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Current Status and Future Needs in Diagnostics and Vaccines for High Pathogenicity Avian Influenza

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Abstract: Since 1959, 32 epizootics of high pathogenicity avian influenza (HPAI) have occurred in birds. Rapid detection and accurate identification of HPAI has been critical to controlling such epizootics in poultry. Specific paradigms for the detection and diagnosis of avian influenza virus (AIV) in poultry vary somewhat among different countries and industry compartments depending on specific needs and resources. Importantly, since HPAI and low pathogenicity (LP) AI of the H5 and H7 subtypes are reportable to the World Organization for Animal Health (OIE), diagnostic procedures are implemented for regulatory purposes and are harmonized to some degree. Most current tests are adequate and have been in use for some time, therefore they have been well validated and presently there is no reported new technology that will completely replace the current tests. However, some modifications, updates or additional tests could be beneficial. The element of AIV diagnostics that is most in need of improvement is in determining the hemagglutinin and neuraminidase subtype specificity of antibody to AIV. Most HPAI epizootics have been eradicated using traditional stamping-out programs, but beginning in 1995, five epizootics have added vaccination as an additional, interim control tool. From 2002-2010, >113 billion doses of AI vaccine have been used in poultry; 95.5% as oil-emulsified, inactivated whole AIV vaccines and 4.5% as live vectored vaccines. The majority of vaccine has been used in the four H5N1 HPAI enzootic countries (China [91%], Egypt [4.7%], Indonesia [2.3%], and Vietnam [1.4%]) where vaccination programs are directed to all poultry. The 10 other countries/regions have used less than 1% of the vaccine, administered in a focused, risk-based approach. Some vaccine "failures" have resulted from antigenic drift of field viruses away from the vaccine viruses, but most have resulted from failures in the vaccination process; i.e. failure to adequately administer the vaccine to at risk poultry resulting in lack of population immunity. China, as the major AIV vaccine user, will drive innovation and commercialization of new vaccine technologies, but because of the low-cost to manufacture the current high quality inactivated whole AIV vaccines, such vaccines will continue to dominate the market for the next 10 years.

Introduction

Since 1955, when the etiology of fowl plague was determined to be influenza A virus, 32 epizootics of high pathogenicity avian influenza (HPAI) have occurred in avian species, mostly domestic poultry [1]. Historically, HPAI virus (HPAIV) has caused sporadic epizootics in domestic poultry when a low pathogenicity (LP) avian influenza virus (AIV) is introduced from the wild bird reservoir and allowed to circulate in poultry, mutating from low to high virulence [2]. However, since 1996, the H5N1 HPAIV has become enzootic in poultry within several countries which has necessitated two main changes within HPAI control and eradication strategies; 1) development and implementation of rapid diagnostic tests to accelerate diagnosis before the virus spreads, which permits a quicker stamping-out action leading to eradication, and 2) addition of vaccines and vaccination as a control tool to manage clinical disease, prevent human infections and maintain food security, especially in economically disadvantaged countries.

Diagnostics

Background and state of diagnostic science

Although specific diagnostic paradigms and work flow may vary depending on needs, the same tests are used almost universally for avian influenza virus (AIV) detection, antibody detection, and subtype identification of AIV from poultry (Fig. 1). Specific procedures for each of these tests are recommended by the World Organization for Animal Health (OIE) (ww.oie.int), and Food and Agriculture Organization Animal Influenza (OFFLU) network, which afford some level of harmonization. All of the current procedures have been in place for many years.

The speed with which HPAI is eradicated is greatly dependent upon the identification of the index case and elimination of the disease before it spreads to additional premises. This was not possible using the traditional diagnostic tool of virus isolation, and necessitated a broad clinical case definition along with quarantine and depopulation of poultry within 0.5-3 km zone in order to compensate for delayed diagnosis. However, with the advent of molecular diagnostics, specifically the real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay, a diagnosis of AI could be made within 3 hours of sample submission. The AIV rRT-PCR test was developed in 2000-2001 by USDA, and field tested in the H7N2 LPAI live poultry market depopulation program during December 2001 in New York and New Jersey [3] and, again during the winter/spring 2002 in Virginia commercial poultry industries [4]. The first field use of rRT-PCR against HPAI was in Chile (H7N3) during late spring of 2002, and in Hong Kong (H5N1) during September 2002 (D. Suarez, personal communication, 8-20-2012). A conventional reverse transcriptase polymerase chain reaction (cRT-PCR) assay was used during the 2003 Dutch H7N7 HPAI outbreak to allow rapid decisions for control [5].

