transistors [186], electrochemical biosensors [187], gene biosensors, and impedance biosensors. The use of novel materials has reduced biosensor manufacturing costs while continuously improving sensitivity. In the future, detection technologies for H5N1 subtype AIV are expected to become more convenient, rapid, accurate, sensitive, and cost-effective. Cross-disciplinary applications of multi-field technologies will be another trend in the development of detection technologies.

Table 3. Technologies Available for H5N1 Detection.

Detection Type	Technology	Target(s)	Sensitivity	Time Required	Application Setting	Limitation	References
Virus Isolation	Chicken embryos	HA	Medium	2–3 days	Laboratory	Require BSL-3 facilities	[188]
	MDCK cells	HA	Medium	2–3 days	Laboratory	Require BSL-3 facilities	[188]
Nucleic Acid Molecular Detection	RT-PCR	M, HA, NA, NP	High	3–6 h	Laboratory	High diagnostic costs and stringent technical requirements	[144,189]
	NASBA	HA, NA	High	3–5 h	On site	High diagnostic costs	[142,189]
	LAMP	HA	High	1–2 h	On site	Complicated primer design	[142,189]
	RPA	HA	High	0.5–1 h	On site	Primer design remains a bottleneck	[157,190]
	Gene chip	HA	High	3–6 h	On site	High detection costs	[175,191]
	NGS	Complete virus genome	High	5 days	Laboratory	High costs and complex operation	[192]
Antigen Detection	Colloidal gold immunochromatography	HA, NP	Low	30 min	On site	Not suitable for the early-stage infection diagnosis	[193]
	Dot-ELISA	HA, NP	Medium	1–3 h	On site	Effective in high-risk avian influenza scenarios with high viral load	[169]
	Fluorescence Immunodetection Technique	HA, NA, NP	Medium	0.5–2 h	On site	Fluorescent dyes have poor stability and insufficient fluorescence intensity	[175,194]
Serological Detection	ELISA	HA, NA, NP	Medium	2–3 h	On site	Lack reproducibility and potential cross-reactivity	[171,175]
	HI	HA	High	3 days	Laboratory	Potential cross-reactions may occur between subtypes	[175]

Notes: HA, NA, NP, and M refer to specific viral proteins: Hemagglutinin, Neuraminidase, Nucleoprotein, and Matrix protein, respectively. Sensitivity levels are categorized as High, Medium, Low and not provided directly in some cases. The "Time Required" is indicative and might vary based on specific conditions and available infrastructure.

4. Status and Challenges of H5N1 Therapeutics Development

The treatment of severe influenza presents numerous challenges, and the prompt initiation of antiviral treatment at the onset of the disease is considered a crucial strategy for reducing mortality.

Currently, the main therapeutic drugs for H5N1 include adamantanes (such as amantadine and rimantadine), neuraminidase inhibitors (NAIs) (including oseltamivir, zanamivir, and peramivir), RNA polymerase inhibitors (such as favipiravir), and polymerase acidic endonuclease inhibitors represented by baloxavir [195]. These antiviral drugs target different stages of the viral replication cycle (see Figure 1), thereby alleviating symptoms, reducing complications, and enhancing patient outcomes [196].

