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Review

Insight into Alternative Approaches for Control of Avian Influenza in Poultry, with Emphasis on Highly Pathogenic H5N1

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Abstract: Highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 causes a devastating disease in poultry but when it accidentally infects humans it can cause death. Therefore, decrease the incidence of H5N1 in humans needs to focus on prevention and control of poultry infections. Conventional control strategies in poultry based on surveillance, stamping out, movement restriction and enforcement of biosecurity measures did not prevent the virus spreading, particularly in developing countries. Several challenges limit efficiency of the vaccines to prevent outbreaks of HPAIV H5N1 in endemic countries. Alternative and complementary approaches to reduce the current burden of H5N1 epidemics in poultry should be encouraged. The use of antiviral chemotherapy and natural compounds, avian-cytokines, RNA interference, genetic breeding and/or development of transgenic poultry warrant further evaluation as integrated intervention strategies for control of HPAIV H5N1 in poultry.

Keywords: influenza; H5N1; control

Abbreviations

AIV= avian influenza virus, ChIFN-α = chicken interferon alpha, ChIL = chicken interleukin, ECE= embryonated chicken eggs, HA = hemagglutinin, HPAIV = highly pathogenic avian influenza virus, IFN = interferon, LPAIV = low pathogenic avian influenza virus, Mx = myxovirus, NA = neuraminidase, NAIs = neuraminidase inhibitors, rFPV = recombinant fowl pox virus, RIG-I = retinoic acid-inducible gene I, RNA = ribonucleic acid, RNAi = RNA interference, siRNA = short-interfering RNA, SPF = specific pathogen free, TLR = Toll-like receptors

1. Introduction

Influenza A virus, the only *orthomyxovirus* known to infect birds, are negative-sense, single-stranded, enveloped viruses contain genomes composed of eight separate ribonucleic acid (RNA) segments encode for at least 11 viral proteins. Two surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA) are playing a vital role in attachment and release of the virus, respectively [1]. The 17 HA and 10 NA subtypes of avian influenza viruses (AIV) are classified according to their pathogenicity for poultry into low pathogenic AIV (LPAIV) result in mild or asymptomatic infections and highly pathogenic AIV (HPAIV) causing up to 100% morbidity and mortality [2,3]. To date, some strains of H5 or H7 subtypes fulfilled the defined criteria of high pathogenicity which potentially evolve from low virulent precursors [4]. Constant genetic and antigenic variation of AIV is an intriguing feature for continuous evolution of the virus in nature [5]. Gradual antigenic changes due to acquisition of point mutations known as “antigenic drift” are commonly regarded to be the driving mechanism for influenza virus epidemics from one year to the next. However, possible “antigenic shift or reassortment” of influenza virus occurs by exchange genes from different subtypes is relatively infrequent, however it results in severe pandemics [6].

HPAIV H5N1 is responsible for magnificent economic losses in poultry industry and poses a serious threat to public health [7,8]. Measures to control the virus in domestic poultry are the first step to decrease risks of human infections [9,10]. Enhanced biosecurity measures, surveillance, stamping out and movement restriction as basic principles for control of HPAIV H5N1 epidemics in poultry [11] has not prevented the spread of the virus since 1997 [12,13]. Recently, vaccines have been introduced in some developing countries as a major control tool to reduce the overwhelming socioeconomic impact of HPAI H5N1 outbreaks in poultry [13]. Different types of inactivated vaccines and to lesser extent recombinant live virus vaccines are already in use that decrease shedding of the virus, morbidity, mortality, transmissibility, increase resistance to infection, lower virus replication and limit decrease in egg production [2,14].

Nevertheless, several challenges facing the efficiency of the vaccine to control the HPAIV H5N1 outbreaks have been reported: (1) Vaccine is HA subtype specific and in some regions where multiple subtypes are co-circulating (*i.e.*, H5, H7 and H9), vaccination against multiple HA subtypes is required [15]. (2) Vaccine-induced antibodies hinder routine serological surveillance and differentiation of infected birds from vaccinated ones requires more advanced diagnostic strategies [16]. (3) Vaccination may prevent the clinical disease but can’t prevent the infection of vaccinated birds, thus continuous “silent” circulation of the virus in vaccinated birds poses a potential risk of virus