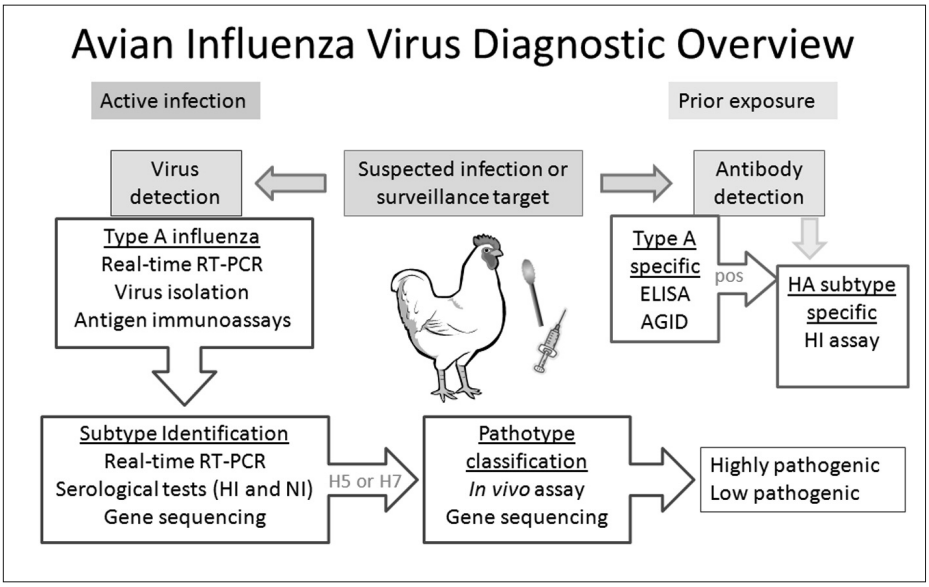

Fig.1. Cartoon of the diagnostic flow for avian influenza virus (AIV) and antibody to AIV. Virus detection tests are conducted with oral swabs, cloacal swabs or tissues. The 3 primary virus detection tests are virus isolation, reverse transcription polymerase chain reaction (RT-PCR) and antigen immune-assays. If a specimen is positive with any of these tests, the subtype may be identified with serological tests, RT-PCR or gene sequencing. If the isolate is determined to be an H5 or H7 subtype virus, the pathotype is characterized by in vivo testing and gene sequencing. Antibody assays to evaluate prior exposure may be type specific (i.e. for type A influenza), such as enzyme linked immunosorbent assay (ELISA) or agar gel immune-diffusion (AGID), or may be subtype specific (often for H5 or H7) such as hemagglutination inhibition (HI) assay.

The strengths and weaknesses of each diagnostic method are well known and each has been periodically modified to achieve the best results, which includes balancing the logistical workflow as well as sensitivity and specificity. In addition to the OIE and OFFLU recommended tests, which are public domain, commercial kits are available for rRT-PCR, antigen detection and ELISA.

The characteristics of the perfect diagnostic assay are: fast, cheap, simple to conduct/easy to use, robust, sensitive and specific. No current test meets all of these criteria. Numerous novel methods and technologies, such as new instruments, (e.g. biosensors [6-9]), new approaches (e.g. microarray [10]) and modified versions of current tests (e.g. microsphere based serology [11-13]) have been reported, but none have been validated, which is partly because none have offered enough of an advantage over the current methods to justify the time and expense of validation. Most of the recent work in AIV diagnostics has been to refine current technology like molecular methods which have a continual need to be updated to account for sequence changes in the virus in the field. Initial validations of some have been reported [14-17].