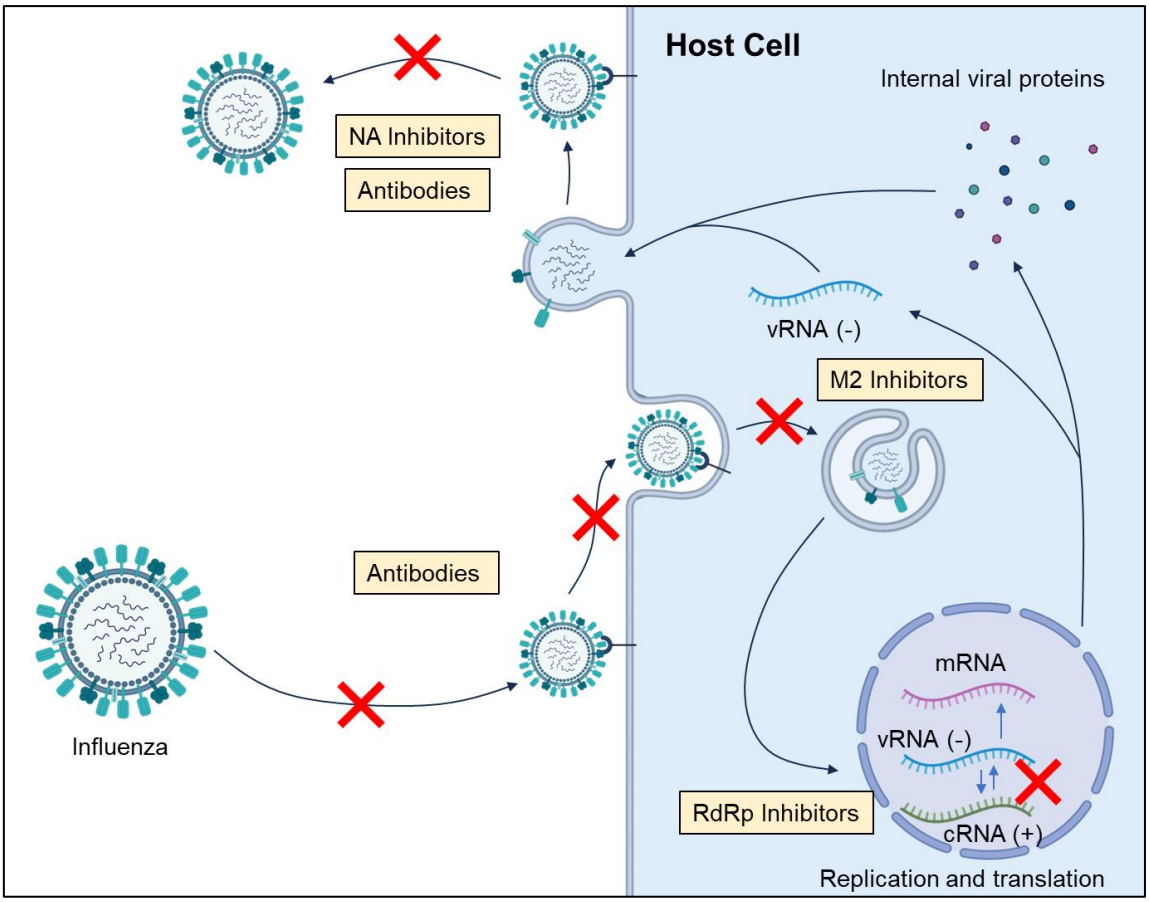

Figure 1. Influenza Virus Lifecycle: Therapeutic Intervention Stages.

Adamantanes inhibit the formation of M2 protein ion channels, thus hindering viral replication. However, due to increased resistance levels from over 50 years of use, adamantanes are no longer recommended for treating influenza A virus infections [195].

Favipiravir, an RNA polymerase inhibitor, was approved in 2014 (Japan) and in 2016 (China) for the treatment of influenza infections. It has demonstrated higher efficacy compared to oseltamivir and the potential to prevent the emergence of resistant strains [195]. However, it has some limitations such as poor solubility, embryotoxicity, and teratogenic risks.

Until 2018, NAIs were the only class of antiviral drugs approved by the FDA. These drugs can shorten symptom duration, reduce viral load, and decrease infection time [38]. In 2018, the FDA approved a new antiviral drug, baloxavir marboxil, for the early treatment of uncomplicated influenza in outpatient individuals aged 12 and above. This drug provides a valuable alternative for treating influenza virus infections resistant to NAIs [197,198].

Rapid viral mutation can lead to drug resistance, diminishing treatment efficacy. Passive immunity is a new strategy for preventing and treating H5N1 virus infections [199]. Limited clinical data indicate that convalescent plasma can effectively treat severe H5N1 infections [200,201]. Several preclinical studies have demonstrated that convalescent plasma can prevent and treat H5N1 infection [202,203]. Plasma from recovered patients could provide a timely and effective treatment for patients during outbreaks and pandemics. Antibody-based therapies, particularly monoclonal antibodies (mAbs), have been found to be promising treatment options for H5N1 virus

infections. Thus, developing human monoclonal antibodies targeting H5N1 has been a focus of passive immunotherapy research. Tan et al. [204] demonstrated that humanized H5 antibodies exhibit positive activity against a broad range of H5N1 strains across HI, viral neutralization, and immunofluorescence assays. Another approach is involves the use of polyclonal antibodies, which can recognize multiple epitopes on pathogen surfaces, offering better protection at a relatively lower cost [205].