spread among poultry flocks and spillover to humans [17–19]. (4) Immune pressure induced by vaccination on the circulating virus increases the evolution rate of the virus and accelerates the viral antigenic drift to evade the host-immune response [20–24]. (5) After emergence of antigenic variants, the vaccine becomes useless and/or inefficient to protect the birds and periodical update of the vaccine is required [20,25–28]. (6) Vaccine-induced immunity usually peaks three to four weeks after vaccination and duration of protection following immunization remains to be elucidated [29]. (7) Maternally acquired immunity induced by vaccination of breeder flocks could interfere with vaccination of young birds [30–34]. (8) Other domestic poultry (*i.e.*, ducks, geese, turkeys), zoo and/or exotic birds even within the same species (*i.e.*, Muscovy *vs.* Pekin ducks) respond differently to vaccination which have not yet been fully investigated compared to chickens [35–42]. (9) Concomitant or prior infection with immunosuppressive pathogens or ingestion of mycotoxins can inhibit the immune response of AIV-vaccinated birds [43–46]. (10) And last but not least, factors related to vaccine manufacturing, quality, identity of vaccine strain, improper handling and/or administration can be decisive for efficiency of any AIV vaccine [2,29].

Therefore, presence of new alternative and complementary strategies target different AIV serotypes/subtypes/drift-variants should be encouraged. This review aims to give an insight into possible alternative approaches for control of AIV in poultry particularly against the HPAI H5N1 subtype.

2. Antivirals

2.1. Chemotherapy

The use of chemotherapeutic agents for control of AIV in poultry was concurrently studied just after discovering their anti-microbial effects [47,48]. However, during the last three decades more attention was paid to the commonly used antivirals, M2 blocker and neuraminidase inhibitors (NAIs), in control of human influenza viruses to be used in eradication of AIV in poultry.

2.1.1. M2 Blockers (Adamantanes)

Amantadine hydrochloride and rimantadine are two M2 blockers which interrupt virus life cycle by blocking the influx of hydrogen ions through the M2 ion-channel protein and prevent uncoating of the virus in infected host-cells [49–51]. The prophylactic activity of **amantadine** in poultry was firstly studied by Lang *et al.* [52] in experimentally infected turkeys with an HPAIV H5N9 isolated in 1966 from Ontario, Canada. Optimum prophylaxis was obtained only when amantadine was administered in an adequate, uninterrupted and sustained amount from at least 2 days pre-infection to 23 days post-infection. During H5N2 outbreaks in Pennsylvania, USA in early 1980s, one of control proposals was the use of amantadine as a therapeutic and/or prophylactic approach. Under experimental condition, amantadine given in drinking water was efficacious to decrease morbidity, mortality, transmissibility and limit decrease in egg production [53,54]. Nonetheless, all recovered birds were susceptible to reinfection [52,54–56] and subclinical infection was reported in most of treated birds [52]. Importantly, amantadine lost its effectiveness as amantadine-resistant mutants emerged within 2–3 days of treatment and killed all in-contact chickens. Amantadine-resistant strains were irreversible, stable and transmissible with pathogenic potential comparable to the wild-type virus. Even

more, the resistant mutants replaced the wild-type virus and became dominant [55–57]. It is worth pointing out that several subtypes of AIV including the HPAIV H5N1 that currently circulate in both humans and birds around the world are mostly resistant to amantadine [58–65]. Since the late 1990s, positive selection of amantadine-resistant HPAI H5N1 viruses in poultry in China has been proven to be increased due to extensive illegal application of the relatively inexpensive amantadine by some farmers to control HPAIV H5N1 (and LPAIV H9N2) infections in chickens [62,66–69]. Hence, rapid selection of amantadine-resistant variants threatens the effective use of the drug for control of human influenza epidemics and/or pandemics [70], therefore the extra-label use of amantadine in poultry was banned by all concerned international organizations [71,72]. The second M2 blocker is **rimantadine**. Because of the unavailability of rimantadine in most countries, its use in poultry is not reported until now in the field. However, Webster *et al.* [73] mentioned that rimantadine administered in drinking water was efficacious against HPAIV H5N2 infection in experimentally infected chickens. Nonetheless, the emergence of rimantadine-resistant variants was comparable to amantadine.