Type A specific tests

Virus isolation in embryonating chicken eggs (ECE) and rRT-PCR (or other RT-PCR based methods) are presently used to detect type A influenza virus directly from field specimens. Rapid type specific tests, rRT-PCR and antigen immunoassays (AgIA), may also be used as screening tests prior to processing a specimen for virus isolation. Virus isolation will unlikely be completely replaced since an isolate is necessary for diagnostic confirmation and characterization.

Although current type specific methods for detecting AIV (i.e. methods for detecting influenza A virus) are adequately sensitive and specific, in most cases, there are areas where improvement or additional tools would be beneficial. For example, more efficient and rapid methods for RNA extraction from tissues and cloacal swabs that remove inhibitors and stabilize the RNA would aid rRT-PCR. Virus isolation would benefit from a media (or other method) which could stabilize viable virus in the absence of the cold chain.

Antigen detection methods such as the commercial AgIA are helpful in the field for inexpensive, rapid testing and can be used pen-side, but they lack sensitivity. Improved sensitivity would greatly improve the utility of these tests. Additionally, a Newcastle disease virus (NDV) AgIA test would help with rapid differentiation of AIV and NDV in the field.

Subtype identification

Field specimens are frequently tested for the H5, H7 or other relevant hemagglutinin (HA) subtypes directly by rRT-PCR. Once an isolation is made, the subtype is identified by serology (hemagglutination inhibition (HI) assay and neuraminidase inhibition (NI) assay) and/or by sequencing the HA and NA. Because HI and NI are resource intensive and large reagents sets are needed to identify all 16 HA and 9 NA subtypes, serological subtyping is most often performed only in reference laboratories. In other labs, serological subtyping may focus on only the most critical HA subtypes, H5 and H7, or other relevant subtypes for the region. Sequencing takes longer (up to 3 days), and not all labs are equipped to sequence specimens as the instrumentation is expensive and requires advanced technical skills to use, but sequencing is the most accurate.

Although not widely used, there are H5 specific AgIA kits available commercially. However, most are not validated adequately to demonstrate that they have sufficient sensitivity with all antigenic variants. Rapid H5 and H7 subtype tests, such as AgIA, which can be validated for specificity would be beneficial for rapid diagnosis in the field or initial testing of egg fluid from an isolate.

Pathotype identification

Once the H5 or H7 subtype has been identified in a specimen, the pathotype must be determined. Pathotype classifications are defined by the OIE, therefore the tests are prescribed by the OIE. Currently there are two prescribed tests: in vivo chicken testing and determining the sequence of the HA proteolytic cleavage site

(PCS). Due to the resources needed for each of these tests, and the regulatory nature of pathotype classification, testing is typically only conducted in national or regional reference laboratories. In vivo chicken testing is the most biologically relevant, but it is very resource intensive and the full test takes up to 10 days after the virus has been isolated in a culture system. Sequencing is much faster and may be accomplished with just the RNA from a swab or tissue specimen, however as discussed previously the resources for sequencing are not universally available.

A rapid test for pathotype classification would be very beneficial, but no technology or method had been identified which would fulfill this purpose. RT-PCR based methods have been proposed and reported [18], but have not been validated and it is not clear if it would be accurate with the wide sequence variation among the PCS of both HP and LP isolates. Furthermore, sequence outside of the PCS may affect pathotype due to post-translational modifications (i.e. glycosylation)[19], so any tests which only assay the PCS may not be accurate with all isolates.

Serology

There are two assays routinely used for pan-AIV antibody detection in poultry, ELISA (numerous commercial kits are available worldwide) and agar gel immunodiffusion (AGID) assay. Both are type specific, relatively inexpensive and have similar sensitivity. Because of the rapid speed and simplicity of high throughput, ELISA will likely eventually replace AGID. Additionally, unlike AGID which does not work well with sera from waterfowl, some commercial ELISA kits are not species specific so they can be used with sera from domestic ducks and other poultry, or even wild bird species. On the contrary AGID is cheaper on a per sample basis and since it detects IgM instead of IgY, antibodies can be detected earlier after infection, so the AGID does have some advantages in certain situations. However, the positive AGID tests results drop-off after 3 months as IgM AIV-specific antibody production is switched to IgY, but ELISA can detect antibody beyond this time period.