The team from Xiamen University generated 52 broadly cross-reactive monoclonal antibodies against HA of H5N1 using sequential immunization strategy. Among these, the monoclonal antibody 13D4 successfully protected mice from lethal challenges posed by four H5N1 strains belonging to different evolutionary clades (1, 2.1, 2.2, and 2.3) [206]. The study indicates the presence of common or conserved epitopes among different H5N1 clades. Further analysis of the binding epitope of 13D4 identified four highly conserved amino acids in the target site [207], which explains the 13D4's capability to neutralize all major H5N1 clades isolated from 1997 to 2014. This finding proposes the existence of broadly neutralizing epitopes in H5 viruses, suggesting that the development of broad-spectrum neutralizing antibodies for antiviral treatment holds significant potential to counter rapid viral mutations.

New Drug Development

Recent advancements in antiviral drug development include targeting viral proteins such as polymerases and matrix proteins. Onradivir is a novel small-molecule anti-influenza drug that targets the PB2 subunit of the influenza virus RNA polymerase, effectively inhibiting viral replication. The study demonstrated that Onradivir, when administered within 48 h of the onset of influenza symptoms, significantly accelerates the recovery of adult patients with uncomplicated influenza, particularly in the resolution of fever and reduction of viral replication [208]. In addition, there are efforts to develop broad-spectrum antiviral drugs, such as small-molecule compounds, and immune-modulating treatments like Taurolidine (TRD). For instance, Li et al. [209] identified a small-molecule compound that interacts with HA2, inhibiting IAV infection by blocking membrane fusion. Piceatannol exhibits both anti-inflammatory and antiviral activities, demonstrating significant anti-IAV effects *in vitro* and *in vivo*, thus presenting itself as a promising broad-spectrum anti-influenza candidate [210]. TRD has demonstrated potential as an anti-influenza therapeutic by inhibiting H5N1 replication in MDCK cells, preventing cytokine storm amplification, and modulating NF-κB signaling to reduce inflammation [211].

The currently applied antiviral drugs (see Table 4) exhibit limited efficacy against H5N1 infections and are affected by significant rates of resistance. Therefore, future research should prioritize the development of novel antiviral drugs and treatment strategies, such as gene and antibody therapies, with broad-spectrum activity against the avian influenza H5N1 virus.

This diagram illustrates the various stages of the influenza virus lifecycle, highlighting where therapeutic agents can act. Antibodies and NA inhibitors block viral entry and release, preventing the virus from spreading to other cells. M2 inhibitors target the uncoating of the virus, stopping replication early. RdRp inhibitors interfere with the viral RNA polymerase activity, disrupting the replication and transcription processes. These interventions collectively aim to inhibit virus replication and manage infection.

Table 4. Antiviral Treatment for H5N1 Infection.