2.1.2. Neuraminidase Inhibitors (NAIs)

So far, there are two main NAIs, oseltamivir (Tamiflu®) and zanamivir (Relenza®) have been licensed for influenza treatment in human in several countries [74]. When exposed to NAIs, influenza virions aggregate on the host cell surface preventing their release and allow the host immune system to eliminate the virus [75,76]. In the early 2000s, **oseltamivir** was discovered as a potent and selective inhibitor of the NA enzyme of influenza viruses [50]. It is currently the drug of choice for the treatment of influenza virus infections in human and being stockpiled in many countries in anticipation of a pandemic [77]. Generally, AIV including H5N1 are sensitive to oseltamivir [78] and a small number of H5N1 strains isolated from avian and human origin have been reported to exhibit resistance to oseltamivir [79–84]. Oral application of oseltamivir via drinking water reduced the morbidity, mortality, virus excretion and chicken-to-chicken transmission in HPAIV H5N2 experimentally infected chickens [85]. Oseltamivir was non-toxic for chicken embryos and prevented the replication of an HPAIV H7N1 in inoculated eggs [86]. An effective prophylactic administration of oseltamivir in experimentally infected chickens and ducks with LPAI H9N2 and H6N2 viruses was also reported [87]. Although it is very plausible that oseltamivir-resistance mutants emerge after application in poultry, however none of the few studies conducted to evaluate efficacy of oseltamivir in avian species reported emergence of resistant strains. In nature, oseltamivir-resistant H5N1 viruses isolated from domestic and wild birds emerged probably due to spontaneous mutations rather than exposure to oseltamivir [80,88–90]. Administration of oseltamivir during an outbreak in commercial flocks is extremely expensive but it could be useful to protect valuable birds [86,87]. On the other hand, **zanamivir** is currently approved in 19 countries for the treatment and prophylaxis of human influenza [50]. Although, development of zanamivir-resistance in poultry is rare [91], it is not effective in preventing a severe outcome and chicken-to-chicken transmission of an HPAIV H5N2 in experimental chickens [85].

2.2. Natural Antivirals

2.2.1. Herbs

Unlimited herbs products contain polyphenols, flavonoids, alkaloids or lignans, mostly from traditional Chinese medicine, offer promise as adjuncts or alternatives to the current anti-influenza chemotherapy [92,93]. Generally, complementary medicine for treating or preventing influenza or influenza-like illness in human seems to be cultural practice differs from nation to nation [94–96]. Innumerable herbs species with potential inhibitory effects on replication of influenza viruses using *in-vitro* cell culture methods and embryonated eggs or *in-vivo* mouse models were frequently described [97–123].

In poultry, antiviral and immunoadjuvant effects of several plants and/or its derivatives have been investigated. In addition to its antiviral activity, these extracts often have anti-bacterial, anti-fungal, anti-inflammatory, anti-oxidant and/or analgesic properties which may provide alternative natural broad-spectrum therapy for control of AIV in poultry farms [124–127]. Sood *et al.* [127] found that *Eugenia jambolana* extracts had 100% virucidal activity against HPAIV H5N1 in tissue culture and *in-ovo* inoculated chicken embryonated eggs (ECE). Menthol, eucalyptol and ormosinine probably have inhibitory effect on H5 viruses due to strong interactions ability with the viral HA protein [128]. NAS preparation, a Chinese herbal medicine, prevented H9N2 virus-induced clinical signs in treated chickens; however transmission of the virus to untreated chickens was not interrupted [129]. Likewise, eucalyptus and peppermint essential oils preparations protected broilers against H9N2 virus infections [130,131]. Moreover, application of lyophilized green tea by-product extracts namely catechins in feed or drinking water reduced H9N2 virus replication and excretion in experimentally infected chickens in a dose-dependent manner [132]. In addition, green tea extract was comparable to amantadine in protection of chicken embryos against H7N3 subtype [120]. Catechins alter the infectivity of influenza viruses probably not only by direct interaction with viral HA but also by inhibition of viral RNA synthesis in cell culture [133]. Furthermore, Liu *et al.* [134] found that statin/caffeine combination was as effective as oseltamivir in reduction HPAIV H5N1-induced lung damage and viral replication in mice.

The immunoadjuvant effect of some herbal extracts as feed additives on the humoral immune response induced by inactivated AIV vaccination in poultry has been studied. Oral administration of *ginseng* stem-and-leaf saponins in drinking water or *Hypericum perforatum L.* as a dietary supplement significantly enhanced serum antibody response to inactivated H5N1 or H9N2 vaccines in chickens [135–137]. The *Cochinchina momordica* seed extract, Chinese medicine plant, when combined with an inactivated H5N1 vaccine as adjuvant increased significantly the immune response and daily weight gain of two weeks old chickens [138]. On the contrary, herbal extracts of *Radix astragali*, *Radix codonopis*, *Herba epimedii* and *Radix glycyrrizae* in drinking water did not improve chicken immune response to H5-AIV vaccination [139], likewise diet supplementation with fresh garlic powder had no effect on the humoral immune response of chickens vaccinated with an inactivated H9N2 vaccine [140].