Probably the element of AIV diagnostics where there is the most need for innovation is for methods to characterize the subtype specificity of antibodies to AIV. Cross-reaction among subtypes and inhibition from the NA can make HI assay results difficult to interpret or even inaccurate. A few solutions have been proposed which involve producing the reference antigens for HI assay in vitro using systems which only express the HA and not the NA protein. However, these would be expensive to develop, produce and validate. Other alternatives are using expressed or whole virus proteins with other serological test platforms, such as microsphere assays, which would allow a greater number of antigens to be screened with a single specimen, thus could provide more in depth and possibly accurate data.

Vaccines and Vaccination

Background

Historically, eradication has been the desired outcome and has been achieved

through comprehensive, integrated control programs utilizing: 1) education of farmers and governmental officials in disease control (including behavioral change communications), 2) diagnostics and surveillance to identify infected flocks, 3) enhanced biosecurity (including quarantine of infected flocks, movement controls within the outbreak zone, and cleaning and disinfection of premises and equipment), and 4) elimination of infected poultry through culling or depopulation [1]. However, in 1994, an epizootic of H5N2 HPAI occurred in central Mexico overwhelming the resources of the poultry industries and government necessitating the implementation of a fifth tool, i.e. vaccination, which allowed management of the clinical disease for eventual eradication. Vaccination has since been implemented in Pakistan against H7N3 HPAI during late 1995; 13 Asian, European and African countries against H5N1 HPAI from 2002 to present; North Korean against H7N7 HPAI during 2005; and Mexico against H7N3 HPAI beginning in 2012 [20, 21]. Vaccination has emerged to be a valuable tool for interim management of HPAI, assuring national food security and preserving the livelihood of rural poor, especially in least developed, developing and transition countries [20, 22]. Vaccination did not create the enzootic nature of H5N1 HPAI in China, Egypt, Indonesia or Vietnam; enzootic status was already evident in these countries prior to implementing vaccination. However, routine and improper use of vaccines and vaccination has contributed to prolonging enzootic status of H5N1 HPAI, contributing to virus "persistence" in the field by complicating surveillance and contributing to complacency.

Vaccines in the field

Based on a survey for 2002-2010, over 113 billion doses of AIV vaccine have been used in poultry in 15 different countries/special administrative regions, mainly against the H5N1 HPAI panzootic within Asia, Africa and Europe. China has been the main user with >103 billion doses (90.99%), followed by Egypt (5 billion doses; 4.65%), Indonesia (2.6 billion doses, 2.32%), and Vietnam (1.6 billion doses, 1.43%), with all 4 countries having enzootic status for H5N1 HPAI and using the vaccines in nationwide routine vaccination programs for all poultry [20]. This high number of vaccine doses used in China was linked to correspondingly high poultry populations in China as compared to a much lower populations in Egypt, Indonesia, and Vietnam. The other 11 countries/special administrative regions (Hong Kong, Mongolia, Kazakhstan, France, The Netherlands, Cote d'Ivoire, Sudan, North Korea, Israel, Russia, and Pakistan) have used <1%, and such vaccine use has been limited to preventative or emergency vaccination programs in limited geographic areas or specific poultry species of higher risk, and not in nationwide vaccination programs. In mid-2012, Mexico began using AIV vaccine in response to an outbreak of H7N3 HPAI in the center of the country [21].

Of the 113 billion doses of vaccine used, 95.5% has been inactivated whole AIV vaccines, while 4.5% has been live recombinant virus vaccines [20]. The latter was primarily recombinant NDV (rNDV) vectored vaccine with H5 influenza gene insert, but some recombinant fowlpox virus (rFPV) with H5 or H5 plus N1 gene

inserts has been used. Most recently, a recombinant herpesvirus turkey (rHVT) with an H5 gene insert and recombinant duck virus enteritis virus (rDVE) with an H5 gene insert has been developed, laboratory tested and licensed or a license is pending [23]. A rFPV with an H5 gene insert has been used in Central America since 1998 against H5N2 LPAI and over 2 billion doses have been used through 2005 [24].