Type	Drug	Mechanism of Action	Effectiveness	Drug Resistance	Limitations	References
M2 inhibitors	Amantadine	Inhibits viral replication by interfering with viral entry and uncoating pathways.	More effective in relieving or treating symptoms of influenza A in healthy adults.	High resistance levels (over 90%) in humans, birds, and pigs, due to over 50 years of use. No longer recommended for influenza A.	Effective exclusively against influenza A and only when administered within 48 h of symptom onset.	[212–215]
	Rimantadine		Rimantadine induces fewer adverse effects compared to amantadine.			
Neuraminidase inhibitors	Oseltamivir	Inhibits viral release and cell-to-cell spread	Effective against influenza A and B, reducing symptom duration and severity. Most effective within 48 h of symptom onset.	Resistance incidence of 0.32% in adults and 4.1% in children.	For use in patients with symptoms lasting less than 48 h. Use caution during pregnancy and breastfeeding. Not indicated for children under 18.	[215–218]
	Zanamivir		Can reduce the risk of symptomatic seasonal influenza and shorten hospital stays.	Resistance remains uncommon (<1%) among the circulating viruses.	Requires inhalation delivery, unsuitable for patients with respiratory diseases or very young/elderly patients.	[217,219]
	Peramivir		Shorten hospital stays and reduce the duration of symptoms.	Resistance in ≤1% of GISRS influenza clinical isolates.	Administered intravenously only.	[220]
	Laninamivir		Reduces risk of symptomatic seasonal influenza and shortens hospital stays.	Resistance remains uncommon among the circulating viruses.	Inhalation administration limits clinical usage.	[217,221]
RNA polymerase inhibitors	Favipiravir	Inhibits viral replication by targeting RNA polymerase.	Approved in Japan for novel or reemerging influenza infections unresponsive to existing treatments.	Adequate tolerability but inconsistent clinical benefits in uncomplicated influenza; remains investigational.	Teratogenic and embryotoxic, contraindicating use during pregnancy.	[222]
	Baloxavir		Effective in reducing symptoms and viral replication. Significant improvement in symptoms within one day.	Resistance up to 11% in adult/adolescent and 26% in pediatric subjects.	Teratogenic risk necessitates further scrutiny for clinical deployment.	[217,222,223]
Passive immunity	Antibodies	Blocking infection and inhibiting virus replication.	Preclinical experiments indicate prevention and treatment can effectively reduce the viral load, and show efficacy in severe cases.	Viral variation may lead to antibody failure.	Requires hospital settings; Restricting its use to severe cases and controlled environments; Drug development is time-consuming and costly.	[206,224–229]
	Convalescent plasma		Limited clinical results show efficacy in severe cases.		Requires hospital settings; Relies on donations from convalescent patients, limiting the supply chain; Stringent requirements for transfusion.	[200,201,203,230]

5. Conclusion

Highly pathogenic avian influenza H5N1 has attracted global attention due to its widespread transmission and high mortality rates. The recent crossing of species barriers by HPAI H5N1 to mammals and humans has raised fears that it could become the next pandemic in humans. The continuous evolution and global spread of H5N1 viruses necessitate vigilant surveillance systems for monitoring H5N1 outbreak and appropriate preventive measures including high-performance detection for H5N1 virus, broad-spectrum H5N1 vaccines with long-lasting protection, and more efficient therapeutic drugs for H5N1 infection. This review summarizes H5N1's global circulation and examines the progress and challenges in vaccine development, diagnostic methods, and treatments, offering valuable information to fight H5N1. Effective public health policies—including timely outbreak reporting, vaccination programs, stockpiling and distribution of medical supplies, public health education, and emergency response measures—should also be considered to mitigate the potentially devastating impact of H5N1 on public health and the economy.

In future research, the emphasis should be to develop broad-spectrum, long-lasting vaccines and novel antiviral drugs that can efficiently control the virus's rapid genetic evolution. Furthermore, exploring universal vaccine platforms, which provide comprehensive protection against diverse influenza strains, becomes essential to anticipate and respond to potential pandemic scenarios effectively. Collaborative efforts across multiple scientific disciplines are advocated to accelerate the development of early detection systems and understanding the virus's behavior in different host populations, ensuring prompt and appropriate public health responses.

For public health agencies, practical steps involve enhancing preparedness through coordinated stockpiling of vaccines and antiviral medications, planning efficient distribution channels, and developing rigorous public education campaigns to raise awareness about prevention and treatment strategies. Monitoring at-risk populations, such as individuals working closely with animals in affected areas, should be prioritized to detect and contain outbreaks early. Emergency response measures, which address the logistical challenges of an H5N1 outbreak and protect frontline healthcare workers, should be continuously refined and tested.

Global collaboration between nations, health agencies, and research institutions is critical in pooling resources and expertise to address the multifaceted challenges posed by H5N1. By adopting an integrated approach to research and public health intervention, stakeholders can build resilient systems capable of effectively managing the evolving threat of avian influenza.

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