Yet, some derivatives (*i.e.*, ginseng saponins) require four to six years to harvest and is very expensive on the market [135]. Methods of the extraction and preparation of the crude extracts and its

purity greatly influence the inhibition activity of some herbs against AIV [132,133]. Moreover, batch-to-batch variations due to variable growth conditions at the plantations have been considered a limiting factor for treatment of influenza [124]. Evident that mutation in the H5 gene probably affects inhibitor binding of some herbs was reported [128]. In addition, *in-vitro* experiments and animal models to confirm the direct antiviral activities against influenza virus are limited [141]. Moreover, comprehensive investigations of herb-drug interactions, potential toxicity, heterogeneity of herbs species, plant parts (*i.e.*, aerial *vs.* root) and biochemical data identifying the active components are inadequately described [142].

2.2.2. Probiotics

A number of studies have reported the efficacy of probiotic lactic acid bacteria such as *Streptococcus thermophiles*, several *Lactobacillus* and *Bifidobacterium* species to enhance the immune response and to protect mice against different influenza strains/serotypes [143–152]. Although probiotics are widely used in poultry to improve innate and adaptive immunity [153–155], there is a paucity of information on its ability to ameliorate AIV infections. *Lactobacillus plantarum* KFCC11389P was as effective as oseltamivir to neutralize the H9N2 virus in ECE and slightly reduced amount of tracheal virus excretion in oral-fed experimentally infected chickens [156]. Out of 220 screened bacterial strains, Seo *et al.* [157] found that *Leuconostoc mesenteroides* YML003 had highly anti-H9N2 activity in cell culture and ECE. Decrease cloacal excretion of the virus and a significant increase in the cytokine IFN-gamma in experimentally infected chickens were observed. Ghafoor and co-workers [158] showed that multi-strains commercial probiotic protexin® (various *Lactobacillus* sp., *Enterococcus faecium*, *Bifidobacterium bifidum*, *Candida pintolepesii* and *Aspergillus oryzae*) improved immune response of broiler chickens to H9N2 vaccination and prevented the mortality and morbidity. On the other hand, dual use of *Lactobacillus spp.* or *Lactococcus lactis* as a vector for vaccine production and immunomodulation bacteria has been successfully constructed and protected mice against HPAIV H5N1 [159,160], such experiments should be evaluated in poultry.

3. Molecular Approaches for Control of AIV

3.1. Avian-Cytokines

Chicken cytokines such as chicken interferon-alpha (ChIFN-α), chicken interleukins (ChIL) and Toll-like receptors (TLR) are essential components of chicken's innate immune system which play a vital role against virus infections [15,161–163]. An innovative application of ChIFN-α to antagonize AIV infection in poultry through direct oral feeding or drinking water has received more attention than other components [164–168]. Sekellick *et al.* [169] showed that up to 60% of investigated AIV population belonged to the HPAI H5N9 subtype were highly sensitive to the inhibitory effects of ChIFN-α. Interestingly, both IFN-sensitive and -resistant clones were obtained after passage of the resistant clones in the presence of IFN which indicated that resistance to ChIFN-α was transient and did not result from stable genetic changes. Xia *et al.* [170] cloned the ChIFN-α gene from three different chicken lines and studied their efficacy against H9N2 viruses *in-ovo* and *in-vivo*. Up to 70%

of *in-ovo* treated chicken embryos were protected against H9N2 virus infection in dose dependent manner. Moreover, chickens received ChIFN-α by oculonasal inoculation at one day of age were protected from death upon H9N2 virus infection given 24 hours later. Findings of Meng and co-workers [166] showed that oral administration of exogenous ChIFN-α was effective to prevent and treat chickens experimentally infected with an H9N2 virus. It potentially reduced the viral load in trachea and resulted in rapid recovery of the body weight gain. In another study, White Leghorn (WL) chickens received ChIFN-α in drinking water for 14 successive days augmented detectable humoral anti-influenza antibodies after exposure to a low dose of an LPAIV H7N2 infection [164]. Thus, it has been suggested that regular water administration of ChIFN-α can create "super-sentinel" chickens to detect early infections with few amount of LPAIV [164].