Despite the broad use of large quantities of AIV vaccines against H5N1 HPAI since 2002, vaccine use has not resulted in eradication in the four enzootic countries; i.e. China, Egypt, Indonesia and Vietnam. In addition, there have been reports of AIV vaccine "failures", specifically isolation of H5N1 and/or identification of clinical disease consistent with HPAI in vaccinated flocks or in regions that vaccinate against H5N1 reporting resurgence of HPAI disease cases [22]. Some of these "failures" have been the result of vaccines not protecting against field challenge because of antigenic drift of the field viruses away from the vaccine strains, but more often, the "failures" have resulted from the lack of proper administration of vaccine or inability to vaccinate and produce a protective immune response in > 60-80% of the at risk poultry population; i.e. a failure to achieve population immunity because of the inability to vaccinate poultry properly [25].

Vaccine technology

The vaccine technologies used experimentally and licensed for use in poultry in the field are listed in Table 1. Five different categories of vaccines have been examined in the laboratory as discovery projects or proof of principle: inactivated whole AIV, live AIV, live vector, in vitro produced hemagglutinin, and DNA vaccines. However, licensing and use has only been achieved with a small number of the technologies and products: inactivated whole AIV vaccines (95.5% of vaccine used) and live vectored vaccines (4.5%; avian paramyxovirus type 1/rNDV, rFPV, rHVT and rDVE). When contemplating why more new technologies have not made it into the market place, the new technology should be compared against a list of the ideal traits of AIV vaccines and against practical knowledge of how poultry health problems are resolved (Table 2). In addition, the reader must accept that the ideal vaccine for humans may not be ideal for poultry.

Before any new technologies will be adopted in poultry, the new vaccine must be shown to protect in experimental poultry trials and must provide protection that is equivalent to or better than the "gold standard", oil-emulsified whole AIV vaccine. In commercial poultry, vaccine development and use is driven by economics and thus adoption of new technologies occurs only if it provides an economic advantage that costs less than living with the problem, or partial solution provided by existing products. Historically, the majority of AIV vaccines have been inactivated whole AIV which are produced in embryonating chicken eggs. This mature, established technology has been successfully used for over 40 years to produce trillions of doses of potent and efficacious inactivated or live attenuated vaccines to other poultry diseases such as Newcastle disease, infectious bronchitis, infectious bursal disease, and other viral pathogens. This technology is inexpensive and has produced

Table 1. Experimental and licensed vaccines for high pathogenicity avian influenza in poultry and other avian species (reviewed in [50].

Vaccine Category	Vaccine	Species	Route	AI Subtypes	Challenge Tested	HPAIV	Licensed	Comments	Additional Ref.
Inactivated AIV	Adjuvanted whole avian influenza virus	Chicken (layer & broiler), turkey, duck, geese, other poultry, zoo birds	SQ, IM, in ovo	H5, H7	Yes		Yes	Mostly oil-emulsified; some with aluminum hydroxide. Includes H5 and H7 LPAIV, H5 and H7 HPAIV and H7 reverse genetic LPAIV seed strains. Requires parenteral administration	
Live AIV	Live wild-type LPAI virus	Chicken	IM, IT Spray	H5, H7	Yes		No	Rumors of intentional exposure with LPAIV to protection from HPAIV have been reported in H5N1 and H5N2 HPAI outbreaks in 1990's and 2000's	[32, 33]
	Attenuated LPAI virus	Chicken	Spray	H5, H7	Yes		No	Temperature sensitive mutant or replace HA with ectodomain of NDV HN gene; risk assessment needed for reassortment potential	[34, 35]
Live Vector	rd-Adenovirus	Chicken	SQ, IN, In ovo	H5	Yes		No	rd = Replication defective, only 1 round of replication occurs after injection. SQ and *in ovo* protected	[36]
	Avian leukosis virus	Chicken	IM	H7	Yes		No		[33]
	Avian paramyxovirus type 1 (rNDV)	Chicken	Eye, IN	H5, H7	Yes		Yes	Licensed in Mexico and China	[37, 38]
	Duck enteritis virus (rDVE)	Duck	IM	H5	Yes		No	Submitted for license in mid-2012 for ducks (China)	[23]
	Fowlpox virus (rFPV)	Chicken (layer & broiler), goose, Muscovy ducks	SQ, WW	H5, N1	Yes		Yes	Licensed 1997 for chickens (USA, Mexico); used primarily in Central America against H5N2 LPAI; limited use in China	
	Herpesvirus Turkey (rHVT)	Chickens	SQ	H5, N1	Yes		Yes	Licensed 2012 for chickens (USA, Egypt). Used Egypt	[39]
	Infectious laryngotracheitis virus vector	Chicken	Eye	H7, H5, N1	Yes		No	N1 did not protect	[40, 41]
	att-Salmonella typhimurium	Chicken	Oral	H5, M2e	Yes		No	Attenuated vaccine strain. Failed to protect from HPAIV challenge with single oral immunization	[42, 43]
	Vaccinia	Chicken	IM, IP	H5	Yes		No	Low to no antibody response	[44]
	rd-Venezuelan Equine Encephalitis virus		SQ, *in ovo*	H5	Yes		No	rd = Replication defective, only 1 round of replication occurs after injection	[45]
In vitro produced hemagglutinin	Baculovirus in insect cell culture	Chicken, Muscovy duck	SQ	H5, H7	Yes		No		[46]
	Eukaryotic systems (plants or cells cultures)	Chicken	IM	H5	Yes		No	*Nicotiana* sp.	[47]
DNA	Naked DNA	Chicken	IM	H5	Yes		No	Not financially viable. Improvements needed in promotors and adjuvants to decrease quantity of nucleic acid needed and reduce number doses for protection	[48, 49]

Table 2. Properties of ideal avian influenza vaccines and vaccination methods for poultry.

Desired Property	Current Situation
Inexpensive	Current cost for inactivated AIV vaccine: $0.05-0.10/dose plus cost of administration ($0.05-0.07 per dose for individual handling and injection) [50]
Use in multiple avian species	Most used in meat, layer and breeder chickens, but large quantity also used in ducks; minor amounts in turkeys, geese, quail, etc. [20]
Single dose protection	Most situations require minimum of 2 doses; prime-boost scenario is optimal with additional boost in long-lived birds at 6-12 month intervals [51, 52]
Mass application	95.5% is inactivated vaccine administered by handling and injecting individual birds, with 4.5% as vectored vaccine given by mass spray vaccination (rNDV vector) [20]
Identify infected birds in vaccinated population	Serological differentiation tests are available, but only minor use. Most vaccine applied without using a DIVA strategy [52]
Overcome maternal antibody block	Maternal antibody to AIV hemagglutinin or virus vector inhibits primary immune response. Initial vaccination must be timed for declining maternal antibody titers to allow optimal primary immune response [53], as also is needed decline in active immunity before giving booster vaccinations [54]
Given at 1 day of age in hatchery or *in ovo*	Inactivated vaccine provide poor protection if given at 1 day of age. Vectored vaccines can be given at 1 day of age, but generally require a field boost with inactivated vaccine 10 days or more later
Antigenically close to field virus	The majority of inactivated whole AIV vaccine uses reverse genetic generated vaccine seed strains to antigenically match field viruses [20, 25]

effective, potent vaccines, and lacks the additional cost of royalty payments for patents used and the expense to purchase and use new manufacturing equipment which would accompany implementation of newer vaccine technologies. However, newer technologies will and have been adopted at the higher cost when they addressed one or more traits that make the new vaccine closer to the ideal vaccine (Table 2). For example, the majority of inactivated vaccine doses produced and used in poultry during 2007-2012 have utilized reverse genetic technology to generated AIV vaccine seed strains which are closer antigenically to H5N1 viruses encountered in the field, and these vaccine seed strains have been updated when antigenic variants have developed in the field [25].