Furthermore, oral administration of live attenuated *Salmonella enterica* serovar Typhimurium expressing ChIFN-α alone or in combination with ChIL-18 significantly reduced clinical signs induced by H9N2 virus and decreased the amount of virus load in cloacal swabs and internal organs [171,172]. Likewise, chicken immunized with a recombinant fowl pox virus (rFPV) vaccine expressing both the HA gene of H9N2 virus and ChIL-18 survived challenge with an H9N2 virus and did not excrete any virus in swab samples and/or internal organs in comparison to non-vaccinated birds [173]. Also, rFPV expressing the H5, H7 and ChIL-18 genes produced significantly higher humoral and cellular mediated immune response and protected specific pathogen free chickens (SPF) and WL chickens against challenge with an HPAIV H5N1. Vaccinated birds had no virus shedding and showed significant increase in body weight gain [174]. So far, efficiency of avian-cytokines to limit AIV infection has not been adequately studied in other avian species. The duck IL-18 and IL-2 genes had been identified and shown to have 85% and 55% nucleotide identity to the chicken equivalents, respectively. Intramuscular inoculations of the duck IL-18 or IL-2 enhanced the humoral immune response of ducks vaccinated with H5N1 or H9N2 inactivated vaccines, respectively [175,176]. Likewise, the recombinant goose IL-2 strengthens goose humoral immune responses after vaccination using H9N2 inactivated vaccine [177].

The TLR-3, TLR-7 and TLR9 are other promising chicken cytokines derivatives that showed broad-spectrum anti-influenza virus activity *in-vitro* and *in-ovo* [178–181]. Nevertheless, the cost of mass production of chicken cytokines is still too high to be applied in large-scale in poultry industry [165]. Moreover, protein stability, host-specificity and labor associated with mass administration of chicken cytokines under field conditions require significant improvement [172].

3.2. RNA Interference (RNAi)

RNAi is a natural phenomenon used by many organisms as a defense mechanism against foreign microbial invasion, including viruses, that able to wreak potential genetic havoc of the susceptible host [182]. Short-interfering RNA (siRNA) is approximately 21–25 nucleotides specific for highly conserved regions of AIV genomes. It effectively mediates the catalytic degradation of complementary viral mRNAs and results in inhibition of a broad spectrum of influenza viruses replication in cell lines, chicken embryos and mice just before or after initiation of an infection [183–187]. Tompkins and colleagues [188] found that siRNA specific for the NP or PA genes induced full protection of mice against lethal challenge with the HPAI H5N1 and H7N7 subtypes and markedly decreased virus titers

in lungs. Likewise, prophylactic use of PA-specific siRNA molecule significantly reduced lung H5N1 virus titers and lethality in infected mice [189]. Moreover, siRNA targeting M2 or NP genes inhibited replication of H5N1 and H9N2 viruses in canine cell line and partially protected mice against HPAV H5N1 [190].

In poultry, Li and others [191] showed that the siRNA targeting NP and/or PA genes inhibited protein expression, RNA transcription and multiplication of HPAIV H5N1 in chicken embryo fibroblasts and ECE as well as prevented apoptosis of infected cells. Likewise, chicken cell line transfected with RNAi molecules specific for the NP or PA of AIV showed decrease the levels of NP mRNA and infective titre of an H10N8 quail virus [192]. Also, NP-specific siRNA reduced H5N1 virus replication in cell culture and ECE [186]. Moreover, siRNA molecules targeting the NP, PA and PB1 genes interfered with replication of H1N1 virus in ECE [184].