Because of the economic aspect of poultry vaccine adoption and long-withdrawal period for oil adjuvant, use of inactivated whole AIV vaccines has been limited to valuable, long-lived or specialty poultry within developed countries, but in developing/transitional countries, the lower cost of labor for vaccination and shorter withdrawal periods for oil adjuvants has fueled use of vaccines in the much larger populations of meat chickens and ducks. Experimental studies have

demonstrated the possibility for mechanized in ovo injection for oil-emulsified, inactivated whole AIV vaccines [26], adenovirus vectored vaccines [27], VEE-vectored vaccine [28], attenuated AIV vaccine [29], and rNDV vectored [30] vaccines for low-cost mass administration, but this has not been commercially implemented. However, theoretically, an even better approach would be to develop vaccine technologies for applying vaccines via low cost methods such as spray application for respiratory delivery or per os application for oropharyngeal and upper digestive tract delivery (i.e. feed or water).

However, even with such technological breakthroughs, the next fundamental question is whether vaccination to control HPAI is the best approach, or if other control strategies such as prevention through management biosecurity or, if encountering a HPAI epizootic, if immediate stamping-out is the better approach. In a recent survey, most countries preferred using a stamping-out program without vaccination and indicated vaccination would only be used if the HPAI epizootic was large and stamping-out was not effective at immediate eradication or vaccination could be used as a potential preventative measure for valuable poultry when the threat of an epizootic was high [20]. Historically, this threshold of overwhelming HPAI epizootic and implementation of vaccination has been reached more quickly in least developed and developing/transition countries than in developed countries, as evident within the 15 countries that vaccinated of which 13 were least developed or developing/transition, but only two were developed countries (France and The Netherlands) and the latter two used a small, time-limited targeted vaccination program [31, 20].

Vaccination

Even with use of vaccine seed strains that antigenically match field viruses and with commercial vaccines of high potency, protection in the field can only be achieved if the at risk poultry are immunocompetent and if individual birds receive the vaccine in the proper dose (which may include booster vaccinations) and by the correct administration route. The goal is to achieve population immunity in the at risk poultry population, which is only achieved if >60-80% of the poultry have a protective immune response [20]. From a national perspective using routine vaccination of all poultry, this goal of national population immunity is difficult to achieve because of limited financial and human resources. Of the 113 billion doses of AIV vaccine used 2002-2010, this resulted in only a 41.9% coverage rate among the at-risk national poultry population of the 15 vaccinating countries/regions [20]. Of these 15, five conducted routine vaccination campaigns of all poultry. Based on their individual poultry populations and vaccination protocols, coverage rate for China was 47.1%, Hong Kong was 86.2%, Egypt was 69.9%, Indonesia was 14% and Vietnam was 52.3% [20]. This suggest that only two countries/regions achieved population immunity, i.e. Hong Kong and Egypt, but alternative data which presented higher village poultry population estimates for Egypt suggests the vaccine coverage rate in Egypt was substantially lower, between 27.8-48.6%

[20]. In addition, the use of 1-day-of-age vaccination in broilers in Egypt may have also contributed to poor population immunity. H5N1 HPAI cases in poultry continue in China, Egypt, Indonesia and Vietnam because of the lack of population immunity, but Hong Kong did achieve national population immunity, with only one H5N1 HPAI case in poultry between 2003-2012 [22]. These data suggest that achieving national population immunity in poultry, with its intensive financial and human resource requirements, is not feasible in most countries. Instead, vaccination should be targeted to at risk poultry and/or specific geographic regions, which requires field surveillance and epidemiological data and modeling to design effective vaccination programs. In addition, the historical yearly vaccination campaigns in cattle and pigs for transboundary diseases are not effective with poultry because of the shorter generation times (i.e. chickens and ducks have 5 month generation time), allowing production of large naïve poultry populations between the vaccination campaigns, thus perpetuating the disease. In addition, countries with large semi-commercial and village poultry populations must develop unique programs that will reach the large number of households with low numbers of birds. Initially, expectations were high that a spray vaccination of rNDV would provide single dose, uniform protection in all poultry. Experimentally, rNDV-H5-AIV by respiratory mass application did provide protection from HPAIV challenge in the laboratory, but when deployed to the field, the presence of high levels of maternal antibody to NDV and H5 AIV, inhibited rNDV replication and failed to provide protection with the single vaccine dose. Instead, the rNDV-H5-AIV has best been used as a priming vaccine followed by inactivated booster vaccination. Additional research is need on optimizing vaccination protocols for different poultry species and ages to achieve low cost immunity.

Conclusions

In the review of available literature and field data the following are concluding observations and specific future needs concerning AIV diagnostics and vaccines for poultry over the next 10 years:

- Current diagnostic methods for AIV in poultry are tied to regulatory needs. Virus detection, subtype identification and pathotyping tests are prescribed by the OIE and any changes will need to fulfill regulatory needs.
- Although regulatory considerations harmonize AIV diagnostics, specifics of testing varies among countries and industry compartments to satisfy different needs and to accommodate the availability of resources.
- In order to be implemented new test formats and modifications, tests must:
  - Undergo thorough validation
  - Be practical for the field and veterinary diagnostic laboratory workflow
  - Be cost effective

- Elements of AIV diagnostic programs where improvements are needed (important, but are generally not critical)
  - A method or media to stabilize viable virus for transport in the absence of the cold chain
  - A rapid and high throughput RNA extraction method for tissues and cloacal swabs which removes substances that are inhibitory for rRT-PCR and which preserves the RNA
  - An AgIA for Newcastle disease virus as differential test for AIV, and improved AgIA's for type A influenza which have sensitivity which is near to that of virus isolation or rRT-PCR (currently the difference is 103 to 105 50% egg infectious doses of virus)
  - A sensitive, specific, and well validated AgIA for identification of the H5 and H7 HA subtypes which reacts with all known variants within each subtype.
  - A rapid and inexpensive test to determine pathotype in a biologically relevant manner
  - A method to accurately identify the HA specificity of antibody to AIV (type A influenza). This is a critical need
- Current test are adequate for most AIV testing, however improvements and modifications would be beneficial
- China and other developing/transition countries will be the primary users and will drive commercialization of new technologies for vaccines, with China being the major user, driving innovation and commercialization of vaccine technologies for domestic demand and a modest export market to other developing/transition countries
- Inactivated whole AIV vaccines will dominate the market, but antigenically-relevant, reverse genetic generated AIV seed strains will continue to be developed and used as field viruses drift antigenically away from current AIV vaccine seed strains, and usage of such seed strains will become more focused to specific national/regional geographic needs as H5N1 HPAI became more geographically-isolated and continue to evolve into different genetic subclades or antigenic subgroups
- Developed countries will focus more on preventative measures such as enhanced biosecurity and early detection, with stamping-out of infected and epidemiologically linked premises, while vaccines and vaccination will only have minor usage as preventative tools used in focused, risk-based strategies
- The current routine nationwide HPAI vaccination programs should be converted to risk-based programs based upon real-time field surveillance with vaccine use being focused to the poultry species, region, and production sectors that are at highest risk of HPAI outbreaks and reservoirs; and these vaccination programs should be continuous and age-specific, and not based on seasonal campaign system, designed to achieve 60-80% coverage in at risk poultry populations and provide continual feedback to modify the vaccination program

as local conditions change. DIVA strategies should be incorporated for improved epidemiological surveillance and vaccination systems

- Improved vaccination rates are needed among domestic ducks, which are a large asymptomatic reservoir of H5N1 HPAI virus in Asia. Vaccination of ducks should be linked to control of an economically-significant disease such as duck virus enteritis (DVE), potentially using a recombinant live DVE vaccine with H5 gene insert, thus providing protection from both DVE and H5 HPAI in a single vaccine.
- Additional research and control program needs:
  - New vaccine vector platforms are needed for cost-effective vaccination of short-lived meat chickens which does not require catching and handling of individual birds, possibly achieved by spray, per os or in ovo application, and ideally providing protection after a single vaccination and not inhibited by maternal immunity
  - A mass vaccination system is needed for village poultry with possible development of a thermostable vaccine that can be used within existing NDV village vaccination programs to boost participation and coverage rates
  - More prime-boost vaccination protocols need to be developed to maximize protection at least cost and provide better population immunity
  - Newer adjuvants for inactivated vaccines are needed that will provide shorter withdrawal periods and that can elicit stronger, longer-lived mucosal immune responses
  - A more time-responsive vaccine licensing and registration process is needed by national veterinary biological authorities; for example, the development of a process of updating replaceable "vaccine cassettes" without requirement of resubmitting a full licensing dossier.

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