In contrast to AIV vaccines, siRNA might not require an intact immune system [193] which is very important particularly in developing countries where a number of immunosuppressive agents are endemic in poultry. In addition, siRNA molecules targeting the highly conserved regions in influenza genome potentially remain effective regardless AIV subtype/serotype variations and despite antigenic drift and shift of AIV [193,194]. Moreover, it has also the potential to reduce the emergence of viable resistant variants [10], in this regard combinations of siRNA molecules "cocktail" targeting several genes/regions may be used simultaneously [195,196]. Furthermore, there is no risk of recombination between siRNA nucleotides and circulating influenza viruses, hence siRNA is complementary to the influenza virus genome [10]. Moreover, the siRNA dose required for inhibition of AIV is very low (sub-nanomoles) [195]. Nevertheless, arise of mutants with the ability to evade the inhibition effect of siRNA are not fully excluded [193]. Unfortunately, there is no stretch of conserved nucleotides in the NA and HA genes sufficient to generate specific siRNA due to extensive variations in these genes among AIV from different species [195]. The siRNA molecules are quickly degraded *in-vivo* affording a transient short-term protection and multiple-dose is required [192]. None of the siRNAs must share any sequence identity with the host genome to avoid non-specific RNAi-induced gene silencing of the host cells [195,197–199]. Delivery vehicle of siRNA to the site of infection is a major constraint [200,201] remained to be investigated on flock-level in poultry. There is accumulating evidence that siRNA is efficient to inhibit influenza virus replication *in-vitro*, however *in-vivo* studies still missing. Research studies focus on mass application of siRNA in poultry as a spray or via drinking water are highly recommended [202].

3.3. Host Genetic Selection

The host genetics play a pivotal role in susceptibility to influenza including the HPAIV H5N1 which is frequently studied in mice models as reviewed by Horby *et al.* [203]. Indeed, the impact of host genetic selection on resistance to AIV infections in poultry has not yet been fully determined. The on-going H5N1 virus epidemics have raised concerns in respect to influenza-resistant chickens either by selective breeding or genetic modification.

3.3.1. Natural Resistance

It has been supposed that fast-growing domestic birds have reduced immune competence against several viral diseases and resistant breeds are mostly poor producers [204]. Natural resistance or less susceptibility of some species/breeds of birds to AIV is not uncommon. In an experiment, five chicken lines were infected with an HPAIV H7N1. Three lines showed high susceptibility to the virus while two lines showed some resistance and survived the infection [205]. Swayne *et al.* [206] observed that an LPAIV H4N8 produced more severe lesions in commercial and SPF WL chickens than in 5 week-old commercial broiler chickens suggesting that SPF WL chickens are more susceptible than broilers to this strain. Thomas *et al.* [207] suggested that WL chickens may be more susceptible to an H3N2 virus of swine origin than White Plymouth Rock broiler-type chickens. On the contrary, severe lesions in commercial broiler chickens compared to SPF was observed after experimental infection with a Jordanian H9N2 isolate [208]. Some wild duck species, particularly mallards, are more resistance to HPAIV H5N1 than others [209]. Conversely, dabbling ducks and white fronted goose were more frequently infected with AIV than other wild ducks and geese, respectively [210]. Wood ducks were the only species to exhibit illness or death between different species of experimentally infected wild ducks in a study conducted by Brown and others [211].

3.3.1.1. Myxovirus (Mx) Resistance Gene

Myxovirus resistance gene is an interferon-stimulated gene encodes Mx1 protein that able to interfere with AIV replication by inhibiting viral polymerases in the nucleus and by binding viral components in the cytoplasm. The role of the Mx gene in resistance against influenza viruses including the HPAIV H5N1 in mammals is well defined [212–218]. However, the contribution of avian Mx proteins as antiviral elements in AIV infection in birds is contradictory and worth further exploration. Although intra- and inter-breed/-species Mx variations have been frequently reported [205,219–226], however commercial chicken lines have lower frequencies of the resistant allele compared to the indigenous chicken breeds [219,220,227] probably due to intensive modern breeding techniques [228]. Duck Mx was the first avian Mx protein to be characterized but no antiviral activity against an HPAIV H7N7 when transfected in chicken and mouse cells was obtained [229]. On the contrary, chickens have a single Mx1 gene [230] with multiple alleles [220] encoding a deduced protein with 705 amino acids in length. Notably, results of anti-influenza activity of the Mx1 protein in chickens are contradictory likely due to using variable experimental setups and different AIV strains. Also, a similar disparity has been noted between *in-vitro* and *in-vivo* experiments [205,231].

Phenotypic variation in the antiviral activity of Mx gene has been linked to a single amino acid substitution of asparagine (Asn) at position 631 in resistant breeds or serine (Ser) in sensitive ones [219]. The 631Asn identified mostly in Japanese native chicken breeds screened by Ko *et al.* [219] was associated with enhanced antiviral activity to H5N1 virus in transfected mouse fibroblast 3T3 cells. Conversely, results obtained by Benfield *et al.* [232,233] and Schusser *et al.* [234] indicated that neither the 631Asn nor the 631Ser genotypes of chicken Mx1 was able to confer protection against several LPAIV and HPAIV including H5N1 subtype in chicken embryo fibroblasts or ECE. Similarly, Mx1 631Asn had no effect on viral replication after *in-vitro* infection of chicken embryo kidney cells

with an LPAIV H5N9 [231]. Moreover, transfected chicken cells expressing chicken Mx protein did not induce resistance to HPAIV H7N7 [235]. *In-vivo*, following intranasal infection with an HPAIV H5N2, chickens carry Asn631 allele showed delayed mortality, milder morbidity and lesser virus excretion than 631Ser homozygotes [231]. Conversely, no correlation was observed between Mx-631 genotypes and susceptibility of chickens to an HPAIV H7N1 as indicated by clinical status and time course of infection [205]. Although, one out of six chicken lines infected with an HPAIV H7N1 had lower mortality, the Mx gene was not involved in this variations among tested chicken lines [236]. Additionally, chickens carry the homozygous Mx resistant allele genotype augmented the lowest HI titer after vaccination with an inactivated H5N2 vaccine compared with chickens that carry the sensitive allele [237].

Taken together, resistance or susceptibility to a disease is usually multifactorial in nature and greatly influenced by both the host and the virus, therefore the role of Mx1 gene merits more in-depth investigation [224,234]. *In-vivo* comparative studies using several native breeds from different countries are required to elucidate the role of Mx1 gene in AIV resistance [231].

3.3.1.2. Other Candidate Genes

Apart from the Mx1 gene, resistance or less susceptibility of ducks to AIV infections compared with chickens has been linked to an influenza virus sensor known as retinoic acid-inducible gene I "RIG-I" (a cytoplasmic RNA sensor contribute to AIV detection and IFN production) which is absent in chickens [238–240]. This RIG-I gene as a natural AIV resistance gene in ducks could be a promising candidate for creation of transgenic chickens [238]. Moreover, different genes and cytokines have been expressed after infection of chicken and duck cells with several AIV subtypes including HPAIV H5N1 [241–244]. Additional genetic candidates that contribute to inhibition of AIV replication could be useful in creation of genetically modified chickens such as cyclophilin A [245], ISG15 [246], viperin [247], heat shock cognate protein 70 (Hsc70) [248] or Ebp1 and/or ErbB3-binding protein [249].

3.3.2. Transgenic Chickens

Current advance in molecular biology and genetic manipulation can facilitate the development of influenza-resistant poultry. Increase resistance of cell lines to influenza virus infection using RNA interfering (RNAi) molecules expressed by a lentiviral vector is more efficient transgenic tool than direct DNA injection or oncoretroviral vectors infection [10,250,251]. Recently, creation of AIV built-in resistant chickens by genetic modification has been experimentally proven by Lyall and colleagues [252]. Chickens equipped with a short-hairpin RNA targets the AIV polymerase binding sites have been created and infected with HPAIV H5N1. Although all infected transgenic birds succumbed to the infection however the virus did not spread to the in-contact transgenic and non-transgenic cagemates [252]. Applicability in food production, safety regulations and consumer's preferences are important challenges face development of genetically modified chickens [252,253]. Moreover, AIV is a "master of mutability" and global production of the resistant chickens must be equipped with many decoys target different genes to avoid rapid generation of AIV resistance. In addition, replacement of the commercial flocks with the newly flu-resistant birds is expected to occur

within short period due to globalization of the poultry industry however replacement of backyard birds seems to be more complicated [253].

4. Summary and Perspectives

Epidemics of avian influenza in poultry are a real challenge for the scientific community [12]. Recently, several approaches to control the disease were developed and have yielded promising results. Although beneficial, these approaches face different limitations and restrictions (Table 1). The use of antiviral drugs in poultry could be an ancillary tool to control AIV infections in valuable birds but not in commercial sectors. Fears of kicking out our leading antiviral drugs in control of AIV are increased by adoption of amantadine (and probably oseltamivir) in poultry and transmission of resistant variants to human. On the other hand, limited supply and high costs of oseltamivir preclude its widespread use for poultry. Compliance with other medications, adverse effects and drug residues in eggs, meat and surrounding environment should be investigated. On the other hand, effectiveness of herbal and cytokines-based medications to protect against HPAIV H5N1 should be seriously considered and further investigation *in-vivo* is inevitable.

Molecular approaches including RNAi and transgenic chickens for control of AIV are encouraging. The use of short interfering RNA prevents the replication of AIV seems to be a promising approach; however specificity to the viral genome without interference with the host genome and non-specifically inhibition of cellular gene activity is critical. Delivery to the host, production costs, mass production and application, storage and handling of the final products are important aspects that remain unresolved. Possibility for arise of mutants with the ability to evade the siRNA activity should also be considered. Genetic resistance to AIV determined by only one point mutation in the Mx gene or complex and multigenic host components as recently determined in mice [254] should be firstly confirmed and secondly elucidation of its relation to the productivity of birds and other diseases must be considered.

Although a proof-of-principle to produce transgenic chickens has been recently reported, technical, logistic and social constraints are facing development of chicken resistant to AIV. Stable transmission and expression of the transgene from generation to generation require extensive studies. Regulatory approval, mass production, costs and marketing of commercial AIV resistant pedigree lines, consumer preferences and food safety issues need to be carefully and fully addressed. Overall, mutation of the virus in the face of any control approach remains the real challenge. Influenza epidemics and pandemics will likely continue to cause havoc in poultry and human populations, therefore innovative alternative or complementary intervention strategies need to be developed. The ultimate goal of all control (including alternate) strategies must be the eradication of avian influenza. In this context, alternate approaches might be an aid but should not jeopardize surveillance and current control measures.

Table 1. Advantages and limitations of different alternative approaches for control of avian influenza viruses in poultry.

Approach		**Advantages**	**Limitations**
Antivirals	**Chemotherapy**		
	M2 Blockers (Amantadine and Rimantadine) and Neuraminidase inhibitors (Oseltamivir and Zanamivir)	• Rapid protection • Mass administration (feed, water) • Cost-effective for individual birds (amantadine HCL) • Suitable for all types of birds against all types of AIV	• Hazards of kicking out cornerstone antivirals in case of pandemic • Emergence of resistant mutants • Require long application period to be effective • Expensive in flock level (Oseltamivir) • Residues in meat and eggs was not fully addressed • Compliance with other medical agents need to be considered
	Natural Antivirals		
	Herbs	• Direct antiviral activity • Immunoadjuvant effect • Additional effects as antioxidants, anti-inflammatory, *etc.* • No adverse effects on body weight, egg production	• Extraction is very expensive • Affection with antigenic changes, herb-drug interactions, cytotoxicity and biochemical traits were not fully investigated • Extraction methods, preparation, purity of the crude extracts greatly influence the efficacy. • Batch-to-batch variations are high due to variable plantations conditions. • Animal models of infection are limited
	Probiotics	• Direct and indirect antiviral activity • Immunoadjuvant effect • Dual use as a vaccine-vector and immunomodulator	• Efficacy against AIV particularly HPAIV is still questionable

Table 1. *Cont.*

Approach		Advantages	Limitations
Molecular approaches	**Avian Cytokines**	• Not affected by antigenic changes • Broad spectrum antiviral activities	• Instability • High production costs • No mass production • Field application limitations
	RNA interference	• Inhibition of any influenza subtype/serotype/variant • High specificity to particular strain/subtype/variant • Do not require intact immune system • Use as a prophylactic and/or therapeutic	• Specificity to the viral genome without interference with the host genome and non-specifically inhibition of cellular gene activity is critical. • Delivery to the host, costs, mass production, storage and handling of the final products consider questionable aspects. • Possibility for arise of mutants with the ability to evade the siRNA activity should not be fully guaranteed • Quickly degraded *in-vivo* • Induce a transient & short-term protection and multiple-dose is required • *In-vivo* research studies still missing
	Naturally resistant birds (Myxovirus Mx resistant gene and other candidate genes)	• Few breeds of chickens and ducks can survive challenge with HPAIV in nature	• Results on the contribution of the Mx gene to AIV resistant are contradictory • Resistant breeds are mostly low producer native breeds. • Interrelation of disease-resistance and production should be weighed • Studies have been conducted only on a limited number of native breeds in some countries
	Transgenic birds	• Although all infected transgenic birds succumbed to the infection however the virus did not spread to the in-contact transgenic and non-transgenic cagemates	• Replacement of backyard flocks • Consumer preferences • Food safety • Regulatory approval • Costs of production • Mutations of AIV

Conflict of Interest

The authors declare no conflict of interest.

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